Genetic and biochemical analysis of the synaptic complex of invertase Gin

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ABBREVIATIONS

ad	"till end volume"
Ap	ampicillin
app.	approximately
bp	base pair
BSA	bovine serum albumin
C-terminus	carboxy-terminus
Cm	chloramphenicol
DEB	1,2:3,4-diepoxybutane
DMSF	dimethylsulfate
DMSO	dimethylsulfoxide
DNase	desoxyribonuclease
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
et al.	and others
EtBr	ethidiumbromide
FIS	factor for inversion stimulation
Gin	G inversion protein
H_2O_{dd}	double-distillated water
HRP	horseradish peroxidase
HTH	helix-turn-helix (motif)
hr	hour
IPTG	isopropyl-1-thio-β-D-galactopyranoside
Kan	kanamycin
kb	kilobase = 1000 bp
kDa	kilodalton
l	liter
lacZ	β-galactosidase gene
µ	micro-
M	molarity
MGB	minor groove binding (motif)
min	minute
ml	milliliter
mM	millimolarity
ng	nanogramme
NMR	nuclear magnetic resonance
N-terminus	amino-terminus
OD	optical density
ORF	open reading frame
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylenglycol
r	resistance
rpm	rounds per minute
RT	room temperature
sec	second
SDS	sodiumdodecylsulphate
st-DNA	single-stranded DNA
Tris	Trihydroxymethylamino-methane
Tc	tetracycline
TEMED	tetramethylendiamine
tyrT	tyrosine tRNA gene
U	unit, enzyme activity
UV	ultraviolet light
V	volt
W	watt
w/v	weight per volume
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. INTRODUCTION

1.1 Site-specific recombination

One of the most striking features of the genome is its ability to change. There are two main processes that underlie this ability: mutation and rearrangement (recombination) of genetic material. However, whilst mutation is predominantly a spontaneous event, genetic recombination usually occurs under a strict control of many factors and often employs an exact mechanism. Dependent on this mechanism three distinct types of recombination systems can be discerned: homologous, site-specific (including transposition) and, illegitimate recombination systems.

As the name suggests, site-specific recombination involves an interaction of specific DNA sites. Recombination occurs by a precise exchange of DNA strands between these sites and formation of new recombinant joints. Site-specific recombination is distinguished from homologous recombination primarily by the mechanism of DNA recognition: in homologous recombination the recognition takes place between two homologous DNA sequences and the search for complementarity is performed using a DNA-protein filament (formed e.g. by the RecA protein). In site-specific recombination, the recombination proteins (recombinases) themselves mediate recognition between the two distant DNA sites. These proteins catalyse the DNA strand breakage and reunion without any requirement for a high-energy cofactor. Since the site-specific recombination involves a reciprocal crossover within two short sites of homologous sequence without any DNA synthesis or degradation it is said to be conservative (Campbell, 1981).

Site-specific recombination systems are ubiquitous throughout eubacteria, prevalent in archaea, but occur only rarely in eukaryotes. Complex eukaryotic genomes, however, can be precisely manipulated by applying site-specific recombination systems (Sauer, 1998; Bode *et al*., 2000). The DNA rearrangements by site-specific recombination systems can serve different purposes, including switching between alternate gene expression patterns, resolution of replicated chromosomes, resolution of intermediates in transposition, integration of viral DNA into host chromosomes, differentiation and pathogenesis, and play an important role in spreading of the genetic elements such as transposons, plasmids, bacteriophages and integrons (Nash, 1996). Moreover, the legacies left by site-specific

recombination events have major consequences in the evolution of prokaryotic genomes (Lawrence, 1999).

Site-specific recombinases can be classified into two major families (Table 1.1) based on amino acid sequence homology and catalytic residues, which are either tyrosine or serine (Esposito & Scocca, 1997; Nunes-Duby *et al.,* 1998; Smith & Thorpe, 2002). The tyrosine family of recombinases, sometimes referred to as the integrase family after the prototypical member λ integrase, uses a tyrosine residue to attack the DNA backbone during cleavage. Other well-known members of this family include the Cre recombinase from phage P1 (Austin *et al.,* 1981; Abremski & Hoess, 1984), FLP invertase from yeast (Broach *et al.,* 1982) and the bacterial protein XerC (Colloms *et al.,* 1990). The members of the serine recombinase family, sometimes referred to as the invertase/resolvase family, possess a catalytic serine residue (Smith & Thorpe 2002). Serine family members include the resolvases γδ (Reed *et al.,* 1982), Tn3 (Krasnow & Cozzarelli, 1983) and ISXc5 (Liu *et al.,* 1998), the invertases Gin (Kahmann *et al.,* 1984) and Hin (Zieg & Simon, 1980) and the phage integrases ϕC31 (Kuhstoss & Rao, 1991), R4 (Matsuura *et al.,* 1996) and TP901-1 (Breuner *et al.,* 2001).

Site-specific recombinases

Serine recombinases (invertase/resolvase family)			**Tyrosine recombinases** (integrase family)	
invertases	**resolvases**	**integrases**	**integrases**	**other tyrosine recombinases**
Gin (phage Mu) *(Escherichia coli)* **Hin** *(Salmonella typhimurium)*	**γδ** *(Escherichia coli)* **Tn3** *(Klebsiella pneumoniae)* **ISXc5** *(Xanthomonas campestris)*	**ϕC31** (*Streptomyces lividans)* **R4** *(Streptomyces parvulus)* **TP901** *(Lactococcus lactis)*	**λ** *(Escherichia coli)* **HK022** *(Escherichia coli)* **P22** *(Salmonella typhimurium)* **HP1** *(Haemophilus influenzae)* **L5** *(Mycobacterium smegmatis)*	**Cre** (P1) *(Escherichia coli)* **FLP** *(Saccharomyces cerevisiae)* **XerC** *(Escherichia coli)*

Table 1.1. Classification of the site-specific recombinases and examples of representative family members.

Host organisms are indicated in brackets.

These two families of site-specific recombinases have different structures and mechanisms and very likely evolved separately. Nevertheless, the recombinases of both the tyrosine and

serine families can perform a wide range of distinct recombination reactions (Hatfull & Grindley, 1988; Landy, 1989; Stark *et al*., 1992; Nash, 1996; Grindley, 1997).

Depending on the end product of the reaction three different types of site-specific recombination reactions can be distinguished:
- inversion of a DNA segment between the recombination sites;
- deletion of a DNA segment between the recombination sites;
- insertion (integration) of a DNA segment (or molecule); this latter reaction usually employs recombination sites located on different DNA molecules.

The recombinases of both families catalyse the reaction by a two-step transesterification process, but they differ in their reaction requirements. The enzymes of the serine recombinase family perform intramolecular reactions only. After binding to the recombination sites that can be present either in direct (in the case of resolvases), or indirect (for invertases) orientation, the proteins bridge the two sites in a so-called synaptic complex and then catalyse strand exchange. The recombination sites are cleaved and the half sites are religated to opposite partners (Stark *et al*., 1992; Nash, 1996). Biochemical studies have demonstrated that in the reaction intermediate (Figure 1.1A), the proteins introduce a staggered double strand break leaving two base pair overhangs with 3' protruding ends. In this intermediate the protein becomes covalently attached to the 5' phosphate of the DNA via a conserved serine (Hatfull & Grindley, 1988; Johnson & Bruist, 1989). This mechanism is quite distinct from that of the tyrosine recombinases, which initiate recombination by a single-strand DNA cleavage, exchange and rejoining of one pair of DNA strands. This generates a Holliday junction as a recombination intermediate, which is resolved by a second set of transesterification reactions carried out with the other pair of DNA strands (Figure 1.1B). In this reaction the proteins are covalently attached to the 3' phosphate of the DNA via the active site tyrosine leaving free 5'-hydroxyl ends (Landy, 1989; Stark *et al*., 1992; Gopaul & van Duyne, 1999; Chen *et al*., 2000).

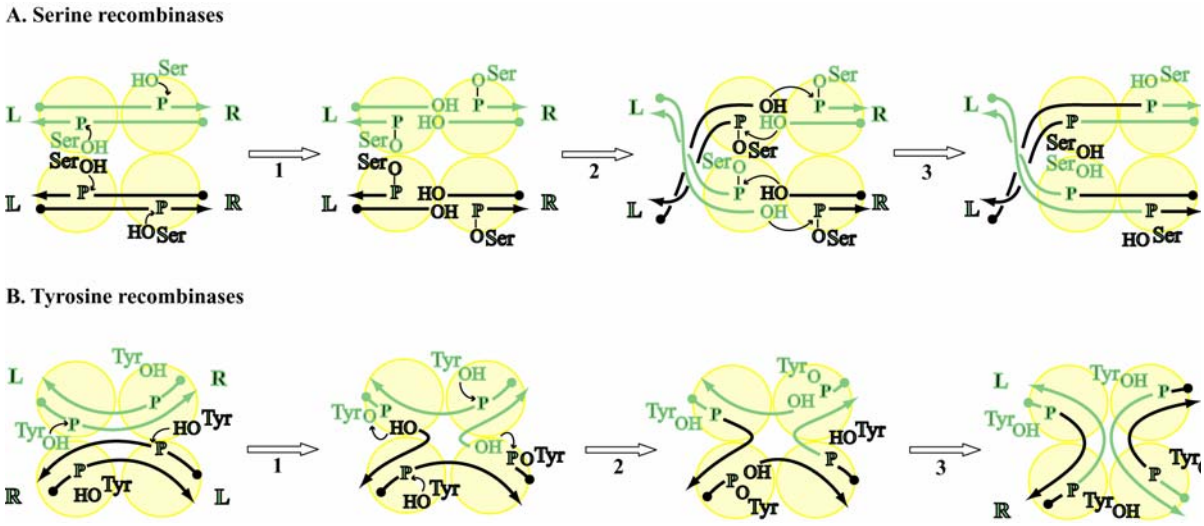


Figure 1.1. Schematic representation of recombination mechanisms employed by serine and tyrosine recombinases.

A. Serine recombinases mediate a coordinated DNA attack to create a 2-bp staggered double-strand break at the centre of each recombinational site. In this process, invertase subunits become covalently joined to each 5' end of the broken strands via an ester linkage at serine (Ser_{OH}). In the next step of the DNA strand exchange, the cleaved DNA strands are positioned into the recombinant configuration and the DNA is religated through reversal of the protein–DNA linkage.
Step 1: two double strand cleavages;
step 2: strand exchange;
step 3: two double strand ligations.
B. Tyrosine recombinases make ordered single strand exchanges between the two recombinational sites: the first pair of exchanges forms a four-way Holliday junction; the second pair resolves the junction to complete the recombination. The nucleophile used for cleavage and formation of the covalent recombinase-DNA intermediate is a conserved tyrosine (Tyr_{OH}). The cleavage sites on each DNA duplex are separated by 6-8 bp with a 5' stagger, and the tyrosine joins to the 3' phosphate.
Step 1: the first single strand cleavages and exchanges;
step 2: the first ligations followed by second single-strand cleavages;
step 3: the second strand exchanges and ligations.
Yellow filled circles represent the subunits of recombinases arranged in tetramers.

1.2 Serine recombinases: resolvases and invertases

Most of the understanding of the mechanism of serine recombinases comes from the studies on the recombinases of the resolvase/invertase family (Hatfull & Grindley, 1988; Stark *et al*., 1992; Grindley, 1994). Four systems have been studied in much detail: the resolution of co-integrates by γδ and Tn*3* resolvases, flagella antigen switching by the *Salmonella typhimurium* Hin invertase and tail fibre switching by phage Mu Gin invertase. The invertases and resolvases are related to each other, about 13% of amino acid residues being common to all members of the serine recombinase family (Hatfull & Grindley, 1988; Sherratt, 1989). Alignment of the amino acid sequences of the three of the most related

proteins of the serine recombinase family is shown in Figure 1.2.

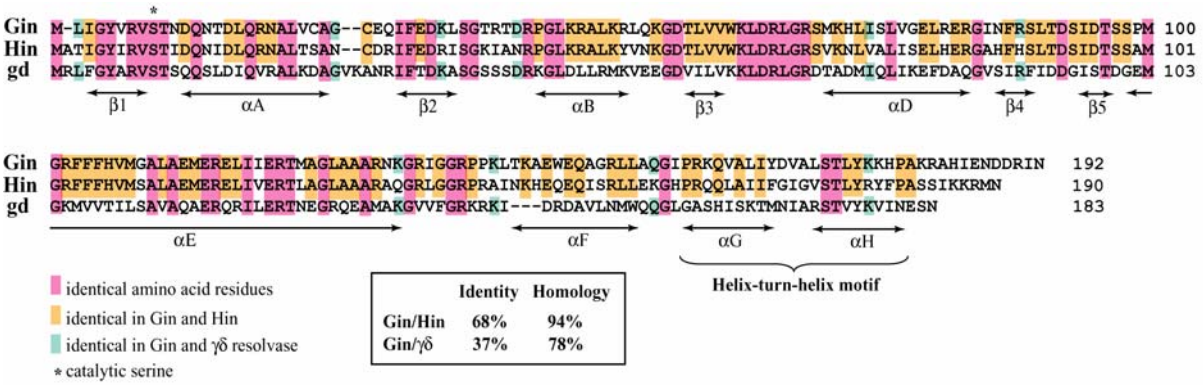


Figure 1.2. **Alignment of the amino acid sequences of the related site-specific serine recombinases.**

Amino acid sequence of the invertase Gin (Plasterk *et al.,* 1983b) is compared to Hin invertase (Zieg & Simon, 1980) and γδ resolvase (Reed *et al.,* 1982).
Residues that are highlighted in colours indicate perfect matches of amino acid sequences among proteins. The secondary structures observed in the crystal structure from γδ resolvase (Yang & Steitz, 1995) are marked underneath the sequence alignment, α for α helix, β for β strand. The asterisk marks the active site residue. Also indicated are the α helices that form the helix-turn-helix DNA-binding motif. Ratio of the proteins identity/homology is indicated according to calculations done by T.Hermann (1996).

Based on biochemical and topological analyses of different resolution and inversion systems, it is thought that both sub-families employ a similar reaction mechanism during DNA strand exchange (reviewed by Stark *et al.,* 1992). However, there are important differences between these systems in the steps that lead to catalysis.

In both resolution and inversion systems, the combined properties of the recombinases and their cognate DNA sites result in strict control over the outcome of the recombination. The resolvases normally recombine two sites only in direct repeat to cause deletion of DNA between the sites and the invertases recombine sites only in inverted repeat to invert the DNA between the sites. Neither type of recombinase will normally recombine two of its cognate sites if they are on different DNA molecules, i.e. they will not be able to catalyse integration. The recombinases detect whether the sites are in the correct orientation by trapping the DNA into specific topologically defined synaptic complexes (Stark & Boocock, 1995). Evidence for these defined complexes comes from the analysis of recombination products obtained *in vitro* with supercoiled DNA substrates. If synapsis occurs by random collision of two recombination sites, as occurs with λ integrase, the

products are knotted or multiply catenated (Pollock & Nash, 1983). The products of resolution are 2-noded catenanes (Figure 1.3), whereas invertases produce mainly unknotted circles (Figure 1.4), indicative of defined synapse topologies in each case (Krasnow & Cozzarelli, 1983; Wasserman *et al*., 1985; Stark *et al*., 1989; Heichman *et al*., 1991).

Resolvases recognise a DNA sequence called *res*, which is composed of three binding sites for resolvase dimers (Figure 1.3). Two *res* sites must be present as direct repeats on the same negatively (-) supercoiled DNA molecule. Only this orientation of sites allows for the formation of a functional synaptic complex, the so-called synaptosome, which entraps three (-) supercoils (Dröge & Cozzarelli, 1989). The resolvase binds to the 2 *res* sites arranged as direct repeats and containing 3 subsites each. One dimer of resolvase binds to each subsite of *res* such that in total the resolvase synaptosome contains 6 dimers.

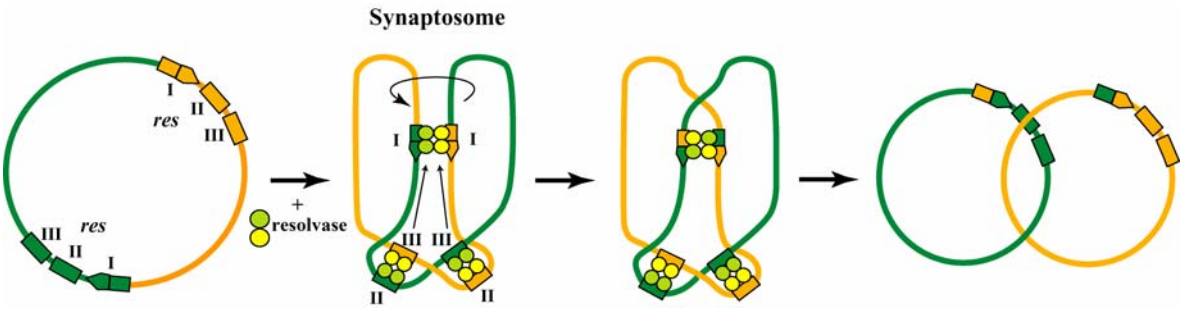


Figure 1.3. The standard substrate for resolution and model of subunit rotation for the catalytic reaction.

Resolvase (indicated with filled green and yellow circles) binds as dimer to subsites I, II and III (filled boxes) in the two *res* sequences to form a catalytic synaptosome. Interactions of the resolvase dimers bound to sites I with dimers bound at sites II and III are indicated by arrows. After cleavage at the subsite I, the DNA strands are exchanged and religated. Catenated DNA products are released upon dissociation of the synaptosome.

The cleavage reaction is catalysed by resolvase dimers bound at subsite I without any need for auxiliary host factors. Once productive synapsis has occurred, the paired site I-bound resolvase dimers are activated for strand exchange (Dröge *et al.,* 1990). There is compelling evidence from mutational and biochemical studies that synaptosome formation and transactivation of strand exchange involves interactions between dimers bound at accessory sites II and III, and between those dimers and the catalytically active ones bound at sites I, respectively. Thus, there is a clear division of labour between resolvase dimers

within the synaptosome: two catalytic dimers bound at sites I are directly involved in strand exchange, whereas at least four other dimers bound to accessory sites II and III play an architectural and transactivating role (Hughes *et al*., 1990; Grindley, 1993; Murey & Grindley, 1998).

Invertases require only two inversely oriented specific sites, termed *gix* in the Gin system, each bound by an invertase dimer and aligned in parallel fashion to form a single catalytic tetramer (Figure 1.4). A highly ordered nucleoprotein complex formed by invertases, the so-called invertasome, contains, in addition, a host protein designated Factor for Inversion Stimulation (FIS). FIS binds to the so-called recombinational enhancer. The enhancer is a key structural element of the invertasome and is composed of two appropriately spaced binding sites for FIS dimers (Kahmann *et al.,* 1985; Johnson *et al.,* 1987). The functional role of FIS and enhancer appears to be similar to that of the resolvase dimers bound at accessory sites II and III (Figure 1.4).

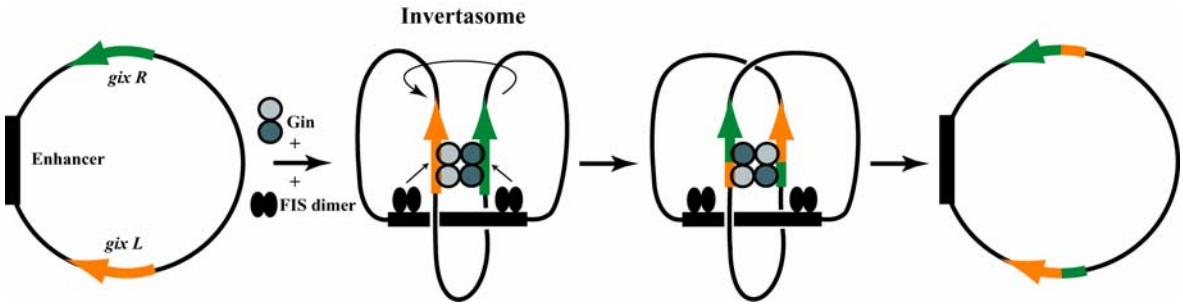


Figure 1.4. The standard substrate for site-specific DNA inversion by Gin and model for the Gin synaptic complex.

The supercoiled plasmid substrate contains two recombination sites, g*ix* L and g*ix* R, and a recombinational enhancer. After Gin (indicated with filled light and dark grey circles) and FIS (filled ovals) dimers are bound at their respective sites, the pairing of these three DNA segment results in invertasome formation. Strand exchange at *gix* sites requires interactions between FIS and Gin dimers, as indicated by the arrows. FIS activates Gin to initiate double-strand cleavage at g*ix* L and g*ix* R followed by exchange of DNA strands and ligation. Resolution of the invertasome after completion of strand exchange results in inversion of the orientation of the DNA segment between g*ix* L and g*ix* R.

1.3 The DNA inversion system of bacteriophage Mu

Site-specific DNA inversion of a 3000 bp DNA fragment, the so-called G segment (Daniel *et al.,* 1973) in phage Mu is catalysed by a nucleoprotein complex involving the phage

encoded invertase Gin and the host protein FIS (Kamp *et al.,* 1978; Kahmann *et al.,* 1985). Two different sets of genes, S and U, involved in the biosynthesis of tail fibres that determine the host range specificity, are expressed from the G region dependent on its orientation (Van de Putte *et al.,* 1980; Grundy & Howe, 1984). The S gene has a constant part (S_c) that lies outside of the invertible region and a variable part (S_v) within it. As shown in Figure 1.5, the inversion of the G region causes alternate expression of S_cS_v and S_cS_v', and of U and U'. As a consequence of this alternate expression, two types of phage particles with distinct tail fibers are produced that are infectious for different host bacteria (Van de Putte *et al.,* 1980; Kamp, 1981).

The *gin* gene is located adjacent to the invertible G segment and is expressed from a promoter that overlaps the *gix* R recombination site (Plasterk *et al.,* 1983a). During the inversion reaction, Gin binds to two identical 34 bp *gix* sites flanking the G segment as inverted repeats (Plasterk *et al.,* 1983a; 1984; Koch *et al.,* 1987). Catalysis of the reaction requires the formation of a synaptic complex of unique topology, in which the recombination sites (*gix* L and *gix* R) each bound by a dimer of Gin, and the recombinational enhancer with two bound FIS dimers, are present (see Figure 1.4). The enhancer sequence is located within the *gin* gene, but it has been shown that the enhancer can function independent of its orientation and distance to the *gix* sites (Kahmann *et al.,* 1985).

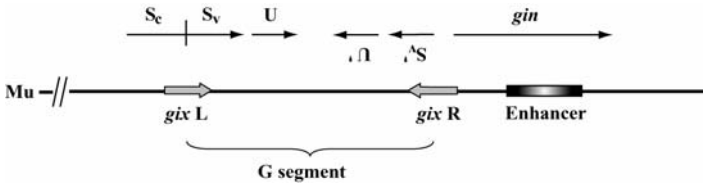


Figure 1.5. Inversion system of the bacteriophage Mu.

Recombination of a G-segment takes place between two identical, 34 bp long *gix* sites (*gix* L and *gix* R), which flank the G segment as inverted repeats. Inversion is catalysed by phage-encoded protein Gin. The *gin* gene maps outside the invertable region. The enhancer sequence is located within the *gin* gene.

In the synapse the two Gin dimers are thought to form a tetramer (Kanaar *et al.,* 1989a; Heichman & Johnson, 1990). This complex is formed at a branch point of a supercoiled DNA molecule, probably stabilised through Gin-Gin and Gin-FIS interactions and entraps two negative supercoils (Kanaar *et al.,* 1988; 1989a).

In the synaptic complex the four DNA strands are cleaved in a concerted manner by Gin, generating a 2 bp staggered cut at the centre of the crossover sites (Klippel *et al.,* 1988a; Johnson & Bruist, 1989; Lim *et al.,* 1992). A tetramer of Gin is thought to represent the catalytically active species since in the cleaved recombination intermediate four Gin monomers are covalently attached to the four 5' phosphate ends before the strand rearrangement (Klippel *et al.,* 1988a; Johnson & Bruist, 1989). The change in the topology of inversion products suggests that after the double-strand cleavage of *gix* sites there is a "simple rotation" of the paired *gix* half-sites by 180^{o} relative to the other paired half-sites (Kanaar *et al.,* 1990), followed by their religation in recombinant configuration (Klippel *et al.,* 1993). As a consequence, the recombination products have a unique topology; they are unknotted and differ from the starting material by a change in linking number (ΔLk) of +4 (Kahmann *et al.,* 1987; Kanaar *et al.,* 1988).

Gin can catalyse the recombination reaction also *in vitro*. The *in vitro* inversion reaction requires a (-) supercoiled DNA substrate, the host factor FIS and Mg^{2+} ions, but no high-energy cofactors (Plasterk *et al.,* 1984). The rotation of the strands is facilitated by the free energy of negative supercoiling, whereas their ligation is aided by the nucleophilic attack of the free 3'-OH-groups on the phospho-serine bonds between protein and DNA (see Figure 1.1A). The FIS protein is thought not only to play an architectural role, but also to stimulate the concerted cleavage of *gix* sites by the Gin tetramer (Safo *et al.,* 1997).

1.3.1 Structure of the Gin invertase protein

The Gin protein of bacteriophage Mu is a member of the sub-family of DNA invertases including Hin, Cin, Pin and Min (Zieg & Simon, 1980; Iida *et al.,* 1982; 1990; Plasterk *et al.,* 1983b; Van de Putte *et al.,* 1984) that show about 60-70% sequence homology and can complement each other when used in the heterologous DNA inversion systems (Plasterk *et al.,* 1983a; Kamp & Kahmann, 1981).

Gin is a 21.7 kilodalton (kDa) protein (Plasterk *et al.,* 1983a) with 193 amino acids. The structure of the protein has not been solved yet. However, the structure of the related $\gamma\delta$ resolvase (Yang & Steitz, 1995) and the C-terminal DNA binding domain of the highly homologous invertase Hin have been determined in complex with the DNA (Feng *et al.,* 1993, 1994; Haykinson *et al.,* 1996; Chiu *et al.,* 2002).

So far several structures of the γδ resolvase have been solved, including the protein complexed with an artificial version of the *res* binding site (Yang & Steitz, 1995) and a recent NMR analysis of a part of the catalytic domain (Pan *et al.,* 2001). These structures have revealed important information about the protein-protein and protein-DNA interactions, but have left some unanswered questions concerning the mechanism of catalysis.

In the crystal structure of the γδ resolvase complexed to the subsite I of the *res* site, γδ binds as an asymmetric dimer (Yang & Steitz, 1995; Figure 1.6). Each monomer has two globular domains (amino-terminal (N) domain, including amino acid residues 1–120 and carboxy-terminal (C) domain, comprising residues 148–183), connected by an extended arm region (residues 121–147). The N-terminal region of the protein consists of four β-sheets packed with four α-helices. Protein–DNA contacts are made via the arm region and the C-terminal domain. The arm region makes extensive contacts in the minor groove of the DNA extending by more than half a turn. The C-terminal domain contains 3 consecutive α-helices that form a helix-turn-helix (HTH) DNA binding motif. This C-terminal HTH domain interacts with the major groove located on the opposite face of the DNA with respect to the catalytic N-terminal domain (Yang & Steitz, 1995). Upon binding γδ resolvase the DNA is bent at a ~60^0 angle.

In this complex, the active site serine residues within the catalytic domain of each subunit are not positioned appropriately and are too far from one another to initiate a concerted site-specific cleavage of DNA (Rice & Steitz, 1994b; Yang & Steitz, 1995). However, they are located in loops that may be more flexible than other parts of the protein. This implies that a conformational change that repositions the active site close to the scissile phosphodiester bonds must precede DNA cleavage and indicates that the obtained site I-resolvase complex possibly reflects an inactive configuration (Yang & Steitz, 1995).

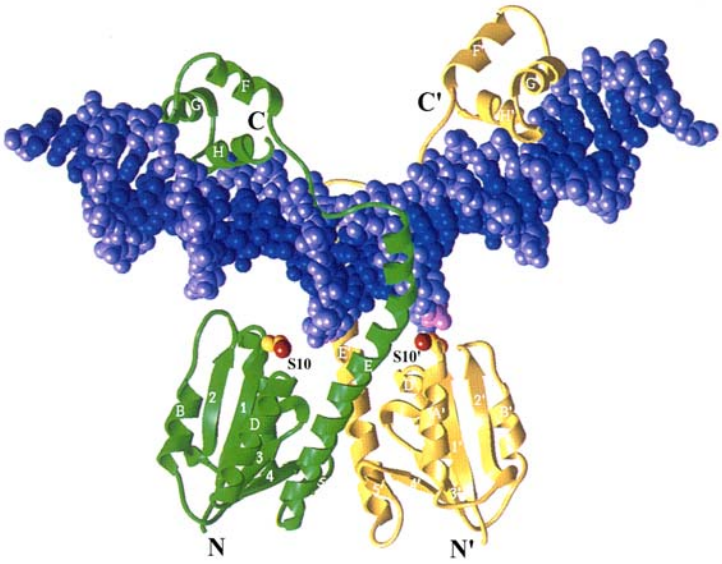


Figure 1.6. The structure of the γδ resolvase dimer complexed with DNA (Yang & Steitz, 1995).

One monomer is highlighted in green and another one in yellow; the active site serine 10 (S10) residues have yellow carbon atoms and red hydroxyl groups. Amino-terminal catalytic (N, N') and carboxy-terminal DNA-binding (C, C') domains are indicated, in which α helices are indicated by letters and β strands by numbers. DNA is represented by the blue ribbon with scissile phosphates highlighted in magenta.

Studies done with a C-terminal 52-mer peptide (amino acids 139-190) of the Hin invertase complexed with a synthetic 13 bp *hix* L half-site DNA provided more information about protein-DNA interactions (Feng *et al.*, 1994; Rice & Steitz, 1994b; Yang & Steitz, 1995; Haykinson *et al.,* 1996). The peptide has three α-helices (F, G, and H), the second and third of which (G and H) form the helix-turn-helix (HTH) motif (Plasterk & Van de Putte, 1984; Bruist *et al.,* 1987b; Dodd & Egan, 1990). As with γδ resolvase, the binding specificity of the Hin invertase results from the interactions of the two structural motifs within Hin with the specific DNA sequence of *hix*. The HTH motif at the C-terminus of Hin comprises Glu 148 through Phe 180 and interacts with the major DNA groove of *hix*. The other is the minor groove binding (MGB) motif that is composed of the three consecutive amino acid residues Arg 140 - Pro 141 - Arg 142 (Feng *et al.,* 1994). The structural model of Hin showed that the N-terminus of the MGB motif lies immediately adjacent to the C-terminal end of helix E. Several reports have emphasised the importance of the MGB motif of Hin in DNA binding. A single nucleotide change in the minor DNA groove of *hix* can abolish binding of Hin, while a base change in the major DNA groove results in a less severe defect (Hughes *et al.,* 1992). According to the studies done by Lee *et al.* (2001), αE helices may

serve to position the MGB motif correctly with respect to the minor groove of the recombination site.

Based on the identified structures of the γδ resolvase (Yang & Steitz, 1995) and the C-terminal domain of Hin (Feng *et al.,* 1993, 1994; Haykinson *et al.,* 1996) a comparative model of the catalytic domain of Gin invertase has been elaborated (Hermann, 1996; Figure 1.7A).

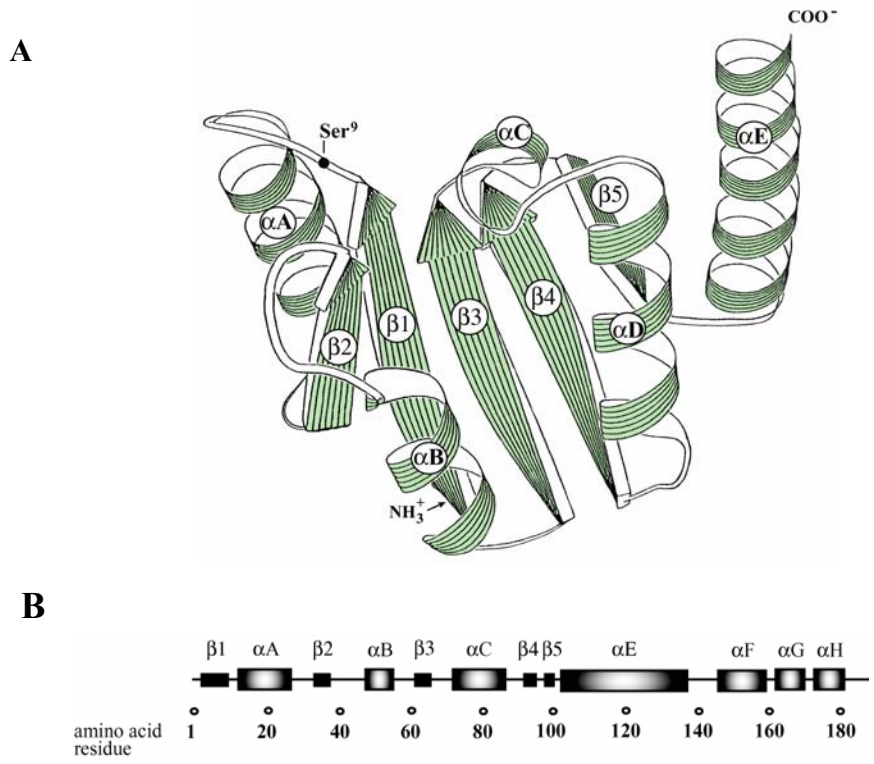


Figure 1.7. Organisation of the Gin monomer.

A. 3D model of the Gin N-terminal catalytic domain (residues M1-I109) based on the identified crystal structure of γδ resolvase (Yang & Steitz, 1995) created by T.Hermann (1996).
Relative orientation of the α-helices and β-sheets and the location of the active site residue serine 9 (S9) are indicated.
B. Secondary structure of the Gin monomer according to the amino acids residues.
The α-helices and the β-sheets are represented as boxes. Numbers indicate the amino acid residues.

1.4 FIS protein, its structure and role in the inversion stimulation

FIS is an abundant *E. coli* protein of 11 kilodaltons (kDa) comprising 98 amino acids and involved in the regulation of many different processes in the cell. Under laboratory conditions FIS is not essential for the viability (Koch *et al.,* 1988; Johnson *et al.*, 1988). FIS

was initially identified as a protein involved in the stimulation of the invertases Gin of phage Mu (Kahmann *et al.,* 1985), Hin of *Salmonella typhimurium* (Johnson & Simon, 1985) and Cin of phage P1 (Haffter & Bickle, 1987). Later it was found that FIS has a role in many other reactions including phage λ excision (Thompson *et al.,* 1987; Ball & Johnson, 1991), regulation of phage Mu transposition (Betermier *et al.,* 1989, 1993; van Drunen *et al.,* 1993), transcriptional activation of rRNA and tRNA operons (Ross *et al.,* 1990; Zacharias *et al.,* 1992; Lazarus & Travers, 1993), ori-C-directed DNA replication (Gille *et al.,* 1991; Filutowicz *et al.,* 1992), regulation of Tn5 transposition (Weinrech & Reznikoff, 1992) and DNA topology (Schneider *et al.,* 1997). In addition, it has been reported that in the absence of a functional *fis* gene, *E.coli* bacteria have reduced growth rates (Nilsson *et al.,* 1992) and show altered morphology (Filutowicz *et al.,* 1992).

The structure of the main part of the protein has been determined by X-ray crystallography (Kostrewa *et al.,* 1991; Yuan *et al.,* 1991). Analysis has shown that FIS is a homodimer each monomer of which is composed of 74 amino acids forming four α helices (A, B, C and D respectively) and a 24 amino acid N-terminus that could not be defined due to low electron density. However, the complete crystal structure of the FIS mutant K36E was resolved by Safo *et al.* (1997) and is shown in Figure 1.8. The structural core consists of four α-helices: A (residues 27–40), B (50–70), C (74–81) and D (85–94), for each subunit in the homodimer structure. This core domain, from residues 27 to 98, is almost structurally identical to that of wild type FIS. The previously unresolved N-terminal structure was shown to contain two short β-strands located at residues 12–16 (β-1) and 22–26 (β-2) which are joined by a hairpin loop (residues 17–21) and form an antiparallel β-sheet protruding from the surface of the otherwise compact molecule. The fact that in the FIS dimer the two antiparallel β-sheets do not have any surrounding residues, suggests that they must be highly flexible in solution.

The two monomers of FIS are interlocked with extensive interactions between them due to which the protein exists as a dimer under different conditions, both in solution and in the crystals (Kostrewa *et al.,* 1991; Yuan *et al.*, 1991; Koch & Kahmann, 1986). The contacts between two subunits in the dimer are formed through extensive Van der Waals interactions and hydrogen bonds. Helix B of one subunit makes contacts with helix A' and helix C' of the opposite subunit as well as with helix D of its own unit. This hydrogen-

bonding network helps to hold the FIS dimer like a rigid body, which undergoes conformational changes only with difficulty.

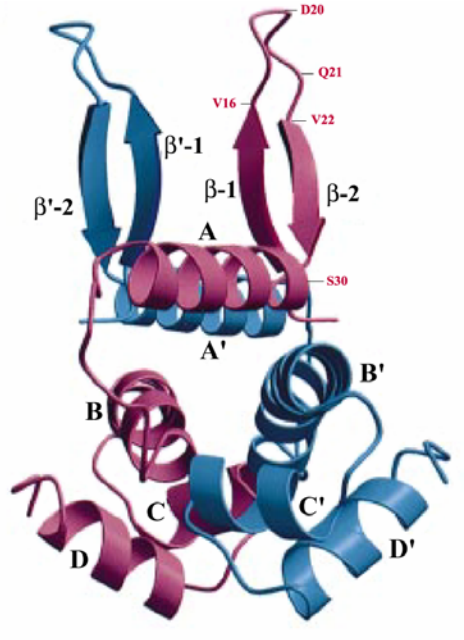


Figure 1.8. Structure of the FIS K36E dimer (Safo *et al.*, 1997).

The four α-helices and two β-strands are labelled as A–D, β-1 and β-2 in the first monomer, which is highlighted in red and A'–D', β'-1 and β'-2 in the second monomer, which is highlighted in blue.

The locations of amino acid residues Val-16 (V16), Asp-20 (D20), Gln-21 (Q21), Val-22 (V22), and Ser-30 (S30), which were shown to be involved in transactivation of Hin-catalysed DNA inversion (Safo *et al.,* 1997; Merickel *et al.,* 1998), are indicated with red letters. Val-16, Asp-20, Gln-21, and Val-22 are within the β-hairpin activating motifs, whereas the two Ser-30 residues are directly across from each other on the A/A' helices.

The last two C-terminal helices (C and D) form an HTH motif, which constitutes the main component of the DNA binding surface. A remarkable feature of the HTH domain is that the helix D contains six positively charged residues (two arginines and four lysines), which are likely to make contact with the negatively charged phosphate backbone of the DNA. This could indicate that FIS recognises DNA through interactions that are predominantly non-specific, which is in agreement with the degenerated consensus 15 bp sequence of the FIS binding site:

(G/T)nnYRnn(A/T)nnYRnn(C/A)

where Y = pyrimidine; R = purine; n = any nucleotide (Hübner & Arber, 1989).

Although FIS binds to highly divergent sequences, the way in which the protein binds to the different 15 bp core sequences is very similar (Pan *et al.,* 1996). Binding of FIS to these sites results in a bending of the DNA by an angle estimated as ≈ 90^{o} (Thompson & Landy, 1988). However, the degree of DNA bending varies for the different FIS binding sites (Gille *et al.,* 1991). The sequences flanking the 15 bp core region appear to be important in

determining the extent of DNA wrapping around the FIS dimer (Pan *et al.,* 1996). The DNA bending induced by FIS is believed to be one of the major functions of this protein.
This is also true with regard to the role of FIS binding at the recombinational enhancer. The recombinational enhancer consists of two essential binding sites for FIS the spacing of which relative to each other is critical for enhancer function (Johnson & Simon, 1985; Kahmann *et al.,* 1985; Johnson *et al.*, 1987). Previous studies suggested that FIS dimers must be associated with both binding sites within the Hin enhancer to form productive invertasomes (Johnson & Simon, 1985; Bruist *et al.,* 1987a; Johnson *et al.,* 1987; Heichman & Johnson, 1990). This was demonstrated by (1) mutagenesis studies showing that removal of either binding site or changes in the spacing between binding sites essentially abolish FIS-activation of inversion; (2) electron microscopy showing the absence of invertasome structures on substrates containing only one FIS binding domain; and (3) stoichiometry measurements indicating two FIS dimers per activated invertasome. It has been proposed that FIS stabilises a tightly bent right-handed DNA loop on binding the enhancer (Travers & Muskhelishvili, 1998).
The studies in the Hin and Gin systems suggested that the enhancer remains associated with the invertasome complex throughout the course of the reaction, though it can be released prior to the ligation step under certain conditions for Hin and more readily for the Gin DNA invertase catalysed reaction (Kanaar *et al.,* 1990; Heichman *et al.,* 1991; Crisona *et al.,* 1994). Thus, whereas the binding of FIS at the enhancer is required to initiate catalysis, it is not required for the final chemical step.

1.4.1 FIS-Gin interactions in the invertasome

As mentioned above, the poor X-ray resolution of the N-terminal 24 amino acids of FIS suggested that this structure might be flexible. The crystal structure of a mutant FIS protein has revealed that previously unresolved amino acids 12–26 near the amino terminus of each subunit form a β-hairpin arm that protrudes over 20 Å from the α-helical core (Figure 1.8) and is responsible for Hin transactivation (Safo *et al.,* 1997; Merickel *et al.,* 1998). Disulfide cross-linking of cysteines introduced at different positions within the β-arms of FIS demonstrated that the arms are mobile in solution. The authors observed that whereas both FIS dimers must be capable of activating Hin, FIS heterodimers that have only one

functional β-arm are sufficient to stimulate both Hin DNA cleavage and strand exchange. Analysis of homodimer and heterodimer mixes of different Hin mutants suggests that FIS must activate each subunit of the two Hin dimers that participate in catalysis. These experiments also indicate that all four Hin subunits must be coordinately activated prior to initiation of the first chemical step of the reaction. These authors also made a surprising observation that the FIS dimers containing the two mobile β-arms linked together by a disulfide bridge at one of the several positions within the β-hairpin loops (e.g., between amino acids 15, 18, and 19) were still able to activate Hin efficiently (Safo *et al.,* 1997). This covalent linkage would prevent simultaneous interaction of the two β-arms of FIS with the two separate regions in the Hin dimer.

Extensive mutagenesis of this region has shown that three amino acids, Val-16, Asp-20, and Val-22, near the tip of the β-arm of FIS are critical for the activation of Hin inversion (Safo *et al.*, 1997; Figure 1.8). Certain solvent-exposed amino acids within the α-helix A may also contact Hin within the invertasome structure (e.g. Ser 30).

FIS mutants either carrying amino acid substitutions in the N-terminal part of the protein, or FIS mutants in which up to 26 amino acids of the N-terminus were deleted, all showed that this region is dispensable for stable DNA binding, but required for the stimulation of the inversion reaction catalysed by Gin (Koch *et al.,* 1991; Osuna *et al.,* 1991; Spaeny-Dekking *et al.,* 1992, 1995c). This suggests that the N-terminus of FIS is involved in interactions with Gin. When however a FIS mutant, lacking the first 29 N-terminal residues, was used in the inversion reaction together with the FIS-independent Gin mutant M114V, the activity of M114V was reduced, suggesting that the two proteins form an additional contact in which the N-terminus of FIS is not involved (Merker *et al.,* 1993). From these results a model was proposed in which the central domain of FIS first stabilises the synaptic intermediate and in a second step of the reaction the N-terminus of FIS interacts with Gin thereby facilitating the subunit exchange by weakening the monomer-monomer contacts in the Gin dimer (Merker *et al.,* 1993).

Additional evidence for such a dual role of FIS comes from the properties of Gin mutants, the activity of which depends on the presence of FIS protein, but does not require the presence of the enhancer (Spaeny-Dekking *et al.,* 1995a). Since those mutants showed no inversion activity when the N-terminus of FIS is deleted, the following conclusion was made: binding of FIS at the enhancer stabilised the formation of an active synaptic complex. For this function the N-terminal part is not required. In a second step the N-

terminal part of FIS interacts with the dimerisation helix of Gin thereby facilitating the subunit exchange. For this action the enhancer is no longer required.

Several Gin mutants have been isolated that are able to carry out the inversion reaction without need for FIS, the enhancer and negative supercoiling (Klippel *et al.,* 1988b). Such mutants showed also less stringent substrate requirements than the wild type protein, since they could promote recombination between direct repeats as well as intermolecular recombination. This suggests that the synaptic complex formed by these mutants is different from that formed by the wild type Gin. From the properties of these mutants it has been proposed that the role of FIS in inversion is to induce a conformational change in the Gin protein, thereby activating this protein to initiate strand exchange, and that the FIS-independent Gin mutants are locked in such an activated configuration. A biochemical analysis of these mutant Gin proteins led to the suggestion that in wild-type Gin system FIS and the enhancer act at a stage after synapsis but before the strand exchange (Klippel *et al.,* 1993). The loss of need for FIS and enhancer in the mutant Gin proteins has been attributed to their ability to partially unwind the *gix* site and to cleave DNA without need for synapse formation (Klippel *et al.,* 1993). All the *gin* mutations that confer a FIS-independent phenotype fall into a single domain that corresponds to the dimerisation surface of γδ resolvase (Sanderson *et al.,* 1990; Hughes *et al.,* 1993; Grindley, 1993; Lim, 1994). These findings indicate that FIS-independent Gin mutants are altered in the interaction surface between the two monomers in the Gin dimer bound at a *gix* site.

Taken together, these data are consistent with a direct interaction between Gin and FIS during the DNA inversion reaction.

1.5 Models of the synaptic complex and mechanism of DNA recombination

The structure of the synapse and the precise nature of the molecular events during strand exchange are not clearly understood. There are two main models that predict different organisation of the synaptic complex and mechanism of recombination.

In the "subunit exchange" model the catalytic domains of the Gin dimers are interacting with each other during synapse formation, whereas the C-terminal DNA binding domains face opposite directions (in other studies also referred as “DNA outside model”). This

model predicts that the Gin monomers rotate while remaining covalently attached to the *gix* half-sites (Kanaar *et al.,* 1990; Heichman *et al.,* 1991).

Such an organisation of the synaptic complex was proposed for γδ resolvase as well – a protein homologous to Gin (Sarkis *et al*., 2001). An important implication of this model is that the crossover sites in the synapse are far apart from one another as they lie outside of the recombinase core, suggesting that there must be substantial movement within the complex to effect recombination (Sarkis *et al*., 2001). Another attractive feature of this model is that the synaptic surface is situated within the 120-residue catalytic domain and it is proposed to be common to all serine recombinases (Arnold *et al*., 1999; Sarkis *et al*., 2001). The experiment described recently (Leschziner & Grindley, 2003) provided strong evidence in support of the idea that the resolvase-subsite I synapse contains the catalytic domains of the recombinase tetramer as its central core, with the two *res* subsites I held on the outside, well separated from each other and crossing to form a local positive node (Leschziner & Grindley, 2003).

The alternative "static subunits" model suggests that the DNA binding domains of opposite dimers face each other with the recombining DNA duplexes packed close together inside the tetramer (“DNA inside”) (Rice & Steitz, 1994a). In this model the interactions between the two *res* subsite I-bound resolvase dimers have not been specified; however, because the DNAs were placed on the inside of the complex, the synaptic interactions most likely involved the DNA binding domains rather than the catalytic domains. A similar mode of synapsis with the DNA inside has been proposed for the invertasome formed by the related recombinase Hin (Merickel *et al.,* 1998; Huang *et al.,* 2003; Figure 1.9). In this model, the *hix* DNA segments are located in the centre of the Hin tetramer and oriented at ~90° with respect to each other.

The "subunit exchange" model nicely explains the experimentally detected topological changes in the substrate as well as extensive knotting of inversion products. Extensive knotting of DNA results from processive recombination leading to multiple rounds of strand rotation and is observed e.g. when the 2 bp spacer regions of the crossover sites do not match each other (Kanaar *et al.,* 1990; Heichman *et al.,* 1991). However, it remains unclear how the nucleoprotein complex is stabilised during the strand exchange, especially when multiple rounds of strand rotation take place.

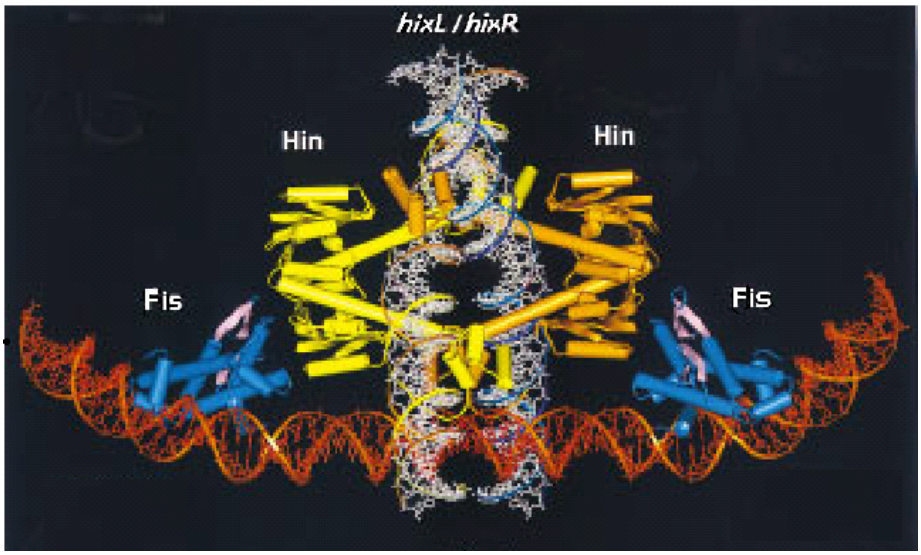


Figure 1.9. Model for the Hin invertasome organisation (Merickel *et al.*, 1998).

Yellow and light orange ribbon diagrams show the Hin dimers complexed with recombination sites on DNA fragments shown in white. Two FIS dimers, depicted in blue with the β-arm activation domains highlighted in pink are bound to an enhancer segment of DNA shown in dark orange.
Proposed model positions the Hin catalytic domains on the outside of the Hin tetramer adjacent to the activating regions of FIS (Merickel *et al.*, 1998). This arrangement of the Hin tetramer is similar to one of the models proposed for the *res* subsite I association in the γδ resolvase synaptosome (Rice & Steitz, 1994a; Yang & Steitz, 1995).

The "static subunits" model meets substantial difficulties in explaining the mechanism by which multiple DNA strand rotation events (observed in processive recombination reactions) could occur within the complex, leading to the complex knotted DNA products (Kanaar *et al.*, 1990; Heichman *et al.*, 1991). This would require severe distortion of DNA within the complex (McIlwraith *et al.*, 1997).
Whatever the exact mechanism, the two models predict different arrangements of the catalytic tetramer in the synaptic complex. The determination of the dimer-dimer interaction interface(s) is therefore crucial to distinguish between these two mechanistically distinct recombination models.

1.6 Arrangement of Gin dimers in the catalytic tetramer: a preliminary model

Direct investigation of the regions involved in the stabilisation of the Gin catalytic tetramer that has been performed previously in our group (Rusch, 1998), takes into account the characterised dimerisation surface of the γδ resolvase (Hughes *et al.*, 1990; Murley &

Grindley, 1998). Genetic studies of γδ resolvase showed that the interactions between two αE helices located in the proximity of the catalytic domains of resolvase monomers, stabilise the dimer both in solution and when bound at the cleavage site (Sanderson *et al.,* 1990; Hughes *et al.,* 1993, Yang & Steitz, 1995). Moreover, investigations in the homologous Hin system also indicate that the αE helices form the dimer interface (Lim, 1994; Haykinson *et al.,* 1996). Importantly, mutations in this region of Gin, Hin and Cin invertases yield either recombination deficient or FIS-independent alleles which suggest alterations in the conformation of the dimer (Klippel *et al.,* 1988a; Hafter & Bickle, 1988; Klippel *et al.,* 1993; Haykinson *et al.,* 1996). Furthermore, the dimer interface has been demonstrated to be critical for FIS-mediated activation of strand cleavage (Haykinson *et al.,* 1996; Deufel *et al.,* 1997). These observations strongly suggest that the conformation of the dimer affects the catalytic activity of the invertase tetramer.

During previous experiments in our group several *gin* mutants with increased capability of pairing the *gix* sites were found and analysed both *in vivo* and *in vitro*. Most of these mutants were recombination deficient and all formed tetramers more efficiently than wt Gin. The mutations were mapped on a three-dimensional model of Gin dimer that was obtained by homology modelling based on the crystallographic structure of γδ resolvase, and a Gin tetramer was modelled by defining the optimal contact surfaces between the dimers (Hermann, 1996; Rusch, 1998).

All mutations were located in domains of γδ resolvase that are involved in catalysis (Sanderson *et al.,* 1990; Rice & Steitz, 1994b; Yang & Steitz, 1995). Homology to the γδ resolvase modelling of the Gin dimer three-dimensional structure indicated that one major class of the mutants (F105L, F105S, A110V, E113V, E117V and I119S) was located in the putative dimerisation domain of Gin, whereas another group (T40S, K62R, D92N) was clustered on the exposed surface of the catalytic domain. Such a distribution of the substitutions resulted in a conclusion that the core of the tetramer is formed by a four helix bundle, whereas the second contact domain, comprising the exposed loop between the ß2 sheet and the αB helix (see Fig. 1.7), is probably involved in the stabilisation of the asymmetric interaction between two of the four Gin monomers. The amino acid T40 is located in the loop connecting the ß2 sheet and the αB helix, and may form contacts to basic residues (K50, R51, K54) in the C-terminal region of helix αB in the opposite Gin subunit. Two mutations in this region (K50R and R51H) were identified, which increased *gix*-pairing (tetramer formation) efficiency *in vivo*. Six previously described mutations in

this region (K34E, S36N, R39G, D41G, P43S, P43L) have been shown to suppress the FIS-independent phenotype of the Gin mutant M114V, rendering the double mutants FIS-dependent for recombination (Merker, 1993). It has been therefore suggested that this region is involved in protein-protein interactions between the Gin subunits during synapse (Merker *et al.,* 1993). In addition, several mutants with substitutions in the corresponding region of γδ resolvase have been identified which are defective in cooperative interactions between resolvase dimers during recombination (Hughes *et al.,* 1990).

Taken together these results are consistent with the involvement of both the αE dimerisation helix and the flexible loop between the ß2 sheet and αB helix of Gin in the stabilisation of the Gin tetramer, whereas the Gin dimer itself is stabilised by interactions between the hydrophobic surfaces of the amphipathic αE helices.

Molecular modelling of Gin dimer-dimer interactions (Hermann, 1996) based on the identified domains suggested such a configuration for the Gin tetramer, in which Gin dimers are aligned side-by-side with their DNA binding domains pointed towards opposite directions (Figure 1.10). The asymmetric interactions between the dimers in tetramer suggested an additional "checkpoint" operating at the stage of an alignment of crossover sites in synapse.

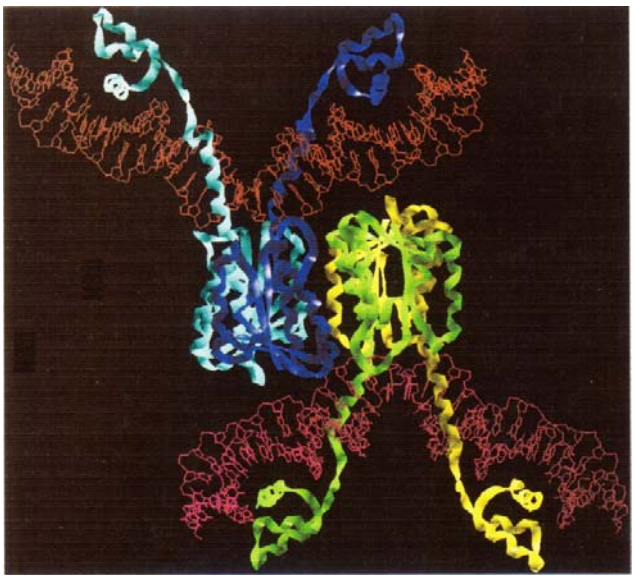


Figure 1.10. Preliminary model of the side-by-side arrangement of Gin tetramer (Rusch, 1998).

Two Gin dimers, depicted one in dark and light blue another in yellow and light green, are complexed with recombination sites on DNA fragments shown as red ribbons.

In the side-by-side model the interactions between the dimers are mostly due to the two patches of charged amino acids located in the outer surfaces of the αE-helices (Figure 1.11). The upper part of the helix is negatively charged whereas the lower part is positively

charged. In fact, the tetramer can be formed by aligning these two patches on the surfaces of the two dimers after rotating one dimer at 180^{o} with respect to another. In this case it is possible to align the positive patch on the one dimer with a negative patch of another. It has been suggested (Rusch, 1998) that in addition to the interactions between hydrophobic residues at the surface of αE, salt bridges between glutamates at positions 113 and 117 and basic residues (H106 and possibly R102) may contribute to tetramer stabilisation. This interaction can be further stabilised by hydrophobic residues that surround these charged patches. Such model of protein-protein interactions correlates with a well-known observation that salt bridges within hydrophobic environment are favourable interactions stabilising protein associations (Rodgers & Sligar, 1991; Xu *et al.,* 1997a, 1997b).

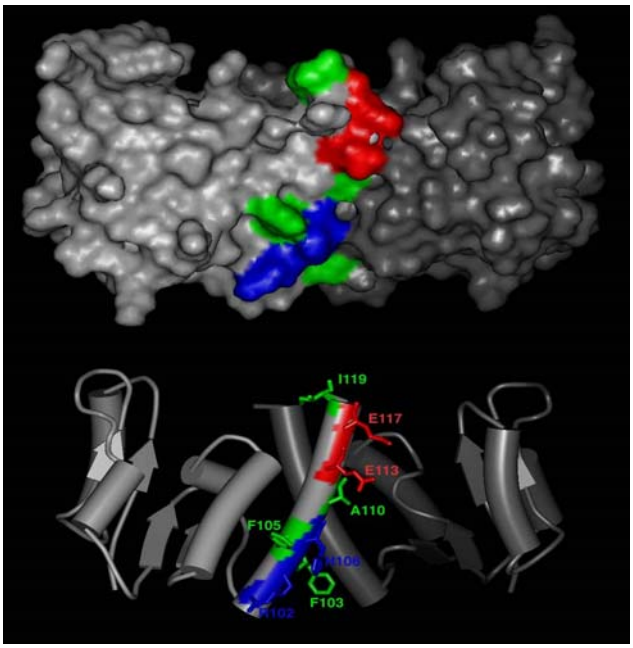


Figure 1.11. Three-dimensional model of the Gin dimer (Hermann, 1996).

GRASP-generated image of the model shows the αE surface electrostatic potential of Gin. Blue indicates positive potential and red indicates negative potential. The hydrophobic residues are shown in green.

The obtained Gin preliminary tetramer model is consistent with the "subunit exchange" model of DNA inversion but also implicates an asymmetry in the interaction between the Gin monomers bound at different *gix* half sites.

Although the "subunit exchange" model nicely accounts for the topological changes observed in the products of recombination, it suggests a simultaneous rotation of cleaved DNA strands with covalently attached invertase monomers (Boocock *et al.,* 1995). Mechanistically, this would require a loosening of contacts between the subunits in dimer and retention of contacts between the monomers of opposite dimers. The extensive contact surface between the opposite monomers in the preliminary Gin tetramer model makes the

retention of such contacts feasible. This is particularly true for the opposed monomers, which form additional contacts by surface exposed flexible loops. However, in this model the catalytic tetramer looses its 4-fold symmetry.

1.7 Modular structure of the recombinases

Another attractive way of studying the arrangement of dimers in a tetramer is the "modular" exchange of regions among enzymes of the same family.

Smith and Thorpe in their recent study (2002) provided an overview of the structural and functional variation in the serine recombinases (Figure 1.12) demonstrating their modular structure. Within this "modules" (regions of conservation) recombinases have a high percentage of homology, indicating their evolutional relatedness.

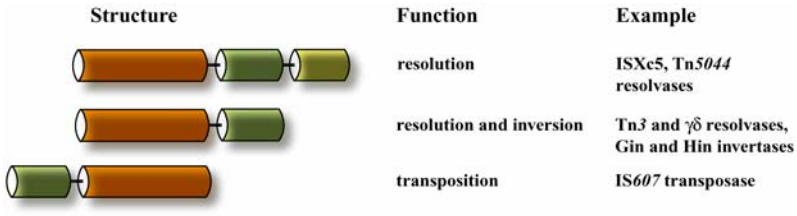


Figure 1.12. Overview of the structural and functional variation in the serine recombinases (modified after Smith & Thorpe, 2002).

The cylinders represent putative domains/regions of conservation of the serine recombinases in which orange is the catalytic domain, green is a DNA binding domain containing an HTH motif and yellow is a conserved region of unknown function.

From phylogenetic analysis of the catalytic domains from 72 serine recombinases they concluded that the structural and evolutionary differences could occur by fusion of an ancestral catalytic domain to unrelated sequences resulting in a family of structurally and functionally diverse proteins.

As it was shown recently (Akopian *et al.,* 2003), catalysis by the N-terminal domain of a hyperactive Tn3 resolvase mutant does not require the presence of its proper C-terminal domain; it can be replaced by the DNA-binding domain of an unrelated protein (in their experiments - a DNA recognition domain from the mouse transcription factor Zif268).

They note, that any specific role for the natural C-terminal domain of resolvase in synapsis of two subsites I, or in catalysis of strand exchange, can now be discounted. The successful substitution of the C-terminal domain with a domain that binds DNA much more tightly (Kim & Pabo, 1998; Abdel-Meguid *et al.,* 1984) also suggests that the mechanism of strand exchange does not require dissociation of the domain from the DNA, as is a feature of some recombination models (Grindley, 2002).

These findings offer an attractive way to study synapse formation by generating modular substitutions between representatives of the different protein families.

1.7.1 The Gin-ISXc5 resolvase chimera

A chimeric protein ISXc5G10 (Schneider *et al.,* 2000) was generated during a collaboration of our group with the group of Dr. P. Dröge (Cologne University) and was studied further in the present work. This chimera contains the residues 1 to 123 of the N-terminal catalytic domain of Gin (corresponding to residues 1 to 126 in γδ resolvase; Yang & Steitz, 1995; see Figure 1.2) including most of the long αE helix that constitutes the dimer interface (Liu *et al.,* 1998). The fusion joined the end of the αE helix of Gin to the flexible arm region and the DNA-binding domain of ISXc5 resolvase (ISXc5 residues 124 to 205, corresponding to residues 127 to 183 in γδ resolvase; see Figure 1.13). About 100 amino acid residues were omitted from the C-terminal extension of the wild-type ISXc5 as these residues proved to be dispensable for resolution activity (Liu *et al.,* 1998).

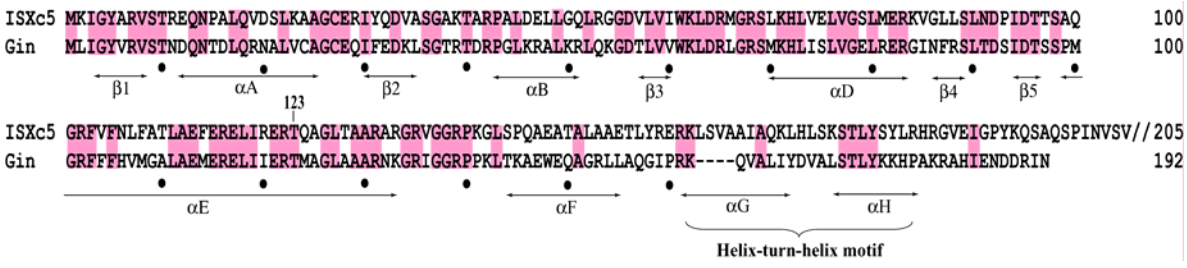


Figure 1.13. Alignment of the amino acid sequences of the ISXc5 resolvase and Gin invertase (Liu *et al.,* 1998).

Site of the fusion (position 123) is indicated. Identical amino acids are shown in red. In the ISXc5 the C-terminal amino acids which were omitted during construction are not shown. The secondary structures are marked underneath the sequence alignment, α for α helix, β for β strand. Also indicated are the α helices that form the helix-turn-helix DNA-binding motif.

Gin, the G10 chimera and the ISXc5 resolvase were all tested on different substrates *in vivo* for their recombination activity (Schneider, 1999; Schneider *et al.,* 2000). It turned out that despite the presence of the large N-terminal catalytic domain of Gin, the G10 chimera was not able to catalyse inversion reaction on the natural Gin substrate containing the *gix* sites either as inverted or direct repeats; it also could not utilise for inversion either the original ISXc5 *res* sites, or the pair of *res* subsites I, presented as direct or inverted repeats. However, the natural substrate for the ISXc5 resolvase could be reproducibly recombined yielding the expected resolution product. Addition of FIS did not affect the resolution reaction by G10 on the substrate containing the recombinational enhancer element (Liu *et al.,* 1998).

However, the substrate containing two *res* subsites I and the recombinational enhancer was efficiently used by Gin for catalysis of inversion reaction in the presence of FIS (Schneider *et al.,* 2000). With this substrate the G10 chimera showed a weak activity, whereas the ISXc5 resolvase was inactive. It was shown in previous studies that the sequence of site I from ISXc5 *res*, where the strand exchange by resolvase occurs, resembles that of the *gix* sites (Liu *et al.,* 1998; Figure 1.14B, C).

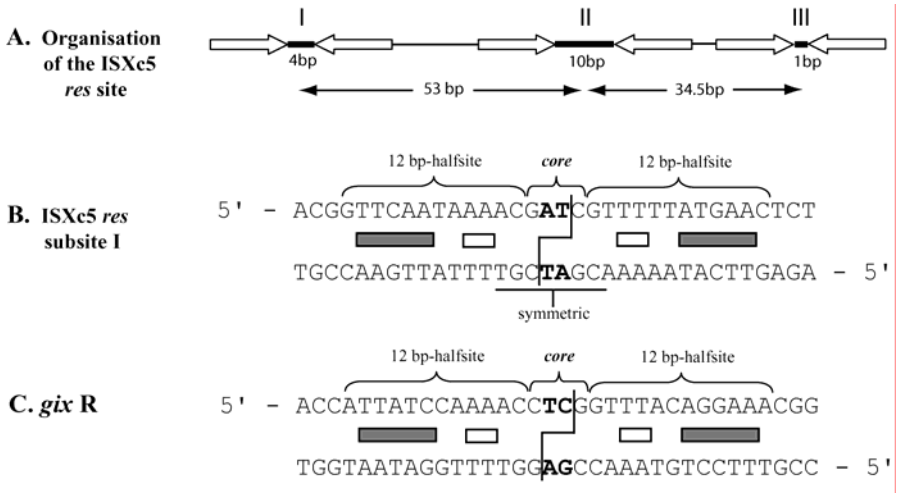


Figure 1.14. Organisation of the *gix* and *res* recombination sites.

A. Schematic representation of the three ISXc5 resolvase binding subsites I, II and III in the *res* recombination site. Each subsite consists of a pair of inverted 12 bp sequences (white arrows) with an internal spacer (black bars) that varies in length for the each subsite (as indicated). The arrows below indicate the different length of the centre-to-centre separation of the subsites.
B. Structure of the ISXc5 *res* subsite I. *Res* subsite contains two inverted 12 bp half-sites separated by the 4 bp symmetric central core region. Regions of the resolvase dimer-DNA interaction are indicated by grey bars for the major groove and by white bars for the minor groove. The enzyme introduces a 2 bp staggered cut in the centre of the symmetric core region indicated by bold letters.
C. Structure of the *gix* R recombination site. Regions of Gin-DNA interactions are indicated as in B. Note that the core region, where Gin introduces a 2 bp staggered cut (indicated in bold), is asymmetric.

Notably, the sequence of *res* subsites I represents an almost perfect palindrome and in contrast to the *gix* sites, the inversion reaction catalysed by Gin on substrate containing *res* subsites I did not depend on their orientation. The efficiency of the inversion reaction was similar to that with a standard substrate containing *gix* sites. As demonstrated earlier with the Hin inversion system (Moskowitz *et al.,* 1991), this result can be explained by the inherent symmetry of the central two A-T base pairs in the core sequence of the *res* subsite I, where the staggered cleavage and strand exchange occurs (Figure 1.14B). This symmetry allows for the formation of a recombinogenic invertasome regardless of the orientation of *res* subsites I in the substrate. Thus, subsites I from ISXc5 *res* can functionally substitute the *gix* sites in the FIS-dependent DNA inversion catalysed by Gin.

Nevertheless, Gin was unable to utilise for recombination the two complete *res* sites (natural target sequences for the ISXc5 resolvase) either as direct or inverted repeats, even in the presence of the enhancer element and FIS. Gin was also unable to recombine the substrate containing a single complete *res* and *res* subsite I oriented as direct repeats. It was shown (Schneider *et al.,* 2000) that Gin recognises both subsites I and III in *res*, but is unable to bind at site II. Sequence comparisons revealed that both I and III subsites exhibit about 53% identity with the *gix* site (Schneider *et al.,* 2000). Furthermore, it appeared that Gin bound at site III interferes with FIS-dependent recombination at sites I. It is possible, therefore, that Gin is unable to catalyse resolution on the substrate containing full *res* site because Gin dimers bound at subsites I and III of *res* interact with each other, or they may compete for interactions with either the dimer bound at the distant *res* subsite I or with the enhancer-bound FIS. In either case, on the substrate containing complete *res* site the Gin dimer bound at the subsite III would act as its own recombinational repressor.

1.8 Goals

A long-standing question concerning the organisation of the synaptic complex formed during site-specific DNA inversion is whether the DNA is on the inside (as suggested by the “static subunits” model) or on the outside (“subunit exchange” model) of the complex. Each model has important implications for strand exchange. A goal of this thesis was to distinguish between these two mechanistically different models using the bacteriophage Mu Gin inversion system. In particular, the aim was to design a strategy proving that the catalytic domains are indeed involved in the formation of the Gin tetramer and to verify the

preliminary model of invertasome proposed earlier in our group by K. Rusch (1998). This would not only help to understand the processes of synapsis and Gin tetramer activation, but also provide a framework for elucidating the mechanism of strand exchange.

Another project was focused on the characterisation of the chimeric recombinase protein ISXc5G10 (containing the N-terminal catalytic domain from Gin and the DNA-binding domain of ISXc5 resolvase). In particular, the aim was to understand the reason for its inversion deficiency, to study the ability of protein to form tetramers and to obtain mutant variants for further illumination of the molecular basis underlying the distinctions between the resolvases and the invertases.

Finally, the question was addressed on the interactions between FIS and Gin during formation of the synaptic complex involved in the initiation of DNA inversion reaction. For this purpose genetic screening was performed for the selection of *fis* mutants that can rescue the impaired recombination activity of a Gin mutant with altered dimerisation interface, Gin H106T. The aim was to confirm that FIS indeed interacts with the dimerisation domain of Gin during tetramer formation and to test whether the position H106 is the site of possible contact.

The ultimate goal of this thesis was to work out a molecular model for the Gin invertase synaptic complex under the consideration of the previous and newly obtained data.

2. RESULTS

Since the invertase Gin is catalytically activated only after the assembly of a synaptic complex containing at least two FIS dimers and the enhancer element, the characterisation of Gin–FIS interactions is crucial for the understanding of the organisation of the invertasome. Thus the analysis of Gin–FIS interactions became the first issue of this study.

2.1 Screening for the Gin H106T activating FIS mutant

It is assumed that there are critical amino acid residues both in Gin and FIS necessary for the formation of the productive invertasome. Their identification can help to understand the interaction between proteins during the recombination reaction and will have implications for the mechanism of inversion. A putative interaction region in FIS was identified in the N-terminus of the protein designated the "mobile β-hairpin arm" (Safo *et al.*, 1997). The region in invertases that is contacted by this flexible N-terminal arm of FIS remains unknown. It was proposed that this region might be constituted by the Gin dimer interface (Merickel *et al.,* 1998). The importance of the dimerisation region was shown also in other related recombination systems (Hughes *et al.,* 1993; Klippel *et al.,* 1993, Haykinson *et al.,* 1996). It was suggested that the mechanism of FIS activation of the DNA cleavage might involve an induced conformational change of the invertase dimer interface that occurs upon the assembly of the invertasome.

To test this hypothesis genetic screening was performed to select for *fis* mutants that can rescue the inversion-deficient phenotype of the mutant Gin H106T carrying a substitution at the dimerisation interface. Position H106 appears to be critical: tyrosine substitution at this position renders the Gin invertase FIS-independent (H106Y), whereas substitution of threonine inactivates Gin (H106T). The analogous mutant of Hin (H107C) is still capable to catalyse FIS-dependent inversion, suggesting that the contact site for FIS is not disrupted by this mutation (Haykinson *et al.,* 1996). Therefore, it was theoretically possible to find a mutant FIS protein, which renders the mutant Gin H106T inversion proficient.

For this purpose mutagenesis of the *fis* gene was performed by error-prone PCR (with a pUHE25-2ΔCm*fis* plasmid as a template) as described (Spee *et al.,* 1993). 2 x 10^6

independent clones were generated. By sequencing it was estimated that about 80% of these carried mutations in *fis*.

The pool of plasmids containing the mutagenised *fis* gene under the control of an IPTG-inducible promoter (pUHE*fis**) was transformed into the tester strain AD1 (CSH50 *fis*::Kan::I^q *oxyR::lacZ*inv) (Deufel *et al.,* 1997). Cells carrying the pUHE*fis** plasmids were then transformed with plasmid pMD3ginH106T carrying the *gin* H106T allele under the control of a temperature sensitive λ_{PL} promoter.

In the AD1 chromosome the *lacZ* gene is flanked by *gix* sites and placed in an "off" orientation with respect to the P_{Cm} promoter. A productive inversion event places the *lacZ* gene in "on" orientation with respect to the P_{Cm} promoter, thus allowing constitutive expression of β-galactosidase (Deufel *et al.,* 1997).

The effect of *fis* mutants was analysed by growing the transformants for 12 hr in the presence of appropriate antibiotics at 28°C on X-Gal plates containing 10 μM IPTG. Gin protein expression was repressed during growth of the cells at 28°C. Shifting the culture to 42°C for 2 hr inactivated the repressor and rapidly led to high levels of protein synthesis. Returning the induced cells to 28°C repressed further recombinase gene transcription while allowing recombination to continue. The recombinational activity was evaluated by the intensity of the blue colour of colonies. Colonies remain white when no inversion occurs, whereas a productive inversion gives rise to blue colonies. From the 900.000 of screened colonies in the mutant pool only one demonstrated the blue phenotype. This phenotype was verified by retransformation of the identified pUHE*fis** plasmid. In the presence of this *fis* mutant allele *lacZ* inversion occurred in 60% of cells. Sequencing revealed that the isolated *fis* mutant carried three mutations leading to amino acid substitutions at positions 3 (E3Q), 6 (V6L) and 14 (S14P).

Previous findings showed that the N-terminal deletion/substitution mutant of FIS Δ4+7 could still promote Gin-mediated inversion, whereas the mutants Δ16 and Δ19 are completely inactive in this process (Spaeny-Dekking *et al.,* 1992). We therefore separated an individual mutation S14P from other mutations in *fis* E3Q/V6L/S14P and tested the *fis* S14P allele alone. The colonies showed the same phenotype as with the triple *fis* mutant, indicating that the S14P mutation alone was sufficient to activate the recombination by GinH106T. When compared the expression levels of the single mutant, triple mutant and wt FIS proteins appeared to be similar (data not shown), suggesting that activation of Gin H106T it is not due to an increased amount of FIS protein.

The *fis* S14P allele was cloned into the pET3a expression plasmid and the 11.5 kilodalton (kDa) FIS S14P-His protein was purified (Figure 2.1). The His-tag at the N-terminus does not interfere the biological activity of the wild type FIS (Merker, 1993).

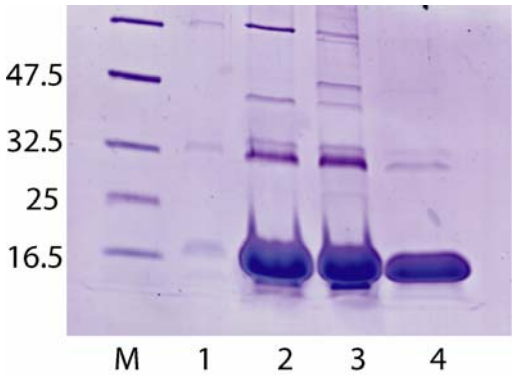


Figure 2.1. Purification of the FIS S14P-His protein.

Proteins were analysed by denaturing polyacrylamide gel electrophoresis and visualised by staining with Coomassie Blue.
M - protein marker (NEB), molecular weight is indicated in kilodaltons (kDa).
lane 1 - aliquot taken during washing step;
lane 2 - elution 1 (with 500 mM imidazole);
lane 3 - elution 2 (with 500 mM imidazole);
lane 4 - elution 3 (with 1 M imidazole).
The concentration of the protein in the last eluate was 5 µg/µl.

2.1.1 DNA binding of FIS S14P

The DNA binding ability of the purified mutant protein FIS S14P was analysed by gel-retardation assay (Fried & Crothers, 1981). For this purpose a radioactively labelled 160 bp PCR fragment comprising three specific FIS binding sites of the upstream activating sequence (UAS) of the *tyr*T promoter was used. FIS forms three distinct complexes with this fragment in a concentration-dependent manner (Lazarus & Travers, 1993). Since the mutant FIS protein contained an N-terminal His-tag, both wt FIS and wt FIS-His were used as controls (Figure 2.2).

Binding of FIS to this DNA fragment generates three complexes of different mobility, corresponding to occupation of one, two and three binding sites of FIS on DNA (complexes F1, F2 and F3, Figure 2.2). The mobility of complexes F2 and F3 (containing two and three of FIS S14P dimers, respectively) was different from that of the wt protein (both with and without the His-tag). The migration of these complexes (but not that of F1) was slower, suggesting that the binding of two or more dimers of the S14P mutant may result in altered DNA bending.

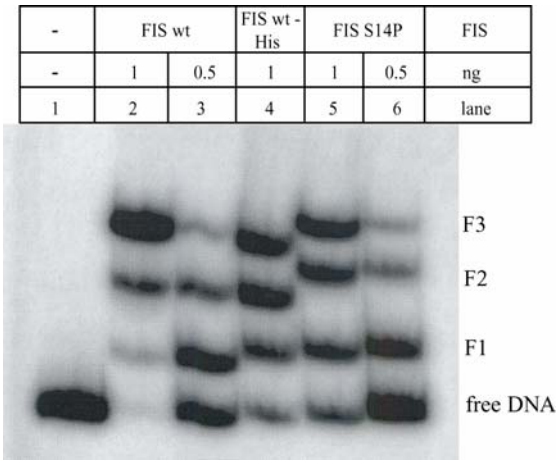


Figure 2.2. Analysis of binding of FIS wt, FIS wt-His and FIS S14P-His proteins to the DNA fragments containing the UAS region of the *tyr*T promoter.

Different concentrations of proteins (as indicated) were incubated with the *tyrT* UAS DNA fragment at 37°C in the binding buffer (see Materials and methods), complexes were separated on a 6% native acrylamide gel and visualised by phosphorimaging.
Lane 1 - free DNA.

Wild-type FIS forms three specific complexes: F1, F2 and F3, corresponding to sequential occupation of three binding sites in the UAS region. Note that the mobility of the F2 and F3 complexes formed by FIS S14P (lanes 5, 6) is slower, than that of complexes with wt FIS proteins (lanes 2-4).

2.1.2 Effect of the FIS S14P on DNA inversion *in vitro*

The stimulatory effect of FIS S14P on inversion catalysed by Gin H106T *in vivo* was also investigated *in vitro*. The supercoiled inversion DNA substrate pAK3 was incubated with different amounts of Gin and FIS in the presence of Mg^{2+}. After the reaction the DNA was cleaved by *Pst*I and analysed by agarose gel electrophoresis to monitor the appearance of a specific band indicative of inversion (Figure 2.3).

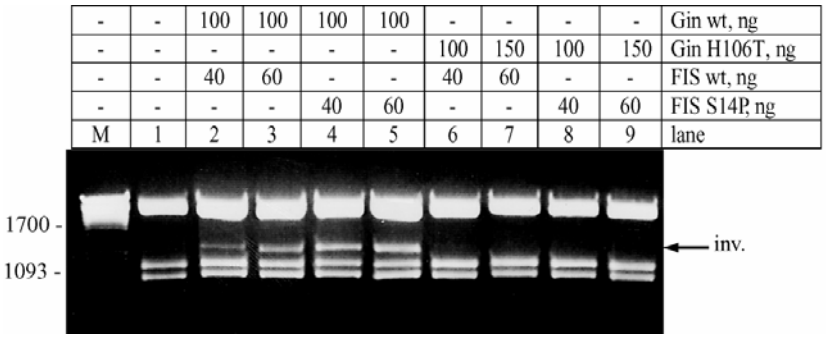


Figure 2.3. Effect of the FIS S14P mutant on a Gin-catalysed inversion *in vitro*.

In vitro inversion reaction was done as described in Materials and methods using pAK3 DNA as a substrate. The DNA was cleaved with *Pst*I and analysed on a 2% agarose gel.
M indicates the λ *Pst*I marker. Lane 1 – control DNA. Arrow indicates the DNA bands that are due to inversion.
Concentrations of the proteins used in the reaction are indicated above each lane.

Measurements of band intensities by using the ImageQuant software showed that purified FIS S14P stimulated inversion by Gin wt up to two-fold, as compared to wild type FIS, but was not able to stimulate inversion by Gin H106T. These observations are consistent with the results of Safo *et al.* (1997) showing that the FIS mutant carrying a substitution at the same position (FIS S14C) had the highest stimulatory effect on Hin inversion among all the obtained FIS mutants.

Thus, although FIS S14P can lead to a 60% productive recombination by Gin H106T *in vivo*, it cannot stimulate inversion by Gin H106T *in vitro*. It was already shown in previous studies that many FIS mutants active *in vivo* are not active *in vitro* and vice versa (Haykinson *et al.,* 1996; Adams *et al.,* 1997). The reason for this discrepancy is not known. It is possible that besides FIS additional factors are required *in vivo* for a productive inversion reaction, which are absent in the *in vitro* reactions. When FIS S14P was tested together with Gin wt *in vivo*, recombination event occurred in 100% of cells (as with FIS wt). The two-fold activation of the wt Gin by the mutant FIS observed *in vitro* cannot be verified *in vivo* since wt FIS already stimulates recombination in 100% of the cells and therefore the increased frequency of inversion is undetectable. Taken together these results are consistent with the importance of the Gin dimerisation surface in mediating the effect of FIS.

2.2 Intermolecular interactions in the Gin invertase catalytic tetramer

As already mentioned in the introduction, Gin binds to its recombination sites as a dimer and becomes catalytically active only after the assembly of the dimers in a tetramer. During previous experiments in our group, several *gin* mutants affected in tetramer formation were detected and analysed both *in vivo* and *in vitro*. Most of these mutants were recombination deficient and all tetramerised more efficiently than wt Gin. The γδ resolvase homology modelling of the three-dimensional Gin dimer structure indicated that one major class of these mutations was located in the putative dimerisation domain of Gin, whereas another group was clustered on the exposed surface loop located between the ß2 sheet and the αB helix of the catalytic domain. This suggested that both the αE helix and the surface exposed loop of Gin are involved in the stabilisation of the Gin tetramer (see Introduction, chapter

1.6). Molecular modelling of Gin dimer-dimer interactions based on the identified domains (Hermann, 1996) suggested a novel configuration for the Gin tetramer, in which the Gin dimers are aligned side-by-side with their DNA binding domains pointing towards opposite directions.

To gain more information on the domain(s) of Gin involved in tetramerisation, cross-linking experiments were performed using a 4Å cross-linker diepoxybutane (DEB) reacting with the NH_2-groups of lysine and SH-groups of cysteine residues. According to previous studies (Spaeny-Dekking *et al.,* 1995b), the lysine residues in the Gin dimer are not close enough to be cross-linked by this reagent.

In order to study the organisation of the Gin tetramer several cysteine substitutions were introduced: L17C (denoted further as 17C), S75C (further denoted as 75C), V107C (denoted 107C), L17C/V107C (denoted 17C/107C) and S75C/V107C (denoted 75C/107C). The positions 17 and 75 were chosen because the preliminary Gin tetramer model predicted a close proximity of the side chains of these residues to their symmetry-related counterparts across a dimer interface. By contrast, the amino acid V107 is involved in the interaction between the monomers in the dimer. Therefore, it was expected that in the double substitution mutants 17C/107C and/or 75C/107C the dimer stabilised by cross-links between 107C-107C could be cross-linked to the opposite dimer via 17C-17C or 75C-75C bridges, yielding a tetramer containing four covalently linked monomers. When assayed by immunoblotting after electrophoresis in SDS-polyacrylamide gels, these species were expected to show an electrophoretic mobility corresponding to a tetramer of Gin (88 kDa), assuming that the molecular weight of Gin is 21.7 kDa (Plasterk *et al.,* 1983b).

The effects of the cysteine substitutions on the inversion proficiency were analysed by transforming the plasmids carrying the *gin* mutants into the inversion tester strain AD1. The efficiency of inversion was monitored by the blue colour of colonies on X-gal indicator plates. Both single substitution mutants Gin 17C and Gin 75C were inversion proficient, whereas Gin 107C was inactive giving rise to white colonies only. Double mutants 17C/107C and 75C/107C were also inactive suggesting that amino acid substitution V107C impairs the recombinational activity of Gin and mutations 17C and 75C cannot rescue this inversion-deficient phenotype. The results obtained *in vivo* were further confirmed by *in vitro* tests (Figure 2.4).

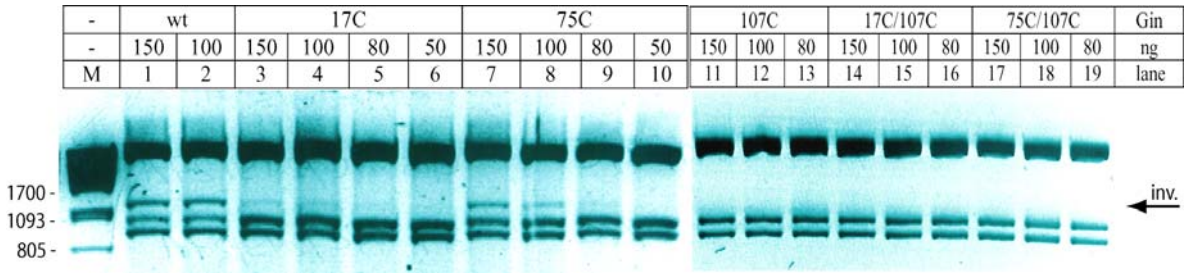


Figure 2.4. Effects of the single cysteine substitutions L17C, S75C, V107C and their combinations L17C/V107C and S75C/V107C on the Gin inversion activity *in vitro*.

Inversion test was performed using the supercoiled pAK3 substrate under standard conditions (see Materials and methods) with 50 ng of FIS protein. DNA was cleaved with *Pst*I and analysed on a 2% agarose gel.
The arrow shows the migration position of the fragments indicative of inversion.
M is a λ *Pst*I marker. Concentrations of proteins are indicated above each lane.
Inversion activity of Gin 17C was reduced (5 fold of the Gin wt activity), but still detectable (lanes 3, 4); activity of a 75C mutant was two-fold less then wt (lanes 7, 8). Gin 107C, 17C/107C and 75C/107C mutants were inversion deficient (lanes 11-19).

Next the cross-linking experiments were performed using the mutant proteins alone or in the presence of substrate DNA and FIS. The reactions were carried out both under reducing (addition of DTT) and oxidising (addition of DEB) conditions. In the case with proteins alone the interactions between the Gin monomers in solution could be detected. In the presence of substrate DNA interactions between the Gin dimers in paired *gix* structures could be detected. In reactions containing Gin, DNA and FIS the interactions in the synapse could be detected.

Incubation of the wt Gin protein with cross-linking agent resulted in a specific product migrating in the gel at the size of the expected dimer (Figure 2.5A). The low level of the Gin wt dimer formation is due to the oxidation of natural cysteine residues C24 and/or C27 (Spaeny-Dekking *et al.,* 1995b). All of the substitution mutants formed covalent dimers in the presence of 50 mM DEB (i.e. under oxidising conditions) independent of DNA and FIS (Figures 2.5B and 2.6). However, the dimer to monomer ratios varied: in the case of wt Gin it was rather low; the highest amount of cross-linked dimer was observed with Gin 107C and double mutants containing this latter mutation. This was expected since the position 107 is situated in the proposed dimerisation surface of the protein. These data are wholly consistent with the results of Lim (1994) and Lee *et al.* (2001) obtained with cysteine substitutions in the dimerisation surface of Hin.

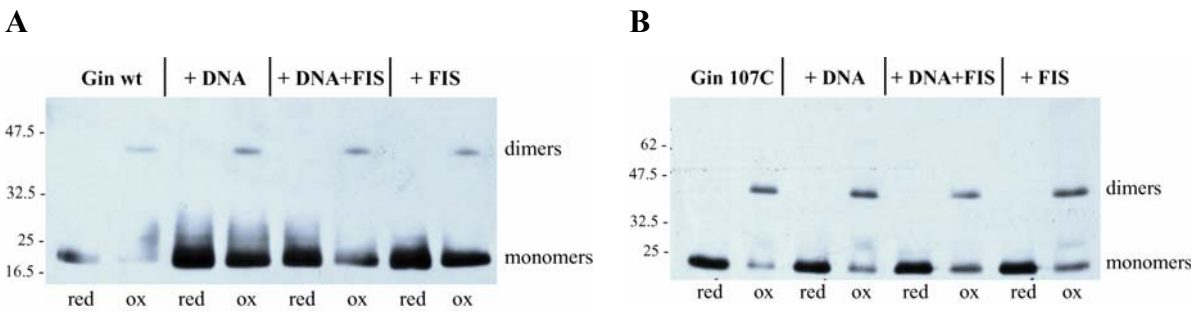


Figure 2.5. SDS-PAGE analyses of the reduced and cross-linked forms of Gin wt (A) and Gin V107C (B).

Proteins were incubated under reducing and oxidising conditions ("red" – 10 mM DTT; "ox" – 50 mM DEB) with or without DNA and FIS and detected by Western analysis using Gin-specific antibodies. The locations of the bands corresponding to the monomeric and dimeric forms of Gin are indicated. The migration of the molecular weight protein marker (not visible on the film) is indicated on the left (in kDa). Note that dimerisation of wt Gin is rather weak, whereas in the case of Gin 107C about half of the protein is in a dimeric form.

As predicted by our model double mutants 17C/107C and 75C/107C were able to form tetramers (88 kDa species) only in the presence of substrate DNA and FIS, suggesting that their formation is due to the interactions between the Gin dimers in the synapse (Figure 2.6C, D). The efficiency of tetramer formation was low, probably due to the transient nature of the synapse.

Unexpectedly also oligomeric bands were detected that migrated as app. 65 kDa species - possibly Gin trimers - formed by double mutants 17C/107C and 75C/107C (Figure 2.6), and less efficiently by single mutant 75C (Figure 2.6A,B) and even less so by 17C (Figure 2.6C). There is no evidence to date that an invertase trimer may be a functionally relevant species. One explanation for the trimer formation would be that the naturally occurring cysteines in Gin (positions 24 and 27) allow trimer formation when combined with single mutants 17C and 75C. The formation of trimers could be facilitated by an increased cross-linking propensity of monomers in the Gin V107C mutant, yet this latter mutant alone did not form trimers (see Figure 2.6C). Notably, although the putative trimers formed more efficiently than the tetramers, their formation was not strictly dependent on the presence of FIS and DNA (compare Figure 2.6A with B).

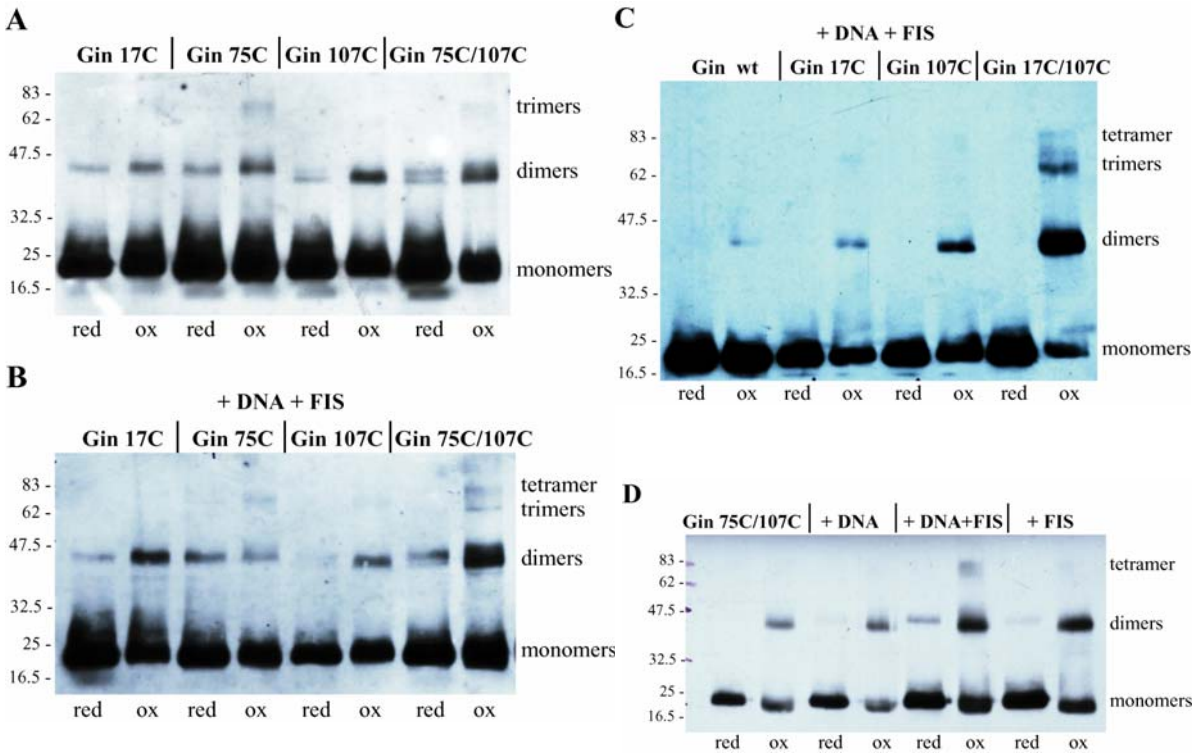


Figure 2.6. Comparison of the cross-linking effect on Gin 17C, 75C, 107C mutants and 17C/107C, 75C/107C double mutants.

Experiment was done *in vitro*; proteins were incubated with or without DNA and FIS, and analysed further by Western blotting using Gin-specific antibodies. Under conditions with 50mM DEB (ox) all mutant proteins were able to form dimers quite efficiently, sometimes even under reducing conditions (**A**, **B**, red). The mutants 17C and 75C alone formed dimers only and a weak band corresponding to the trimer was detectable in the presence of DNA and FIS (**C**). As expected the mutant 107C formed well detectable dimers and no other forms (**A**, **B** and **C**). Note that in the combination of the 107C with the 17C and 75C substitutions the trimers were formed with proteins alone (**A**) but tetramers formed only in the presence of DNA and FIS (**B**, **C)** but not with DNA alone **(D**).

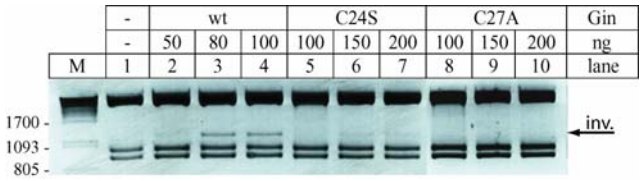
According to Spaeny-Dekking *et al.* (1995b) one or both natural cysteine residues are located at the interface of the Gin dimer, and this places the dimerisation domain to the N-terminal part of the protein. In their experiments, binding of the disulfide-bonded dimers of Gin to a recombination site was strongly reduced, suggesting that the subunits need to reorient in order to stably bind the DNA. In the protein-DNA complex, however, cross-linking of cysteine residues was still possible, indicating that the N-terminal parts of two Gin subunits are also in close proximity when bound to DNA. Therefore, it was necessary to study the role of these natural cysteine residues in the oligomerisation of Gin.

The cysteine residue at position 27 is conserved among all DNA invertases and seems to be important for Gin function because an alanine or tyrosine substitution at this position makes Gin recombination deficient *in vivo* (Spaeny-Dekking *et al.,* 1995b; unpublished data).

Serine substitution at the position 24 leads to the same effect and a mutant carrying substitutions of both cysteines (C24S/C27A) is inversion deficient as well.

Next the Gin mutant proteins carrying C24S and C27A single mutations and the double mutant were purified and tested for their recombination activity *in vitro.* The results obtained were unexpected: Gin C27A was inversion deficient and Gin C24S was able to catalyse a week inversion whereas the double mutant C24S/C27A showed inversion activity comparable to that of the wt Gin (Figure 2.7).

A



B

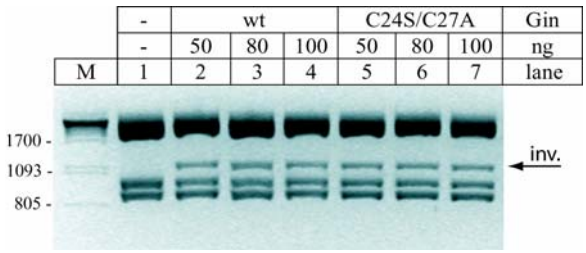


Figure 2.7. Comparison of inversion activity of Gin mutants with single (A) and double (B) substituted natural cysteine residues C24S and C27A.

In vitro inversion reaction was performed with supercoiled pAK3 DNA as a substrate. The DNA was cleaved with *Pst*I and analysed on a 2% agarose gel. Arrows indicate the DNA bands that are due to inversion.
M is λ *Pst*I marker. Lane 1 – control DNA.
Single mutant C24S shows very weak inversion activity at high protein concentration (A: lanes 6, 7) as compared to wt Gin (A: lanes 2-4) and mutant C27A is inactive (A: lanes 8-10). The double mutant C24S/C27A shows inversion activity (B: lanes 5-7) comparable to that of wt Gin (B: lanes 2-4).

The Gin double mutant protein C24S/C27A was analysed further using the cross-linking experiments. The results obtained (Figure 2.8) demonstrated that in the absence of natural cysteine residues Gin C24S/C27A did not produce covalent dimers any more, neither in the absence nor in presence of DNA and FIS. This observation confirms that the covalent dimer formation observed with the wt Gin is due to cross-linking of these natural cysteine residues by DEB (see Figure 2.5A), consistent with the previous report of Spaeny-Dekking *et al.* (1995b).

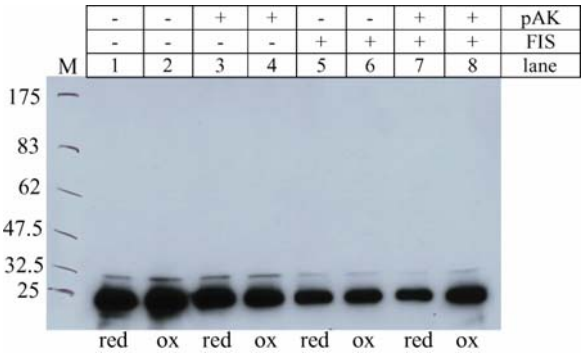


Figure 2.8. Western blot analysis of Gin C24S/C27A cross-linking.

Amount Gin C24S/C27A in the reaction was 100 ng; pAK3 DNA – 500 ng; FIS – 50 ng.
M - protein marker, red – 10 mM DTT, ox – 50 mM DEB.
Gin C24S/C27A cannot be cross-linked under any condition used indicating that the natural cysteine residues are necessary for cross-linking the monomers of wt Gin.

Next the Gin double mutants 17C/107C and 75C/107C carrying in addition the substitutions of the both natural cysteine residues C24S and C27A (this double mutation is referred further as ΔC) were generated, the proteins purified and analysed in cross-linking experiments. The results obtained demonstrated that in the absence of the both natural cysteine residues, the Gin 17C/107CΔC mutant started to form dimers and tetramers more readily, sometimes resulting in slowly migrating multimers (Figure 2.9). However, in contrast to Gin 17C/107C addition of substrate DNA and FIS had no effect on the formation of these complexes by Gin 17C/107CΔC. This latter mutant, as well as the mutant Gin 75C/107CΔC, formed an additional band migrating between the putative trimer and tetramer and independent of the presence of DNA and FIS (designated the ΔC–specific band). In the case of Gin 17C/107CΔC this unspecific band was detectable only under reducing conditions.

In the case of the Gin 75C/107C mutant the putative tetramer was formed in a DNA and FIS-dependent manner but only when the natural cysteines were present. In their absence Gin 75C/107CΔC only formed the ΔC-specific complex both under reducing and oxidising conditions and independent of addition of FIS and substrate DNA. Thus the ΔC double mutant lacking the natural cysteine residues in combination with both either 17C/107Cor 75C/107C mutations demonstrated a loss of ability to form tetramers in a FIS and DNA-dependent way, strongly suggesting that the natural cysteine residues are involved in the interactions between Gin protomers in the synaptic complex.

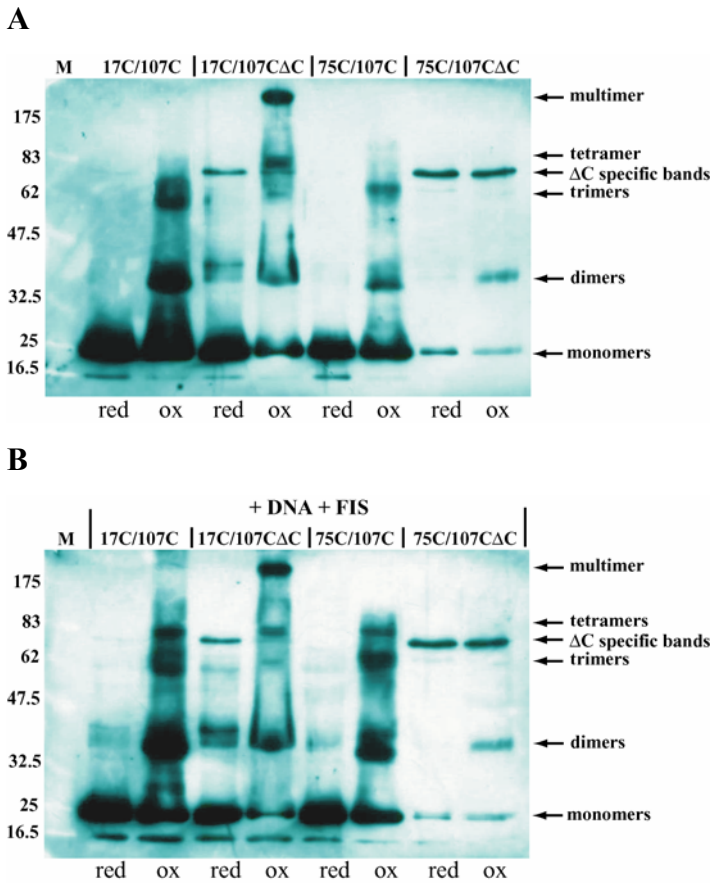


Figure 2.9. Comparison of the cross-linking effects on Gin 17C/107C and 75C/107C mutants with substituted natural cysteine residues (ΔC).

After incubation alone (**A**) and with DNA and FIS (**B**) under standard conditions (se Materials and methods), proteins were analysed by Western blot using Gin-specific antibodies.
M is a protein marker. Molecular weight is indicated in kDa.
Gin 17C/107C and 75C/107C proteins showed dimer and trimer formation when incubated alone and were able to form tetramers when incubated with DNA and FIS, as expected.
Note that in the case of mutant proteins with substituted cysteines additional band appeared, migrating between trimers and tetramers under reducing conditions (indicated by arrow as ΔC specific band). Gin 75C/107CΔC under oxidising conditions also formed dimers and ΔC specific complexes. 17C/107CΔC mutant under oxidising conditions formed dimers and complexes corresponding to the tetramers, but also was multimerised with high efficiency.

2.3 *In vitro* tetramerisation (*gix-gix* paring) assay

To further analyse the importance of the surfaces identified by the cross-linking experiments for the interactions within Gin tetramer, another group of previously characterised Gin tetramerisation mutants (Rusch, 1998) have been studied. The mutation K34E is located on the surface exposed loop between the ß2 sheet and the αB helix of Gin. The Gin K34E mutant was identified as a suppressor of the FIS-independent phenotype of Gin M114V, H106Y and F104V mutants (Merker, 1993), all of which carry the amino acid substitutions in the Gin dimerisation domain. The mechanism by which suppression is achieved relies on an inter- rather than an intramolecular interaction, and in combination these mutations probably affect the structural organisation of the protein molecule in a global way. Data obtained from crystal structure analysis of the γδ resolvase dimer (Yang & Steitz, 1995), when applied to the related Gin (see Figure 2.10) indicate that in Gin the

mutation M114V can affect the interaction of the monomers within the dimer (Sanderson *et al.,* 1990). The mutation K34E and the other identified suppressor mutations for the Gin M114V, for example D41G (Hughes *et al.,* 1990), map to the domain involved in the inter-dimer interactions.

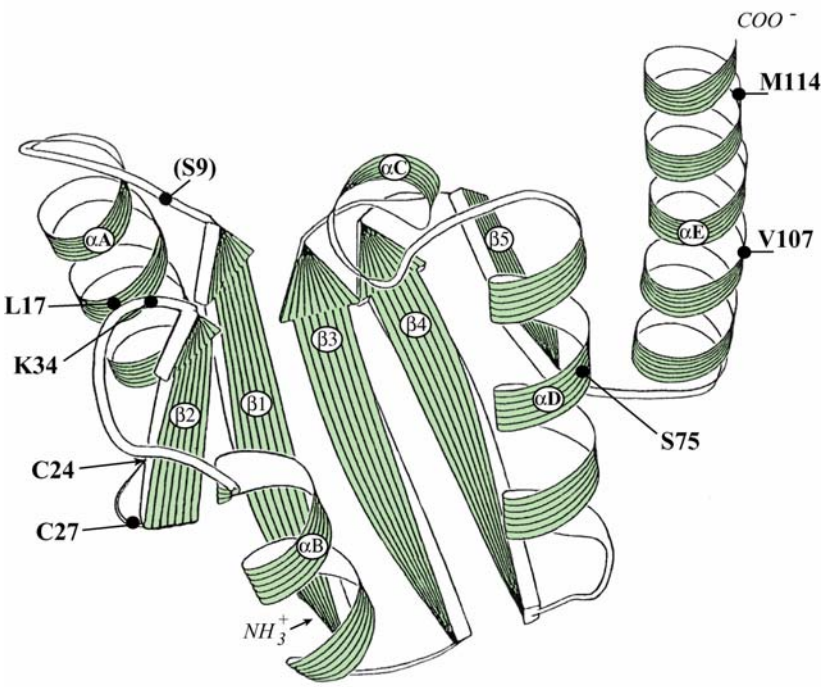


Figure 2.10. Structure of the Gin N-terminal catalytic domain based on identified crystal structure of γδ resolvase (Yang & Steitz, 1995).

Relative orientation of α-helices and β-sheets and the location of the active site residue serine 9 (S9) and studied amino acids are shown.
Amino acid residues analysed in this study are indicated: M114 and V107 are within the dimerisation αE helix; residue L17 is on the αA helix, residues C24 and C27 are located on the loop between αA helix and β2 sheet, K34 – on the flexible loop between β2 and αB helices (surface exposed loop) and S75 – on the αD helix, which is parallel to the αE helix.

Since both the dimerisation domain and the N-terminal surface exposed loop were found to be involved in tetramerisation, single mutants and double mutants, carrying the substitutions in both regions, were compared in an *in vitro* "tetramerisation assay" established by K. Rush (Rush, 1998). In this assay complexes formed by Gin protein were analysed by PAGE using two DNA fragments of different size (156 bp and 44 bp) both carrying a *gix* site, whereby only one of these fragments was radioactively end-labelled by γ-P^{32}. Binding of a Gin dimer to each of these two distinct fragments leads to the formation of complexes of different mobility depending on the fragment size. However, if both the

156 bp and 44 bp DNA fragments are present in the incubation mixture an additional strongly retarded complex appeared. This complex shows identical mobility independent of which of the two *gix* DNA fragments are labelled and thus represented a tetramer species in which the two DNA fragments of distinct lengths are paired by protein interactions between the Gin dimers. In this assay the quantitation of the ratio of tetramer to dimer complexes formed (see Materials and methods) gives an estimate of the tetramerisation efficiency (Rusch, 1998).

The expectation was that the double mutants carrying substitutions in both regions shown to be important for tetramerisation would increase the stability of tetramers containing both DNA fragments with *gix* sites. The tetramerisation assay was modified by adding either the reducing agent DTT or the cross-linking agent DEB to the reactions. These chemicals did not change the nature of the complexes formed, but affected their stability (Figure 2.11). Presence of DTT in the reaction did not affect the dimer complexes but specifically prevented tetramer formation; in contrast, presence of DEB caused stabilisation of the tetramer complexes, most likely by cross-linking the natural cysteine residues of Gin.

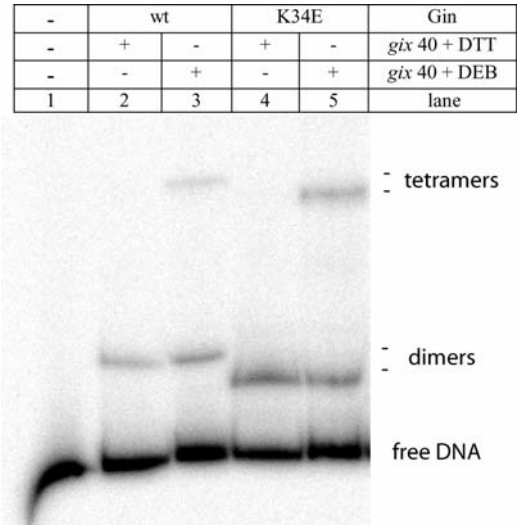


Figure 2.11. Comparison of tetramerisation activities of Gin wt and Gin K34E under conditions with 10 mM DTT and 50 mM DEB.

Lane 1 – 156 bp P^{32}-labelled fragment carrying a *gix* site. Gin wt contains a His-tag, resulting in a different mobility of the dimer and tetramer bands as compared to Gin K34E.
Both proteins formed dimers in the same amount, but K34E mutant was able to tetramerise up to four times more efficiently (lane 5) than wt Gin (lane 3) as quantified by ImageQuant.
Concentration of proteins was 10 ng.

To confirm the previously reported high tetramerisation activity of the K34E (Rush, 1998), the purified Gin wt and Gin K34E proteins were incubated with a radioactively labelled 156 bp DNA fragment carrying the *gix* site under standard binding conditions. Afterwards an excess of the unlabelled 44 bp *gix* DNA fragment was added. Both Gin wt and Gin K34E produce a slower migrating tetramer complex upon binding to both *gix* sites in a native polyacrylamide gel (Figure 2.11). As expected, the tetramers were formed more efficiently with the mutant K34E, than with wt Gin. In this assay no difference was detected between 1

and 5 minutes of incubation time with DEB (Figure 2.12, lanes 5, 6), therefore 1 min of cross-linking was used for all further experiments.

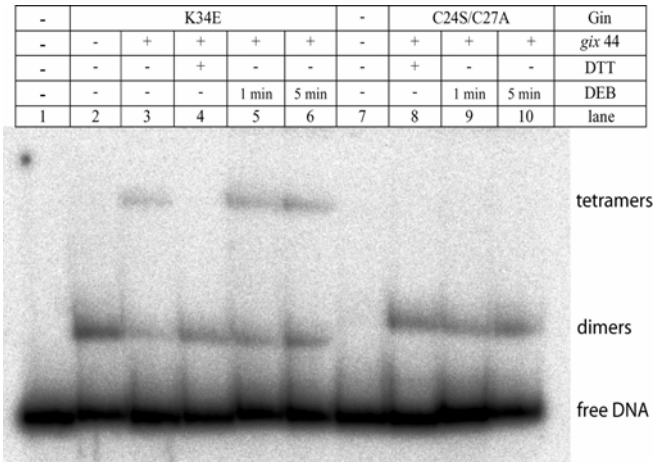


Figure 2.12. Comparison of the tetramerisation activity of Gin K34E and C24S/C27A mutants.

Gin K34E alone readily formed a tetramer between the two DNA fragments (γ-P^{32}-labelled 156 bp fragment and "cold" 44 bp fragment) containing the *gix* sites. The yield of Gin K34E tetramers increased three fold after adding 50 mM DEB in the reaction already after 1 min incubation. Note that His-Gin C24S/C27A does not produce tetramers. With 10 mM DTT both proteins formed dimers (lanes 4 and 8).
Free DNA - lanes 1 and 7. Concentration of each protein was 10 ng.

It was interesting to verify the possible role of the natural cysteine residues in tetramer formation revealed in the previous experiments (see Figure 2.9). With Gin C24S/C27A mutant no tetramer formation was observed under any conditions used, despite of the ability of protein to efficiently form the dimer complexes. This is wholly consistent with the previous observations suggesting that the positions C24 and C27 are involved in the stabilisation of the tetramer.

Gin derivatives carrying single C24S or C27A mutations were also analysed in the tetramerisation assay. Both mutants appeared to be able to form tetramers to different amounts (Figure 2.13), indicating that each of these positions can independently, yet to different extents, contribute to the stabilisation of the tetramer.

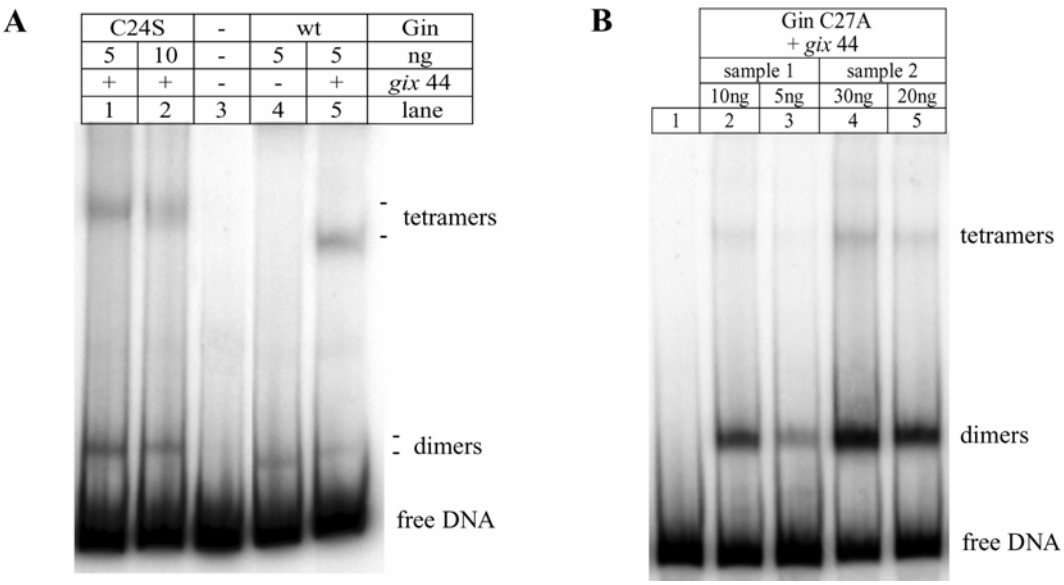


Figure 2.13. Comparison of the tetramerisation ability of Gin wt, His-Gin C24S (A) and His-Gin C27A (B).

Mutant and wt proteins were incubated under standard conditions (see Materials and methods). Gin wt was used as a control. The protein concentrations and the bands corresponding to the dimer and tetramer are marked.
A. Gin wt used in this experiment did not contain a His-tag, which explains a faster migration of complexes formed.
Lane 3 - free γ-P^{32}-labelled 156 bp DNA fragment.
B. For testing Gin C27A two different protein preparations were used (sample 1 and sample 2). In both cases proteins were able to form dimers with a high efficiency, whereas tetramers were barely detectable.
Lane 1- free γ-P^{32}-labelled 156 bp DNA fragment.

Next the effect of the combination of substitutions in the dimerisation surface (M114V) and in the surface exposed loop (K34E) on tetramer formation was investigated. In this experiment Gin M114V alone formed neither dimers nor tetramers, but a complex of intermediate mobility (Figure 2.14A,B: lanes 2, 3, 4). Since Gin M114V has a high DNA bending and nicking activity this unusual migration could be due either to strong bending or to cleavage of the *gix* fragment (Deufel *et al.,* 1997). Gin K34E formed as expected only dimers in the presence of DTT, and both dimers and tetramers in the presence of DEB. The Gin protein with substitutions at both positions (Gin M114V/K34E) formed "usual" dimer and tetramer complexes, alike the Gin K34E. However, by contrast to the latter, the double mutant was able to form tetramers even in the presence of DTT. This indicates that Gin K34E/M114V double mutant formed a stable tetramer that could not be destabilised by DTT. This result is consistent with the involvement of both domains - the dimerisation surface and the surface exposed loop of Gin, in tetramer formation.

A (DTT) **B (DEB)**

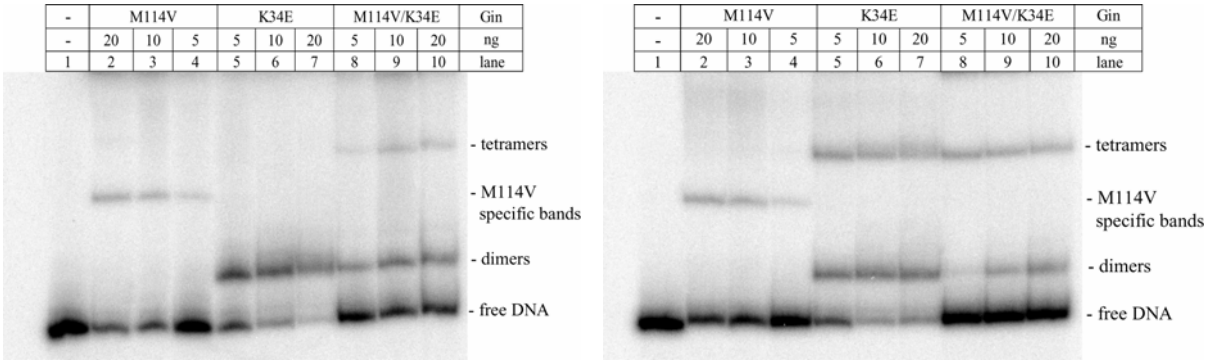


Figure 2.14. Comparison of the tetramerisation ability of Gin M114V, Gin K34E and Gin M114V/K34E mutants *in vitro* under the following conditions:

A (DTT) addition of 10 mM DTT in the reaction;
B (DEB) addition of 50 mM DEB in the reaction.
All experiments were done in the presence of excess of the 44 bp *gix* DNA fragment to allow tetramer formation.
Lane 1 –free γ-P^{32}-labelled 156 bp DNA fragment.
Protein concentrations are indicated above each lane.

Other combinations of substitutions in the dimerisation domain (V107C, M100C) with the mutation K34E were also investigated.

In the absence of DTT and DEB the Gin mutant V107C formed both dimer and tetramer complexes, but only dimers in the presence of DTT (Figure 2.15A, lane 3), whereas with DEB it formed predominantly tetramers with a low amount of dimers (Figure 2.15A, lane 4). Similarly, Gin K34E formed both dimer and tetramer complexes in the absence of DTT and DEB. The Gin K34E dimer complexes formed with similar efficiency under all conditions tested (Figure 2.15A, lanes 5-7; B, lanes 4, 5), whereas the tetramer formation was only slightly facilitated by DEB (Figure 2.15A, lane 7; B, lane 5; C, lane 9) and prevented by DTT (Figure 2.15A, lane 6; B, lane 4; C, lane 8). In the double mutant K34E/V107C, however, the tetramer complex was detected even in the presence of DTT (Figure 2.15B, lane 8), which normally prevents tetramer formation. This indicates therefore, that these two mutations cooperate with each other and stabilise the tetramer, as in the case with K34E/M114V.

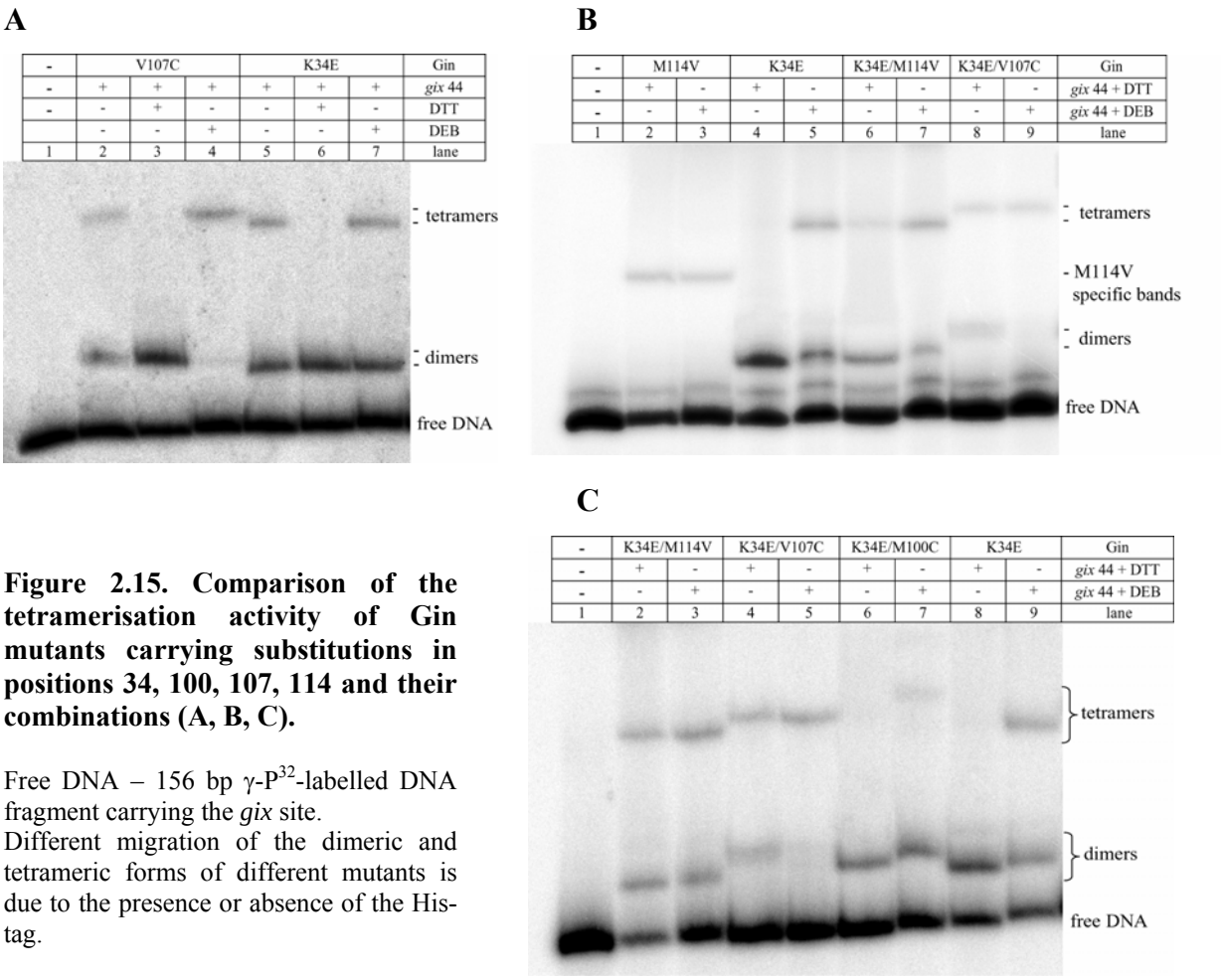


Figure 2.15. Comparison of the tetramerisation activity of Gin mutants carrying substitutions in positions 34, 100, 107, 114 and their combinations (A, B, C).

Free DNA – 156 bp γ-P^{32}-labelled DNA fragment carrying the *gix* site.
Different migration of the dimeric and tetrameric forms of different mutants is due to the presence or absence of the His-tag.

However, using the combination of K34E/M100C mutations the tetramer formation by Gin was not observed when DTT was added (Figure 2.15C, lane 6), and only a weak tetramer was formed when DEB was added (Figure 2.15C, lanes 7). Notably, the position 100 is situated straight at the N-terminal end of the dimerisation αE helix and is probably not involved in the interaction between the monomers.

Taken together these results support the notion that both the αE helix as well as the flexible surface exposed loop between the β2 sheet and αB helix of Gin are involved in the stabilisation of the Gin tetramer.

2.4 Characterisation of the role of the C-terminal DNA binding domain in the synaptic complex formation using the chimeric protein generated by fusion of Gin invertase and ISXc5 resolvase

All the above-mentioned studies aimed at the understanding of the organisation of the synaptic complex by introducing substitutions in the catalytic domain of Gin. The obtained results clearly confirmed the importance of this domain in the formation of the invertasome. However, in the experiments performed in collaboration with Dr. P. Dröge (Cologne University) it became evident that the DNA binding domain also specifically contributes to the formation of the synaptic complex. During this work a number of protein fusions between Gin invertase and the ISXc5 resolvase from *Xantomonas Campestris* has been generated in the laboratory of Dr. Dröge and tested for the recombinational activity (see Figure 2.16). ISXc5 resolvase with a 100 amino acid C-terminal deletion was used (ISXc5ΔC, see Introduction, chapter 1.7.1). The chimeric protein ISXc5G10 consisting of a catalytic domain of invertase Gin and DNA-binding domain of ISXc5 resolvase was most interesting for the future studies (Schneider *et al.,* 2000). It was found that although it contained the entire catalytic domain of Gin invertase, this chimeric protein was no more able to catalyse inversion, but could catalyse deletion, which is a reaction characteristic for resolvases.

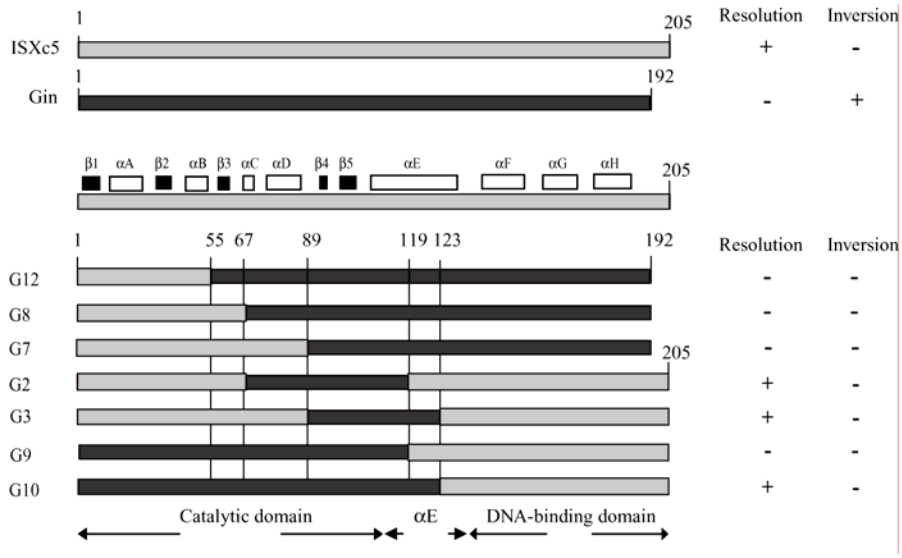


Figure 2.16. Schematic representation of the fusion proteins (modified after Schneider, 1999).

Fusions were done according to the secondary protein structure, identified for the γδ resolvase (Yang & Steitz, 1995). Filled boxes indicate β sheets, empty boxes - α helices. In the mutant G10 the DNA-binding domain of Gin is completely substituted by the corresponding domain of ISXc5 resolvase. This protein is able to catalyse DNA resolution but not inversion reaction.

2.4.1 Mutagenesis of the ISXc5G10 chimera

The fact that *ISXc5*G10 chimera has lost the ability to catalyse inversion indicated that the DNA binding domain is important for determining the directionality of reaction (and probably for the organization of the synaptic complex). This property of the chimera was used for identification of the residues that could be crucial for the determination of the directionality of the reaction. The idea was to test whether by introducing substitutions in the ISXc5 C-terminal DNA binding domain of G10 it would be possible to convert the chimeric resolvase into an invertase.

For this purpose a test system for screening the G10 mutants capable to catalyse inversion has been designed. The reporter plasmid pE3 was constructed by cloning the *lacZ* gene in "off" orientation with respect to the *tyrTD* promoter on a pTyrTD plasmid (Auner *et al.,* 2003). The plasmid also contained the Gin recombinational enhancer element. Since the G10 chimera can bind *res* subsites I but not the *gix* sites (see below), the inversion substrate contained the *lacZ* gene flanked by two ISXc5 *res* subsites I instead of *gix.* In contrast, Gin itself has been previously shown to be able to bind and catalyse inversion on a DNA inversion substrate containing two *res* subsites I (Schneider *et al.,* 2000).

A PCR random mutagenesis of the DNA binding domain of the ISXc5G10 chimera was carried out and the *g10* chimera mutants were cloned in pMD3E (Deufel *et al.,* 1997) under the control of the temperature-sensitive λ_{pL} promoter. The pool of the mutant plasmids was obtained and transformed into the cells containing the reporter plasmid pE3. Transcription of the mutant *g10* chimera genes was induced by heat-inactivation of the λ_{ts} repressor. The wild type allele of the *g10* gene did not yield any blue colonies and served as a negative control, whereas with wt *gin* all colonies were uniformly blue (positive control).

After screening of app. 20000 colonies about 20 mutants were identified, which demonstrated inversion activity comparable to that of wt *gin*. Strikingly, with these mutants after retransformation a great variation in the colour of colonies on individual plates has been observed, whereby the pattern appeared to be hardly reproducible. Therefore, to quantitate the activity of the identified mutants β-galactosidase assays were performed. During these experiments it was discovered that the ISXc5 *res* subsite I can serve as a weak promoter providing a potential -10 element for transcription initiation (sequence is written below). Therefore in these mutants the *lacZ* gene was transcribed constitutively independent of its orientation with respect to the *tyrTD* promoter.

-10

TTTTGCAACGGTTCAATAAAACGATCGTTTT**TATGAA**CTCTGATGGGGCGA

AAAACGTTGCC**AAGTTAT**TTTGCTAGCAAAAATACTTGAGACTACCCCGCT

-10

In the case of efficient inversion catalysed by wild type Gin the influence of this background promoter activity was negligible. In the case of the G10 chimera the binding to the sites was tight enough, to prevent any transcription and so the colonies appeared white. But with mutants having weak DNA binding and weak inversion activity it was not possible to distinguish the inversion-dependent β-galactosidase expression from that driven by the *res* subsite I "promoter".

To avoid the influence of the *res* I subsites on transcription a new inversion substrate p17i has been constructed on the basis of the pTyrTD plasmid. In p17i construct one *res* I subsite was located within *lacZ* as a translational fusion with the *lacZ* ORF. The other *res* I subsite was located at the end of the *lacZ* gene, so that only the 3´-end of the *lacZ* gene was inverted with respect to the promoter (see Materials and methods). In p17i the insertion of the *res* subsite I in frame with the *lacZ* ORF did not affect the β-galactosidase activity, and functional β-galactosidase could be expressed only after productive inversion event between two *res* subsites. Cells carrying the p17i reporter were transformed with the pool of G10 chimera mutant plasmids. Unfortunately, after screening of about 200.000 colonies no inversion proficient ISXc5G10 mutants were found. It is possible, that amino acid substitutions not only in the DNA binding domain but also in other domains of the ISXc5G10 chimera are necessary to restore the inversion activity, for example in the αE helix. Despite the failure to identify inversion proficient mutants, in this part of work an inversion test system was developed where the "artificial promoter" effect of ISXc5 *res* subsite I could have been avoided. This system can be used for further screening of inversion proficient mutants after the random mutagenesis of the whole ISXc5G10 chimera gene.

2.4.2 Cluster substitutions in the G10 chimera's DNA-binding domain

Since no inversion proficient G10 chimera mutants were found after random mutagenesis of the DNA-binding domain, a separate class of mutants of the G10 protein was generated by

extensive substitutions in the DNA-binding domain. Using the PCR-megaprimer method large regions of the chimera's C-terminal domain corresponding to secondary structure elements (α-helices and spacers between them) were substituted by corresponding regions of Gin (Figure 2.17).

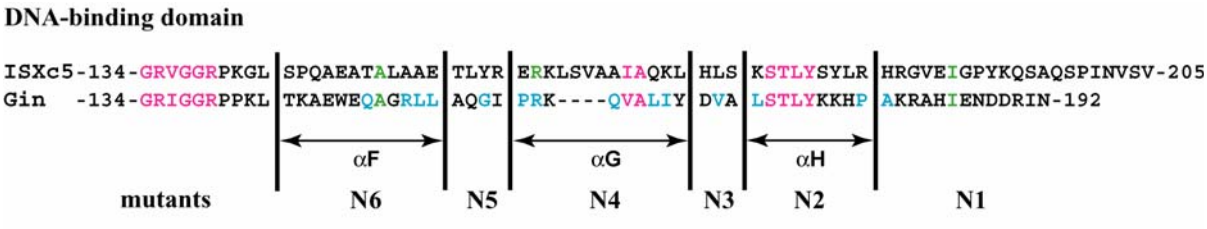


Figure 2.17. Comparison of the amino acid sequences of the DNA-binding domains of ISXc5 resolvase and Gin invertase.

Residues that exhibit a high degree of identity in invertases and resolvases are indicated in red, residues that are identical in resolvases are indicated in blue, and those that are shared between Gin and ISXc5 are shown in green. The secondary structure elements (α helices) observed in the crystal structure of γδ resolvase (Yang & Steitz, 1995) are marked with arrows underneath the sequence alignment. Also indicated are the names of substitution mutants that were generated corresponding to the secondary structure elements.

Mutant genes were cloned into the pMD3E expression vector and plasmids were transformed into the *E. coli* cells containing either the inversion reporter plasmid p17i, or the resolution test plasmid pFres-dir for testing inversion and resolution activity *in vivo*. As positive controls pMD3E plasmids carrying the wt genes either of ISXc5 resolvase and Gin invertase were transformed to the same strains. The effect of the substitution mutations was analysed by growing the transformants in the presence of appropriate antibiotics at 28°C on X-Gal plates for 12 hr and shifting them to 42°C for 2 hr for the inactivation of the transcriptional repressor (see Materials and methods). The inversion activity on the p17i substrate was evaluated by the intensity of the blue colour of colonies; the resolution activity on the pFres-dir was identified by restriction analysis. None of the generated mutants showed either inversion or resolution activity, independent of the extent of the regions that were substituted (summarised in Figure 2.18). These mutants were also not able to utilise the *gix* sites for inversion when transformed into the *E. coli* reporter strain AD1.

In parallel also other mutants of the G10 chimera were generated in which the Gin part of the chimera was extended towards the ISXc5 HTH domain (Chim-135, Chim-145 and Chim-155; Figure 2.18). Unfortunately, when tested for catalysis of inversion or resolution

in vivo, they all did not show any activity. In addition the inversion activity of the Ch162/198 double mutant found as a "false positive" in the previous screening procedure performed with the pE3 reporter plasmid was tested. This double mutant had resolution activity, but as expected was not able to catalyse inversion.

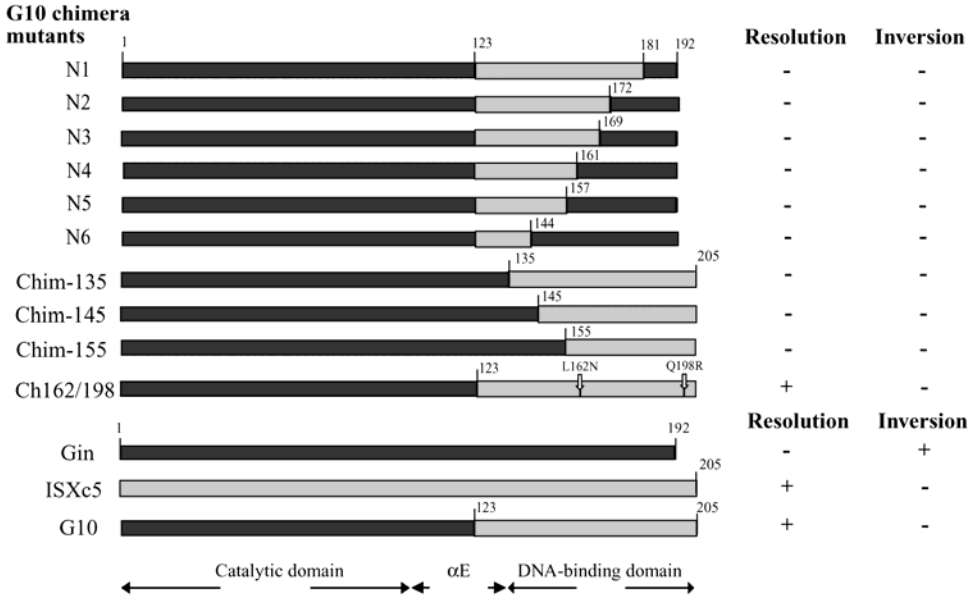


Figure 2.18. *In vivo* recombination activity of the G10 chimera mutants with substitutions in the DNA-binding domain.

Mutants N1-N6 were generated by substitutions of the sequences corresponding to the secondary structure elements: every α helix and spacers between them of G10 Chimera were substituted one by one by the same of Gin.
In the mutants Chim-135, -145 and -155 the Gin N-terminal part was extended from position 123 in G10 to the positions 135, 145 and 155 respectively. Amino acid substitutions in the mutant Ch162/198 are indicated by arrows.

Taken together these results demonstrated that neither random mutagenesis nor large substitutions in the DNA binding domain alone are sufficient to generate an invertase from the G10 chimeric resolvase.

2.4.3 Tetramerisation activity of the G10 chimera

In further experiments dedicated to the analysis of the inversion deficiency of G10 chimera, the emphasis was on the specific influence of the DNA-binding domain which is derived from ISXc5 on the catalytic domain which is derived from Gin. More compellingly, the

tetramerisation properties of the protein were studied. The rationale was that, if the DNA-binding domain of ISXc5G10 has no influence on tetramerisation, then the effects of substitutions in the catalytic domain on the formation of tetramers should be similar to Gin. Therefore, the same substitutions that were found to affect the tetramerisation of Gin were generated in G10 chimera in order to study the interaction between the regions that appeared crucial for tetramer formation.

First of all, the ability of G10 to form tetramers was compared to the Gin wt tetramerisation efficiency (Figure 2.19). Quantitation of the tetramer to dimer ratios by ImageQuant software demonstrated that the G10 chimera formed tetramers with at least two-fold higher efficiency than Gin.

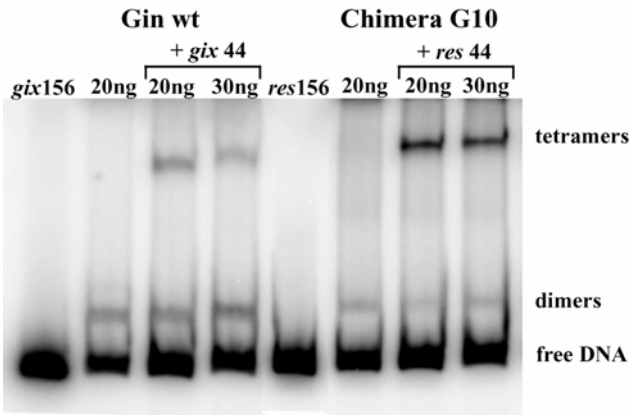


Figure 2.19. Comparison of the tetramerisation efficiency of G10 chimera and wt Gin.

Increasing amounts of proteins were incubated under standard conditions with 156 bp γ-P^{32}-labelled DNA fragments carrying *gix* site or *res* subsite I (as indicated). "Cold" 44 bp fragments containing *gix* site or *res* subsite I were added to the reactions as indicated. Complexes were separated on a 6% native acrylamide gel and visualised by phosphorimaging.

Second, amino acid substitution mutants G10 K34E, G10 V107C, G10 M114V and their double combinations were generated and tested for the recombination activity *in vivo*. None of these mutants were able to catalyse either inversion or resolution on any DNA substrate (either with *gix* or *res* subsites I, data not shown). Despite the remarkable ability of Gin M114V mutant (Klippel *et al.,* 1988a, b) to recombine without requirements for FIS, enhancer and DNA supercoiling, the same mutation in G10 could not make this protein active for the inversion with the substrate p17i.

Next, these mutant proteins were purified and used in the *in vitro* tetramerisation assay (Figure 2.20). When the proteins were incubated without any addition, low levels of dimeric complexes were formed (lanes 3, 6, 9). When incubated in the presence of DEB (lanes 2, 5, 8, 11) all proteins formed complexes that corresponded to the tetramer with high efficiency, while the dimeric forms were barely detectable. In reactions with all tested proteins addition

of DTT stabilised dimeric complexes and prevented the tetramer formation in the case of wt G10 chimera (lane 4) and G10 M114V (lane 12). However, addition of DTT did not prevent the G10 K34E/M114V protein to form tetramer complexes (lane 10), and had almost no effect on tetramer formation by the G10 V107C mutant (lane 7).

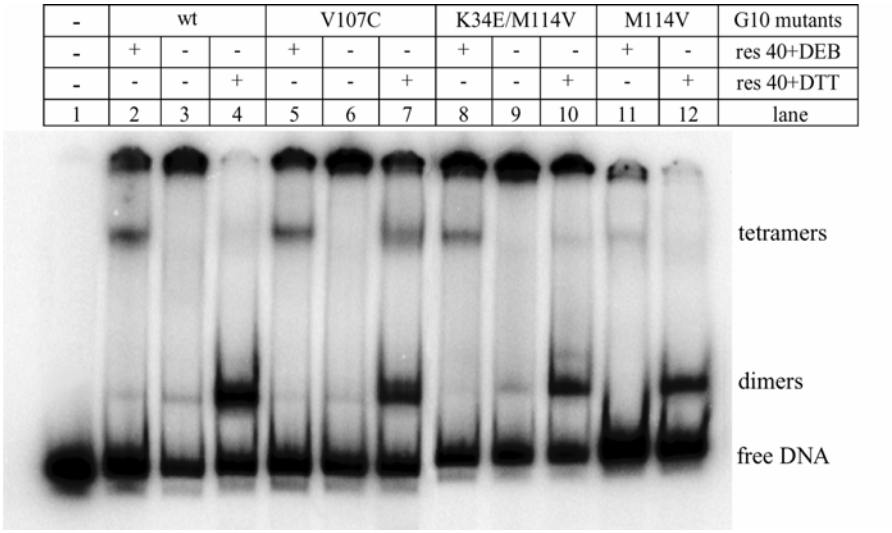


Figure 2.20. Comparison of G10 chimera N-terminal mutants in the tetramerisation assay.

All proteins were incubated with the 156 bp γ-P^{32}-labelled DNA fragment carrying *res* subsite I. Cold 44 bp fragment containing *res* subsite I was added to the reaction mix alone or in the combination with DTT or DEB. Concentration of all proteins was 20 ng.

The obtained results show that although the G10 chimera is characterised by a higher overall tetramerisation capability than Gin, the substitutions that enhance the tetramer formation by Gin have a similar effect in G10 chimera. One significant distinction was the behaviour of the V107C mutation, which can efficiently counteract tetramer destabilisation by DTT in the context of G10, but not in the context of Gin (compare Figure 2.15A, lane 3 and Figure 2.20, lane 7). Since all the generated substitutions were located in the N-terminal catalytic domain of G10 (which is identical to Gin), these results indicate that the observed differences are due to distinct organisation of the DNA-binding domains of Gin and G10 chimera.

2.4.4 Generation and testing of reciprocal chimeras

Since the tetramerisation assays showed distinct effects of the DNA binding domains on the ability of Gin and G10 chimera to form tetramers, several additional chimeric recombinases - designated as reciprocal chimeras (RC) - were generated by fusion of the catalytic domain of ISXc5 resolvase (residues 1 to 123) to a DNA-binding domain of Gin (residues 124 - 192). In the mutants RC 100 and RC 111 the Gin part was extended towards the N-terminal domain (residues 100 to 192 and 111 up to 192 were from Gin, respectively), such that in the RC 100 mutant the entire αE helix originated from Gin, whereas in the RC 111 mutant a part of αE helix (which shows no similarity among invertases and resolvases, see Introduction, Figure 1.2) originated from ISXc5 resolvase. Also mutants with one, two or three additional amino acid insertions at the site of fusion in the RC protein were generated (Figure 2.21: RC-4; RC-2 and RC-9). Finally, since the region 119 - 123 contains just one different amino acid at the position 120 between Gin and ISXc5 resolvase (120R - 120I), correspondingly (see Introduction, Figure 1.13), also a R120I substitution in the RC protein was generated.

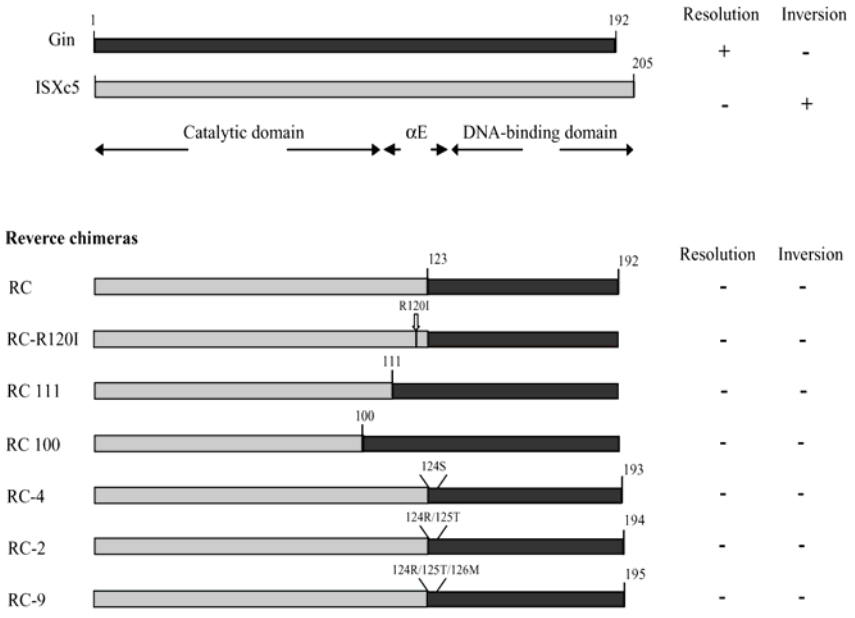


Figure 2.21. Schematic representation of the genes of generated reciprocal chimeras and results of their *in vivo* recombinational activity.

With a standard substrate, resolvase acts efficiently only on full *res* sites oriented as direct repeats contained within the same DNA molecule (Reed, 1981; Krasnow & Cozzarelli,

1983). Since the reciprocal chimera and its derivatives had the catalytic domain from resolvase, it was interesting to test their ability to recombine a substrate containing two full *res* sites. To study the recombinational activity of the reciprocal chimera and its derivatives *in vivo*, the substrates containing ISXc5 full *res* sites either as direct (pFres-dir) or inverted (pFres-inv) repeats, as well as the p17i substrate containing *res* subsites I as inverted repeats, were used.

After transformation of the recombinant pMD3E plasmids together with resolution substrates containing full *res* sites into CSH50, cells were grown for 12 hr at 28°C and shifted to 42°C for 2 hr to activate transcription. Plasmid DNA from single colonies was isolated and resolution was monitored by cleavage of the DNA with *Xmn*I, followed by gel electrophoresis. The specific singly linked catenane products of resolvase reaction are detectable by the appearance of the 3853 bp and 3565 bp product fragments. These products were observed only in reactions with ISXc5 resolvase and G10 chimera and only with the natural pFres-dir substrate (Figure 2.22). None of the mutants did show any activity (data not shown).

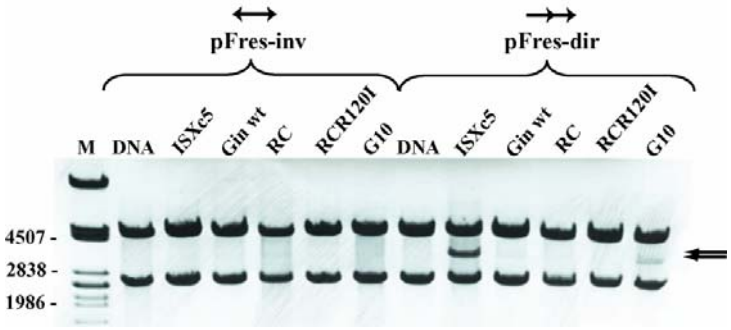


Figure 2.22. Comparison of the *in vivo* resolution activity of different recombination proteins on the substrates containing ISXc5 full *res* sites either as direct (pFres-dir) or as inverted (pFres-inv) repeats.

Fragments corresponding to the 3853 and 3565 bp resolution products are indicated by arrows. The *Xmn*I restriction site in pMD3E is unique resulting in a linearised fragment of ~ 5800 bp. M indicates the λ *Pst*I marker. The test was performed in *E. coli* strains expressing FIS and described in Materials and methods.

In contrast, when the RC proteins were tested *in vivo* employing the inversion substrate p17i carrying two *res* subsites I as inverted repeats, recombination activity was detected only in the case of wt Gin and Gin MV114. Both G10 and RC chimeras, as well as all the reciprocal chimera mutants, were inactive (Figure 2.23).

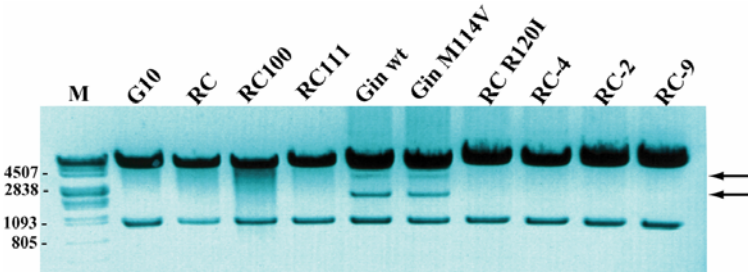


Figure 2.23. Comparison of the *in vivo* inversion activity of the recombination proteins on the substrate containing two ISXc5 *res* subsites I as inverted repeats (p17i).

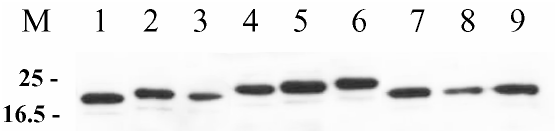
Inversion test was performed in *E. coli* strains expressing FIS and described in Materials and methods. To measure recombination efficiency, the plasmid DNA was digested with *Aat*II restriction enzyme that distinguished the orientation of the invertible segment and subjected to agarose gel electrophoresis. *Aat*II restriction site in pMD3E is unique resulting in a linearised fragment of ~ 5800 bp.
Arrows indicate fragments corresponding to the 4252 and 2063 bp inversion products.
M indicates the λ *Pst*I marker.

Taken together these results show that by substitution of the DNA-binding domain in ISXc5 resolvase by the same domain of Gin it was not possible to generate mutants that are able to catalyse either resolution or inversion.

It was recently shown that a functional resolvase with a designed DNA sequence recognition can be created by fusing the catalytic domain of a hyperactive mutant of Tn3 resolvase to a DNA recognition domain from the mouse transcription factor by a short linker peptide (Akopian *et al.*, 2003; see Introduction, chapter 1.7). This indicates that the flexible region between the catalytic and the DNA binding can play a crucial role in the organisation of a productive synaptic complex.

Therefore, in the next approach to confer more flexibility to the protein, linkers of different length (10, 14, 16 amino acids) were introduced between the N- terminal and C-terminal domains at the positions 124 and 125 (into a modified site for *Bsi*WI restriction endonuclease) of G10 chimera and RC chimera genes, respectively. All these linker mutant proteins were tested *in vivo* for recombination activity on substrates for inversion or resolution. However, also these mutants appeared to be inactive in recombination (data not shown).

Notably, none of these insertions resulted in a reduced expression of the mutant proteins as judged from a Western blot analysis of extracts derived from the *E. coli* strains containing plasmids with different mutant genes (Figure 2.24). All the mutant proteins were present in the cells in roughly similar amounts, comparable to Gin, G10 and RC. This indicates that the proteins were not destabilised as a result of the linker insertions.



1. Gin wt
2. G10 chimera
3. RC rev.chimera
4. G10 + 10
5. G10 + 14
6. G10 + 16
7. RC + 10
8. RC + 14
9. RC + 16

Figure 2.24. Western blot analysis of the linker mutant's protein expression level.

Cell extracts were loaded on a 15% SDS-PAA gel and analysed by Western blot using Gin antibodies. RC proteins are presented in a less extent, which can be due to the absence of some antigens determinants in the N-terminal part of the protein, which is from resolvase.
M is a protein marker.

2.5 Interaction of the catalytic domains during tetramer formation

2.5.1 Characterisation of the DNA binding properties of the recombination proteins

According to the "preliminary model" (Rusch, 1998) Gin dimers are interacting via their catalytic domains during a tetramer formation, whereas the same interactions for the invertase Hin are suggested to involve DNA-binding domains (Merickel *et al.,* 1998). To distinguish between these two different organisations of the synaptic complex a new strategy of studying the interacting surfaces was designed. Since G10 chimera consists of a catalytic domain of Gin and a DNA-binding domain of ISXc5 resolvase, it is possible to directly test which of the protein domains are involved in the interaction during formation of a synaptic complex. In short, the ability of a Gin dimer to form a tetramer with a dimer G10 chimera was investigated. As a necessary control for this experiment first the DNA-binding properties of the proteins and in particular, their specificity of recognition of the *gix* and the *res* sites was analysed. In addition, to avoid "false positives" due to formation of Gin-G10 heterodimers, the ability of Gin and G10 chimera to form heterodimers was analysed using both the *gix* and the *res* sites.

2.5.1.1 Binding of the proteins to the *gix/res* sites of different lengths

First, each protein was tested for the *gix/res* binding specificity using the γ-P^{32}-labelled DNA fragments of different lengths (156 bp and 44 bp). Gin and Gin-His proteins were promiscuous, capable to bind both the *gix* and *res* sites on 156 bp DNA fragments. The RC chimera, which contains the Gin DNA binding domain, was binding to both sites as well. In contrast, the G10 chimera and ISXc5 resolvase efficiently bound *res* subsites I but were not able to bind *gix* sites, as expected (Figure 2.25).

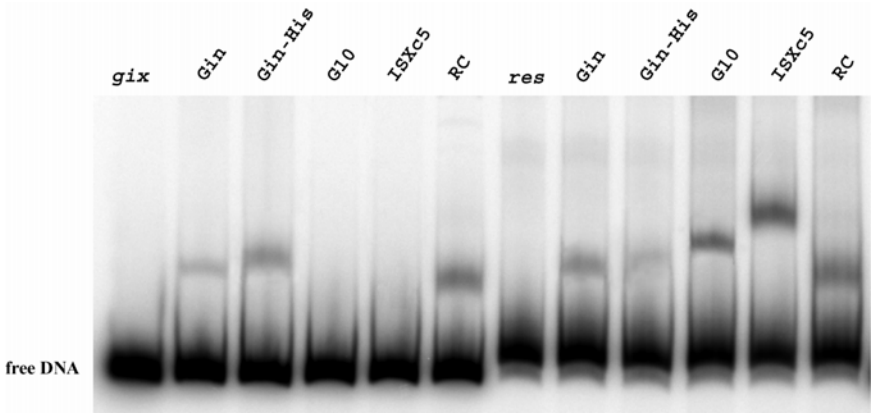


Figure 2.25. Binding of the recombinase proteins to the long fragments containing *gix* or *res* sites.

156 bp γ-P^{32}-labelled fragments carrying *gix* or *res* sites (as indicated) were used. Incubation of the fragments with different proteins resulted in the formation of protein-DNA complexes of different mobility dependent on protein size.

Similarly, when tested with the short 44 bp DNA fragments, neither G10 chimera nor ISXc5 resolvase showed binding to the *gix* DNA fragment, but both were able to bind the *res*-subsite I DNA fragments with similar efficiency (Figure 2.26). Gin again was able to bind both the *gix* and the *res* sites.

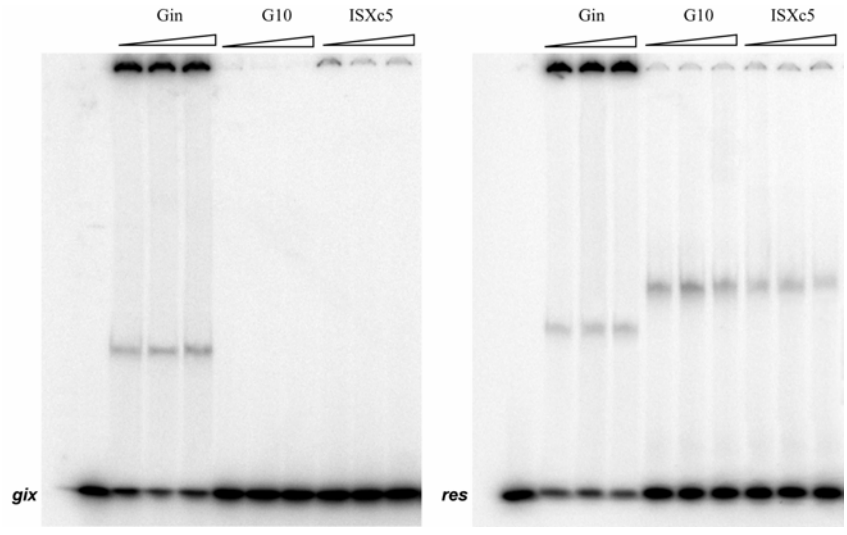


Figure 2.26. Binding of the recombination proteins to the short fragments containing *gix* or *res* sites.

44 bp γ-P^{32}-labelled DNA fragments carrying *gix* or *res* sites (as indicated) were used. The incubation of the DNA fragment containing *res* subsite I with increasing amount of purified proteins resulted in the formation of protein-DNA complexes of different mobility depending on protein size.

2.5.1.2 Interactions with ISXc5 *res* subsite I

Next the binding of proteins to their specific sites were analysed in more detail using the DNase I footprinting. The complexes formed on binding of Gin, G10 chimera, ISXc5 resolvase and RC reverse chimera to the 156 bp DNA fragment containing *res* subsite I and Gin and RC proteins bound to the 156 bp DNA fragments containing *gix* sites were investigated. Both fragments were uniquely radioactively labelled at one end.

With the radioactively labelled *res* subsite I fragment regions that were protected from DNase I cleavage, or showing increased cleavage were observed, indicating enhanced occupation of the *res* site with increasing concentrations of ISXc5 and G10 proteins (Figure 2.27A, lanes 4, 5; B, lanes 4, 11).

Although *res* subsites I can be efficiently used by Gin for catalysis of inversion (Schneider *et al.,* 2000), the DNase I protection pattern by Gin indicates a weak interaction with the *res* site. Similar result was obtained with RC protein (Figure 2.27A, lanes 2, 3 and 6, 7; B, lanes 1-3 and 12-14).

The obtained results suggest that with *res* subsites I the nature of the protein-DNA interactions in the case of resolvase or G10 is different from that of Gin and RC. Strong DNase I hypersensitivities induced by ISXc5 and G10 suggest that the binding of proteins induces a bend in the *res* sequence, as observed for the interactions of Gin M114V with the *gix* recombination site (Mertens *et al.,* 1988).

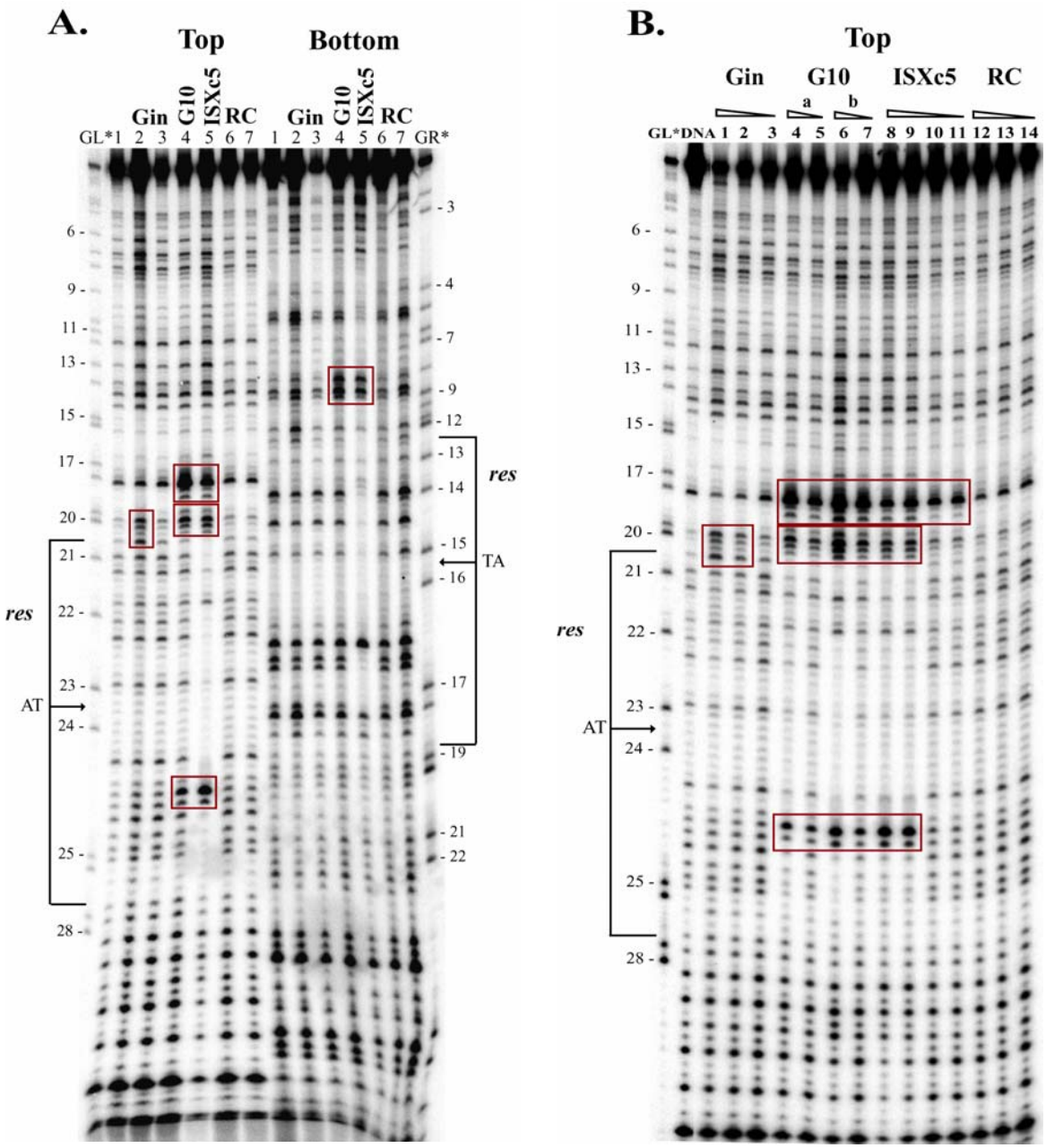


Figure 2.27. DNase I protection assay of the Gin, G10 chimera, ISXc5 resolvase and RC chimera binding to ISXc5 *res* subsite I.

A. A linear DNA fragment containing ISXc5 *res* subsite I was 5'-labelled by γ-P^{32}ATP either on the top or bottom strand (corresponding Maxam-Gilbert G-ladders are indicated as G-L* and G-R*). Proteins were used in following concentrations: Gin: 200-100 ng (lanes 2, 3); G10: 200 ng (lane 4); ISXc5: 200 ng (lane 5); RC: 200-100 ng (lanes 6, 7) with 1 ng of the *res*-containing DNA fragment. Lane 1 – free DNA. *Res* subsites I are indicated by vertical lines; arrows in the *res* site point to the central 2 bp where strand cleavage and exchange occurs (AT; TA). Regions of hypersensitivity are marked by the red squares and are completely similar in the case of G10 and ISXc5.

B. The DNase I cleavage protection patterns of different concentrations of purified Gin (200-150-100 ng), G10 chimera obtained from two independent protein preparations (a - 200-150 ng; b – 250-200 ng), ISXc5 resolvase (250-200-150-100 ng) and RC reverse chimera (250-200-150 ng). The top strand of the DNA fragment was radioactively labelled. Lane marked as DNA is free DNA without protein.

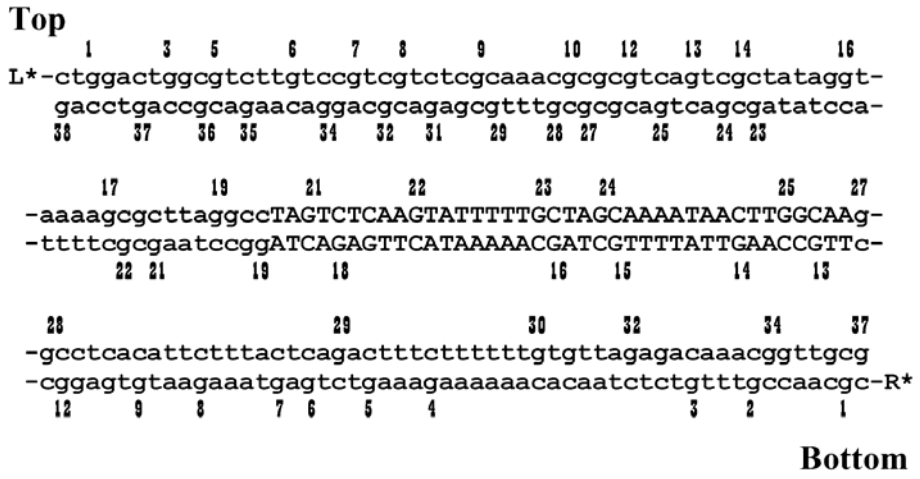


Figure 2.28. Sequence of the DNA fragment containing ISXc5 *res* subsite I used for the DNase I footprinting assay.

The sequence of the 38 bp *res* subsite I is written in capitals. Numbers above and below the sequence indicate the positions of G´s in the G-ladder and correspond to the positions of bases on the top and bottom strands in the footprints shown in Figure 2.27.

2.5.1.3. Interactions with the *gix* site

We also compared the DNA binding properties of the Gin and RC proteins to the fragments carrying *gix* sites by DNase I footprinting assay. Since it was already shown that neither ISXc5 resolvase nor G10 chimera could bind the *gix* site, these proteins were not used in this test. The footprinting results demonstrated that Gin at high concentrations protected the *gix* site, most noticeable on the bottom strand (Figure 2.29, lane 9). In contrast the RC chimera did not show any significant protection at any concentration used, despite the fact that the protein was able to bind the *gix* site in band-shift experiments (see Figure 2.25). The conditions for protein binding in these two assays are quite different, but nevertheless the presence of the ISXc5 catalytic domain in the RC chimera could impair the DNA binding capability of the Gin HTH domain. This may also explain the inability to generate any recombination-proficient RC mutants.

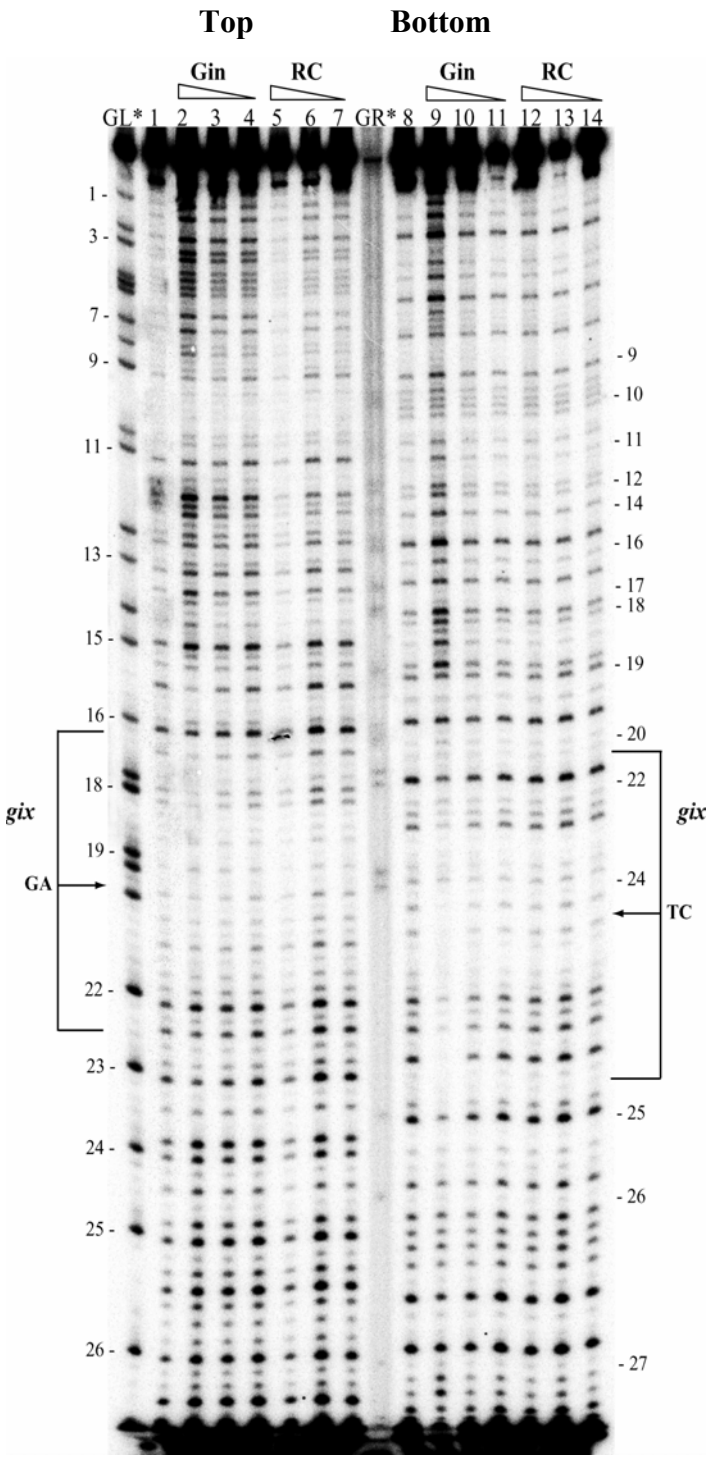


Figure 2.29. DNase I footprinting analysis of Gin- and RC-*gix* interactions.

Increasing amounts of proteins were incubated with a γ-P^{32}ATP uniquely 5'-labelled PCR fragment containing the *gix* site. After DNase I treatment the DNA was loaded on a denaturing polyacrylamide gel and visualised by phosphorimaging. The lanes marked GL and GR are Maxam-Gilbert G-ladders for the top and bottom strands, respectively.
Lanes 1 and 8 – free DNA. Lanes 2-4 and 9-11, decreasing amounts of Gin ranging from 200 to 50 ng.
Lanes 5-7 and 12-14 – decreasing amount of RC ranging from 300 to 100 ng.

Top - top strand, bottom - bottom strand labelling. *Gix* sites are marked with vertical lines; the region where cleavage takes place is indicated by arrows.

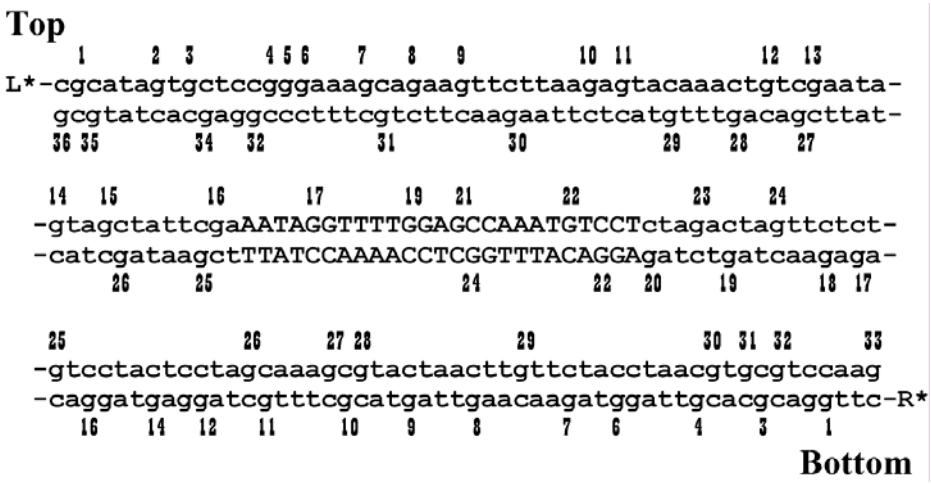


Figure 2.30. The nucleotide sequence of the 156 bp DNA fragment containing the 25 bp *gix* site used in the footprinting experiment.

The sequence of the 25 bp *res* subsite I is written in capitals. Indications are as in Figure 2.28.

2.5.2 Formation of heterodimers

In the next step it was necessary to test a possibility of heterodimer formation between Gin and the G10 chimera. It was shown previously that the Gin dimer could be formed on the DNA by combining different monomers (for example, one monomer containing a *myc*-tag and another without). This leads to differences in the mobility of the dimers in band-shift experiments: the dimers consisting of two Gin monomers with *myc*-tags migrate slower, than the dimers of Gin monomers without *myc*-tags, whereas the heterodimers demonstrate an intermediate mobility (Merker, 1993; Figure 2.31).

Figure 2.31. Scheme of a heterodimer formation in a band-shift experiment.

When the two proteins, one of which is tagged, and the other not, are mixed in solution prior to incubation with the *gix* DNA, three complexes could be observed. The distribution of these complexes shows a ratio of 2:1:2. The upper complex corresponds to the bound dimer of the tagged Gin variant, the lower to the dimer of untagged Gin and the intermediate complex to the heterodimer of both molecules bound to the *gix* site.

Either Gin and Gin-His, or Gin and ISXc5G10 proteins were mixed in different ratios and incubated with a radioactively labelled 44 bp DNA fragment carrying a *res* subsite under binding conditions. Both Gin-His and G10 chimera alone formed a distinctively slower migrating protein-DNA complex in comparison to Gin wt (Figure 2.32A). At certain concentrations of Gin and Gin-His heterodimers containing both proteins were formed. When G10 chimera and Gin wt proteins were mixed and incubated at different concentrations, heterodimers could not be detected, indicating that dimerisation of monomers of Gin and G10 upon binding to the *res* was not possible.

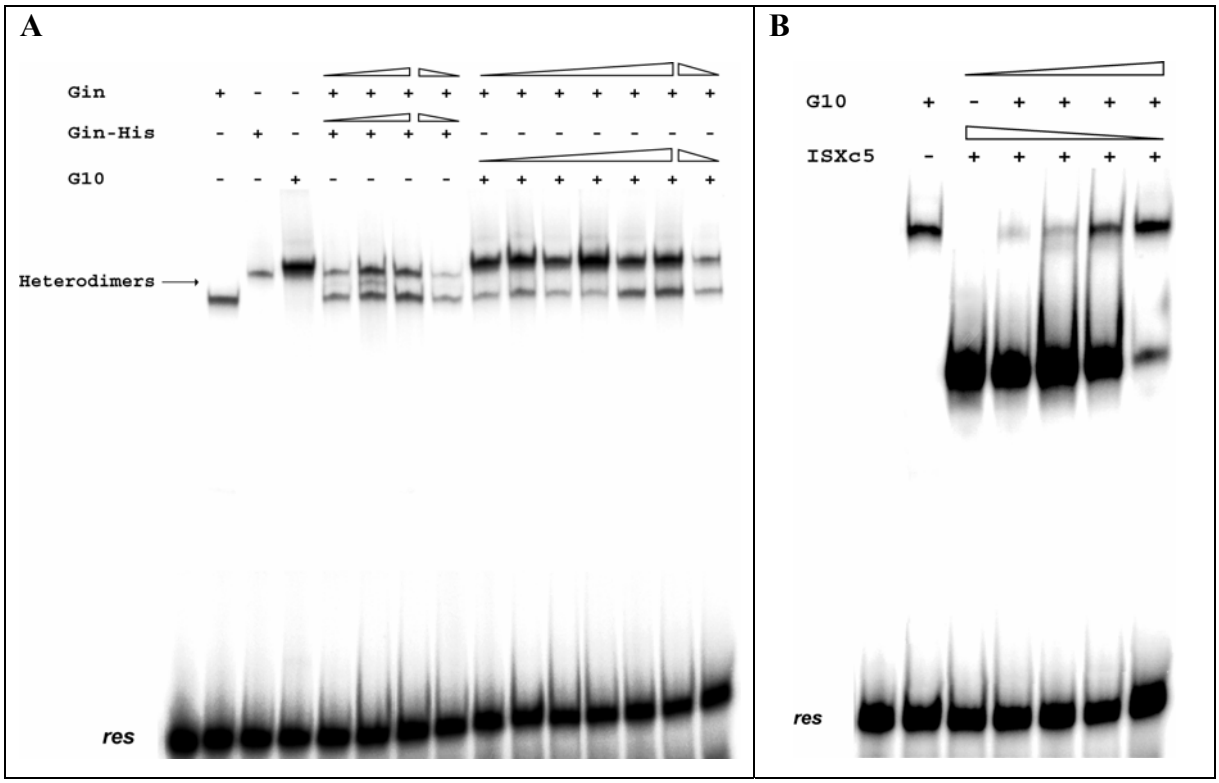


Figure 2.32. Correlation of the heterodimers formation: (A) between Gin wt/Gin-His and Gin wt/G10 chimera; (B) between G10 chimera and ISXc5 resolvase.

Protein/DNA complexes were analysed using a 44 bp 5'-labelled DNA fragment containing ISXc5 *res* subsite I. Amounts of proteins were 4 ng – 50 ng. Complexes were subsequently analysed by electrophoresis through a 5% non-denaturing polyacrylamide gel. Heterodimer formation occurred only with Gin wt/Gin-His. No heterodimers formation between ISXc5 resolvase and G10 chimera was observed. The position of the unbound DNA fragment is indicated (*res*).

2.5.3 Interaction between catalytic domains – the "beads experiment"

After all these control experiments have been carried out, the nature of the interacting domains of G10 chimera and Gin were directly tested in an experiment using the *gix* DNA fragments immobilised on streptavidin-covered magnetic beads. The scheme of the experiment is presented in Figure 2.33.

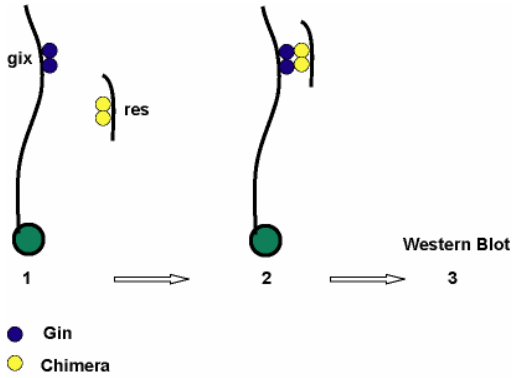


Figure 2.33. Schematic representation of the "beads experiment".

For the experiment short 44 bp DNA fragments carrying an ISXc5 *res* subsite I and long 156 bp biotinylated DNA fragments containing a *gix* site were used. The 156 bp *gix* fragments were immobilised on the streptavidin beads and incubated with Gin protein. Afterwards His-tagged G10 chimera or ISXc5 resolvase proteins were added, each preincubated with an excess of the short 44 bp *res*-fragment.

After incubation followed by extraction of unbound material, complexes could be obtained containing tetramers. The composition of the tetramers could be analysed by Western blotting using His-tag-specific antibodies.

The experiment was based on the fact that neither G10 chimera, nor ISXc5 resolvase can bind the *gix* site (chapter 2.5.1.1), therefore the question was whether they can be attached to the immobilised DNA containing the *gix* site via interactions with a Gin dimer prebound at the *gix* site.

Both purified G10 chimera and ISXc5 resolvase contained His-tags and therefore could be recognised by His-specific antibodies. The Gin protein used in this experiment did not contain any tag. However, as a positive control for this experiment also a Gin protein with a His-tag was used. Biotinylated 156 bp DNA fragments containing *gix* site were immobilised on the streptavidin coated magnetic beads and incubated with Gin protein in the binding buffer at 37^{o}C for 10 min followed by 3 washing steps to remove unbound protein. As a result stoichiometric complexes were obtained where one Gin dimer was bound to the *gix* site of each fragment. Then G10 chimera and ISXc5 resolvase proteins each presaturated with the 44 bp DNA fragment carrying the ISXc5 *res* subsite I were added. After incubation at 37^{o}C for 30 min the supernatants were removed and the beads were washed 3 times in order to remove all unbound protein and 44 bp *res* DNA. In the next step the complexes were released from the beads by incubation of the samples at the

95°C for 5 min in the buffer containing SDS and β-mercaptoethanol. The beads were separated from the reaction mix by short centrifugation. The supernatants obtained were loaded on a 15% protein SDS gel and proteins were analysed after Western blotting using the His-tag-specific antibodies (Figure 2.34).

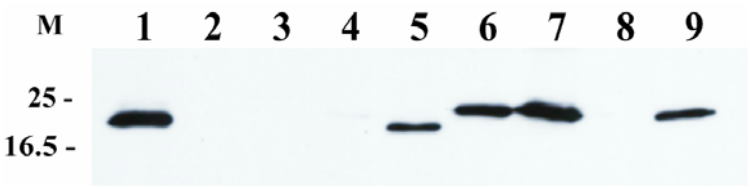


Figure 2.34. Results of the Western blot analysis of protein composition in the beads experiment.

1 - o-*gix* + GinHis	**4 - o-*gix* + ISXc5**	**7 - G10 supernatant**
2 - o-*gix* + Gin wt	**5 - o-*gix*-Gin + GinHis-*res***	**8 - o-*gix*-Gin + ISXc5-*res***
3 - o-*gix* + G10	**6 - o-*gix*-Gin + G10-*res***	**9 - ISXc5 supernatant**

o-*gix* indicates the samples where DNA fragments carrying *gix* sites were immobilised to the magnetic streptavidin particles;

o-*gix*-Gin – indicates Gin bound to the *gix* site of the DNA fragments; ***res*** indicates that added proteins were already preincubated with the *res*-containing short DNA fragments;

supernatant – indicates supernatants obtained in the first washing step after addition of G10- and ISXc5 resolvase - *res* subsite I complexes to the reactions. M – is a protein size marker.

The obtained results suggested that both Gin-His and chimera G10-His could be captured by the Gin bound at the immobilised DNA, whereas ISXc5 resolvase could not (Figure 2.34, lanes 5, 6 and 8). Since the catalytic domains are similar both in Gin and G10, but different in Gin and ISXc5, this result strongly suggests that in the tetramer the Gin and G10 dimers interact via the catalytic domains. The possibility of formation of Gin-G10 heterodimers, or of direct binding of the G10 chimera or ISXc5 resolvase proteins to the *gix* DNA was ruled out in our control experiments (Figure 2.34, lanes 3 and 4; see also Figure 2.32).

3. DISCUSSION

A main step in site-specific recombination reactions involves the formation of a properly arranged synaptic complex in which the recombining DNA duplexes are brought in close proximity. The topological changes of the DNA during the recombination reaction catalysed by DNA invertases are well studied (Kanaar *et al.,* 1988, 1989a, 1990), but little is known about the interactions between the proteins involved in the inversion reaction. The recombination enzymes bind DNA as dimers, but acquire enzymatic activity as tetramers only. Therefore the understanding of the organisation of the recombinase tetramer is of central importance. By now there are two models that predict different interactions of the recombinase monomers within the tetramer.

Merickel *et al.* (1998) have proposed a molecular structure for the Gin-related Hin synaptic complex, in which the paring of the *hix* sites (i.e. the interaction of the dimers in the tetramer), is mediated by the Hin DNA-binding domains. This model encounters difficulties in explaining how each DNA duplex can rotate for 180^{o} inside the complex, while remaining covalently attached to the protein. Evidences for such an attachment came from the experiments done by Boocock *et al.* (1995), in which the proteins and DNA sites cross-linked to each other remained active for recombination, thus proving that in the recombination reaction DNA binding domains remain attached to sites during all reaction steps. In the tetramer model elaborated in our group (in the present work referred to as the "preliminary model", Rusch, 1998) the Gin dimers are interacting via the catalytic domains. The molecular modelling of the Gin tetramer (Hermann, 1996) by using the three dimensional structure of the homologous γδ resolvase suggested that the Gin dimers are aligned side-by-side with their DNA binding domains pointing towards opposite directions.

If the crossover points are on the inside of complex in relatively close contact with each other, strand transfer could potentially involve movements of cleaved DNA ends within a relatively fixed protein cage, as was observed with Cre and Flp. This is particularly attractive for the serine recombinases that must rely entirely on the recombinase to hold the synapse together, since these recombinases make double-strand breaks. On the other hand, if the DNA is on the outside with the recombination sites separated by the synapsed catalytic domains, then there seems to be no easy way to exchange DNA strands (following

recombination site cleavage) without large accompanying movements of protein subunits or catalytic domains.

The aim of this thesis was to discern between these two models and to reveal the protein-protein contacts involved in the site-specific recombination reaction catalysed by the invertase Gin.

3.1 Gin-FIS communication

In the synaptic complex formed between the two inverted repeats bound by invertase molecules and the enhancer sequence bound by FIS, the three DNA sites are brought in close proximity (Kanaar *et al*., 1988; Kanaar *et al*., 1989b; Heichman & Johnson, 1990). As evidenced by many studies, FIS is directly involved in the establishment of the proper protein-DNA structures, assembly of the complex and activation of the recombination reaction – probably by serving as a recombinational trigger.

Previously it was shown that the recombination by Hin invertase αE helix cysteine mutants was no longer sensitive to oxidation once FIS–Hin interactions have occurred in an invertasome complex (Haykinson *et al.*, 1996). These data imply that activation by FIS induces directly or indirectly a conformational change within the Hin dimer interface.

To prove this notion in this work an inversion-deficient mutant Gin H106T carrying a substitution in the dimerisation domain was chosen as a target for activation by FIS. The position 106 appeared to be crucial during invertasome formation also because the substitution H106Y makes Gin FIS-independent. The H107C mutant of Hin (analogous to H106 in Gin) is still capable to catalyse inversion, suggesting that the contact site for FIS is not disrupted by this mutation (Haykinson *et al.,* 1996). By using a genetic test system a mutant FIS S14P was identified that provided for 60% stimulation of inversion by Gin H106T *in vivo*, but was inactive *in vitro*. Differences between the *in vivo* and *in vitro* results obtained with FIS S14P and Gin H106T can be explained by the absence of certain factors (e.g. the nucleoid associated protein HU) in our *in vitro* reactions that can facilitate recombination *in vivo* (Haykinson & Johnson, 1993). Since the efficiency of inversion critically depends on the supercoiling level of DNA (Lim & Simon, 1992), it is also possible, that the supercoiling level of the DNA substrate used *in vitro* was suboptimal for the inversion reaction catalysed by Gin H106T.

It is not clear at present whether there is a direct interaction between Gin H106T and FIS S14P via these mutated positions, or perhaps the effect is mediated by the enhancer sequence, because FIS S14P is likely to bend the DNA in a different way (Figure 2.2). Although the stimulation of H106T inversion *in vitro* was not detected, the two-fold stimulation of the Gin wt inversion indicates that FIS S14P is able to affect the nucleoprotein complex in a way that makes recombination more efficient. Analyses of FIS binding to both specific and nonspecific sites have shown that FIS induces DNA bends of various degrees upon binding DNA (Betermier *et al.,* 1994; Pan *et al.,* 1994, 1996). FIS-induced DNA bending may play a more direct role in FIS-dependent stimulation of site-specific recombination or transcription. Bending of the DNA at the exact positions (sites) of the recombinational enhancer may be required to direct the topological changes of supercoiled DNA domains in the synaptic intermediate structure (Perkins-Balding *et al.,* 1997). During studies in the Hin-inversion system, Osuna *et al.* (1991) showed that amino acid changes in FIS that affect DNA bending reduced recombinational enhancer activity by approximately 50-fold, as measured by the rate of DNA inversion. It is therefore possible that the small difference in the DNA bending angle associated with each site may position the N-terminal domains of FIS S14P in a more favourable orientation for interactions with Gin. Notably, the mutation S14P in FIS is positioned exactly in the region shown to be important for the stimulation of recombination (Spaeny-Dekking *et al.,* 1992).
Taken together, the obtained results are wholly consistent with the effect of FIS on the dimerisation domain of Gin and the proposed ability of FIS to change the dimerisation domain conformation (Merickel *et al.,* 1998).

3.2 Gin surfaces involved in the interactions of dimers in tetramer: mutations that affect the tetramer formation are situated in the catalytic domain

In the assembly of the synaptic complex two different Gin-Gin contacts can be expected during tetramer formation: one involved in formation of the Gin dimers which bind to the recombination sites and the other one involved in stabilisation of the tetramer in the synaptic complex.
In the highly homologous γδ resolvase dimer the subunit contacts are due to interaction of the long amphipatic αE helices, which in the dimer wrap around each other in a coiled-coil

configuration forming a parallel bundle (Sanderson *et al.,* 1990; Rice & Steitz, 1994a). This interface is partially conserved among resolvases and invertases with respect to the primary sequence, but highly conserved with respect to the distribution of hydrophobic and charged amino acids, crucial for the dimerisation via the hydrophobic face of the two αE helices (Sanderson *et al.,* 1990; Rice & Steitz, 1994a). The region of αE helix that forms the dimerisation interface contains mainly hydrophobic amino acids both in Gin and γδ resolvase (Spaeny-Dekking *et al.,* 1995b). Several studies carried out on the related to Gin invertases Cin and Hin indicate that this same domain is involved in the subunit dimerisation (Klippel *et al.,* 1988b; Hafter & Bickle, 1988; Klippel *et al.,* 1993; Lim, 1994; Haykinson *et al.,* 1996).

Investigations of the Gin-related site-specific recombinases also suggest that a quaternary change in the dimer interface accompanies catalytic activation. When disulfide cross-links were formed between cysteines introduced into the amino-terminal end of the αE helix of γδ resolvase and Hin, thereby restricting movement between subunits, catalytic activity was abolished, though resolvase or Hin binding to DNA was unaffected (Hughes *et al.,* 1993; Lim, 1994; Haykinson *et al.,* 1996).

Previous studies identified two separate domains in Gin, which play a role in tetramerisation. These represent the putative dimerisation helix αE and an exposed loop between the β2 sheet and αB helix of Gin (Merker *et al.,* 1993; Rusch, 1998). Several mutations in these domains strongly affect the stability of the tetramer formed both *in vivo* and *in vitro* (Rusch, 1998). In this study, assuming that there are two separate surfaces of interaction, mutations in the αE-helix and surface exposed loop between β2 and αB were generated, which potentially could interact according to the proposed model. By testing the mutants of Gin, we have confirmed the previous observations that the putative dimerisation domain of Gin monomers is also involved in the interaction between the Gin dimers. In particular, we found that the cross-linking of the monomers in dimer via a substitution of cysteine at the position 107 stabilises the tetramer. Furthermore, when we combined the mutations in the dimerisation αE-helix (V107C, M114V) with a mutation in the surface exposed loop between β2 and αB (K34E), the yield of the tetramer was increased (Figure 2.15), suggesting that these two regions cooperate in stabilising the tetramer.

Finding that the same domain is involved in the stabilisation of both the dimer and the tetramer of invertase is also consistent with the otherwise disparate observations that zwitterionic detergents disrupt the Hin dimeric interactions and stimulate the cleavage

activity, but inhibit the religation of DNA strands in recombinant configuration during synapse (Haykinson *et al.*, 1996). It is conceivable that the destabilisation of the largely hydrophobic interactions stabilising the tetramer at the step following the strand-cleavage would inhibit relegation of the cleaved strands.

According to the preliminary Gin tetramer model amino acid side chains at the positions L17 and S75, which are located on two opposite sides of the Gin monomer, could closely approach each other in the tetramer. It is noteworthy, that the regions around the positions L17 and S75 are almost similar for Gin and γδ resolvase as judged by distribution of the hydrophobic and hydrophilic amino acids, although the identity of the amino acid residues is rather low. The hypothesis was that substitution of cysteines at these positions in combination with similar substitutions in the dimerisation αE-helix could be used for generation of the covalently cross-linked Gin tetramers. Therefore cysteine substitutions were introduced at both positions (L17C and S75C) and the proteins were tested both alone and in combination with substitutions in the E-helix (V107C) for interactions during the synapse by using cross-linking experiments with DEB. Before cross-linking the proteins were incubated either alone to investigate the interactions in solution, or in the presence of supercoiled inversion substrate DNA and FIS to allow synapse formation. In control reactions the cross-linking by DEB was prevented by addition of the reducing agent DTT. The results of these experiments are summarised in the Table 3.1.

In summary, the cross-linking experiments indicate that the double mutants L17C/V107C and S75C/V107C could form tetramers with the same efficiency, but only when DNA and FIS were added to the reaction. This suggest that presence of DNA and FIS can provide for additional interactions bringing these positions in close neighbourhood, and this specific dependence indicates that these are interactions characteristic of synapse.

Table 3.1. Summary of the results obtained during cross-linking experiments. Comparison of the Gin wt and Gin mutant's inversion activities and their ability to form high-order complexes.

Gin	Inversion		Dimer formation		Tetramer formation		Trimer formation	
	in vivo	*in vitro*	red	ox	red	ox	red	ox
wt	+	+	-	+	-	-	-	-
V107C	-	-	-	+	-	-	-	-
L17C	+	-/+	-/+	+	-	-/+	-	-/+
S75C	+	+/-	-/+	+	-	-/+	-	-/+
L17C/V107C	-	-	-/+	+	-	+	-	+
S75C/V107C	-	-	-/+	+	-	+	-	+
C24S/C27A	-	+	-	-	-	-	-	-
L17C/V107C/ΔC	-	-	-	+	-	+	+*	+*
S75C/V107C/ΔC	-	-	-	+	-	-*	+*	+*

* Note that complexes formed by Gin L17C/V107C/ΔC and S75C/V107C/ΔC mutants (indicated as trimers) had a distinct mobility. These complexes were designated as ΔC-specific bands (see Results), and their formation was DNA and FIS independent.

However, these studies revealed in addition the importance of the natural cysteine residues C24 and C27 for the Gin-Gin interactions in synapse. In the absence of these natural cysteines the proteins have lost their specific dependence on FIS and substrate DNA and formed dimers and higher order complexes migrating between the putative trimers and tetramers in the gel (Figure 2.9A,B). It was shown previously that the natural cysteines C24 and C27 are responsible for the cross-linking of Gin wt in solution resulting in formation of Gin dimers (Spaeny-Dekking *et al.,* 1995b). However, the dimerisation domain proposed for Gin dimer bound at the DNA is the αE-helix, and strong cross-linking of the Gin V107C mutant observed in this study supports this notion (Figure 2.5B). The results obtained with the C24/C27 double mutant indicate that these positions are crucially involved in the interactions during the synapse. Additional support for this notion was obtained in experiments using the tetramerisation assay with *gix* sites on linear fragments. While the Gin C24S and Gin C27A proteins were able to form both dimers and tetramers, the double mutant C24S/C27A showed no tetramer formation, although it was able to dimerise efficiently. These observations strongly suggest that the natural cysteines play a crucial role in the organisation of synapse. Surprisingly, the Gin C24S/C27A double mutant

was still able to catalyse inversion, although only *in vitro*, but not *in vivo*. This suggests that the natural cysteines are important but not essential for the recombination reaction.
One possible explanation of this phenomenon is that the efficiency of recombination may crucially depend on the stability of the Gin tetramer (Table 3.2).

Table 3.2. Summary of the results obtained in the *in vitro* tetramerisation (*gix*-*gix* paring) assay and inversion activity of the tested proteins.

Gin	Inversion		Dimer formation		Tetramer formation	
	in vivo	*in vitro*	red	ox	red	ox
wt	+	+	+	+	-	+
K34E	-	-	++	+	-	++
M114V	++	++	-*	-*	-*	-*
K34E/M114V	+	+	+	+	+	++
V107C	-	-	++	-/+	-	++
K34E/V107C	-	-	+	+	+	+++
C24S/C27A	-	+	+	+	-	-

* Note that M114V mutant formed specific complex which was some kind of intermediate between dimeric and tetrameric forms (see Results).

In the genetic screen performed by K. Rusch (1998) the selection was for mutants forming more stable tetramers in comparison to wt Gin. All these mutants, including the mutants carrying substitutions in the dimerisation αE-helix, were recombination-deficient. Consistent with this observation, all the αE-helix dimerisation interface Hin mutants tested by Lee *et al.* (2001) showed better *hix*-paring activity than wild-type and were able to form an invertasome, but were unable to cleave the DNA. In contrast, the mutations of the same interface, such as e.g. M114V, apparently destabilise the dimer, impair the ability to form tetramers and render Gin FIS-independent (Klippel *et al.,* 1988a; see also Figure 2.14). However, the combination of M114V with the "tetramerisation-up" mutation K34E renders the protein FIS-dependent and stabilises the tetramers. Other mutations found as M114V suppressors (such as K34E, S36N, R39G, D41G, P43S and P43L) were inversion-deficient and all were able to tetramerise more efficiently than wild-type Gin (Merker, 1993; Rusch, 1998). Similar mutant *gin* alleles that can suppress the FIS-independent phenotype have been isolated by Spaeny-Dekking *et al*. (1995a). The identified mutations are located in a

short region of ten amino acids in the region of catalytic domain that is involved in dimer-dimer contacts in γδ resolvase (Hughes *et al.,* 1990). Since FIS is thought to stimulate conformational rearrangements that in particular affect the αE-helices (Haykinson *et al.,* 1996) it is conceivable that all the "tetramerisation-up" mutants are impaired in conformational flexibility required for the rearrangement of the tetramer. The mutation of natural cysteines, which themselves are likely stabilising the tetramer, therefore could affect the conformational flexibility. But it is also unlikely that the function of these cysteines in the inversion reaction is to form disulphide bonds, since in the cytoplasm of *E. coli* cells the cysteines are maintained in a reduced state by the action of thioredoxin reductase (Dermann *et al.,* 1993). Also the homologous invertases Hin and Cin contain a serine residue at the position corresponding to C24 in Gin, showing that a cysteine residue at this position is not important for the inversion activity.

In addition to the natural cysteines, the residues at the positions 17 and 75 are also making a close approach, because both substitutions in combination with V107C mutation were required to stabilise the cross-linked tetramers in the synaptic complex (Figure 2.9). The amino acid residues at positions 17, 24, 27 are located on the exposed surface comprised by αA helix and the β2 sheet adjacent to the surface exposed loop between the β2 sheet and the αB helix. Since amino acid substitutions at these positions affect the tetramer formation, this entire large region, situated on the side of the molecule opposite to that of the dimerisation αE-helix, is very likely to be involved in the dimer-dimer interactions. Amino acid residue at the position 75 is situated on the outer surface of αD helix, which is parallel to the αE dimerisation helix and faces the same direction as those residues on the αE helix (positions 110, 113, 117) that affect tetramer formation (Rusch, 1998). However, it appears that in the absence of natural cysteines, the organisation of putative tetramers (ΔC specific complexes) is different. Not only they have lost the dependence on FIS and substrate DNA, but also show increased resistance to the reducing agent DTT (Figure 2.9). Notably, in addition to synapse-specific tetramers, oligomer complexes (putative trimers) independent of FIS and DNA form quite efficiently also in the presence of natural cysteines. The nature of these complexes is not clear, but they represent probably a trimer consisting of one monomer of Gin attached via a 17C or 75C cross-link to a Gin dimer, cross-linked via 107C.

Taken together these data suggest that there are several potential interacting surfaces allowing oligomerisation of Gin and that FIS and DNA select the proper Gin-Gin

interactions in the synapse. These involve the interactions of the exposed surface comprised by loop between the αA helix and the β2 sheet where the natural cysteines are located. This specific tetramer complex can be further stabilised by cross-linking via 107C in combination with either 17C or 75C substitutions, both of which are located in the vicinity of putative tetramerisation domains. However, in the absence of the natural cysteines this selectivity is lost and Gin oligomerises by using other interactions. The residues 17C/107C and 75C/107C play a role in these interactions again, although we cannot exclude that in contrast to wild-type Gin, in the Gin C24/C27 double mutant the natural lysines become available for cross-linking by DEB. The ΔC specific complex is formed with a high efficiency as judged by the ratio to Gin dimers (Figure 2.9). These observations imply that the presence of natural cysteines prevents certain unproductive interactions, which have a potential to cause oligomerisation of Gin in a FIS and DNA independent manner.

The pertinent question is whether the tetramer studied in this work is relevant to the synaptic complex. The *in vivo* screening performed by K. Rusch (1998) with a DNA substrate containing *gix* sites separated by a short (83 bp) spacer was devoid of recombinational enhancer. The phenotype of the mutants identified by genetic screening has been confirmed by *in vitro* tetramerisation assay using linear *gix* fragments. In the present study we confirmed the "tetramerisation-up" phenotype of several previously isolated mutants using the same assay. However, in contrast to the previous study, in this work the investigation of the tetramer complexes was carried out also using naturally supercoiled DNA inversion substrate with recombinational enhancer and FIS, which are the prerequisites for productive synapse formation. With this approach we not only confirmed the role for the previously identified tetramerisation domains using substitutions in the vicinity of the putative tetramerisation domains (17C and 75C), but also revealed a new region comprised by the flexible loop between the αA helix and the β2 sheet, which could not be identified in the previous work.

Taken together the results of cross-linking experiments demonstrate that the synapsed tetramer can be stabilised by combining the effects of the substitution 17C located on the αA helix, the substitution 75C located on the αD helix, and the substitution V107C located on the αE helix. This indicates that extensive contacts are clustered in two structurally separated large regions of the catalytic domain, which cannot simultaneously interact in the tetramer without involving strong rearrangements of protein contact surfaces within the tetramer (see Figure 2.10 and Figure 3.1).

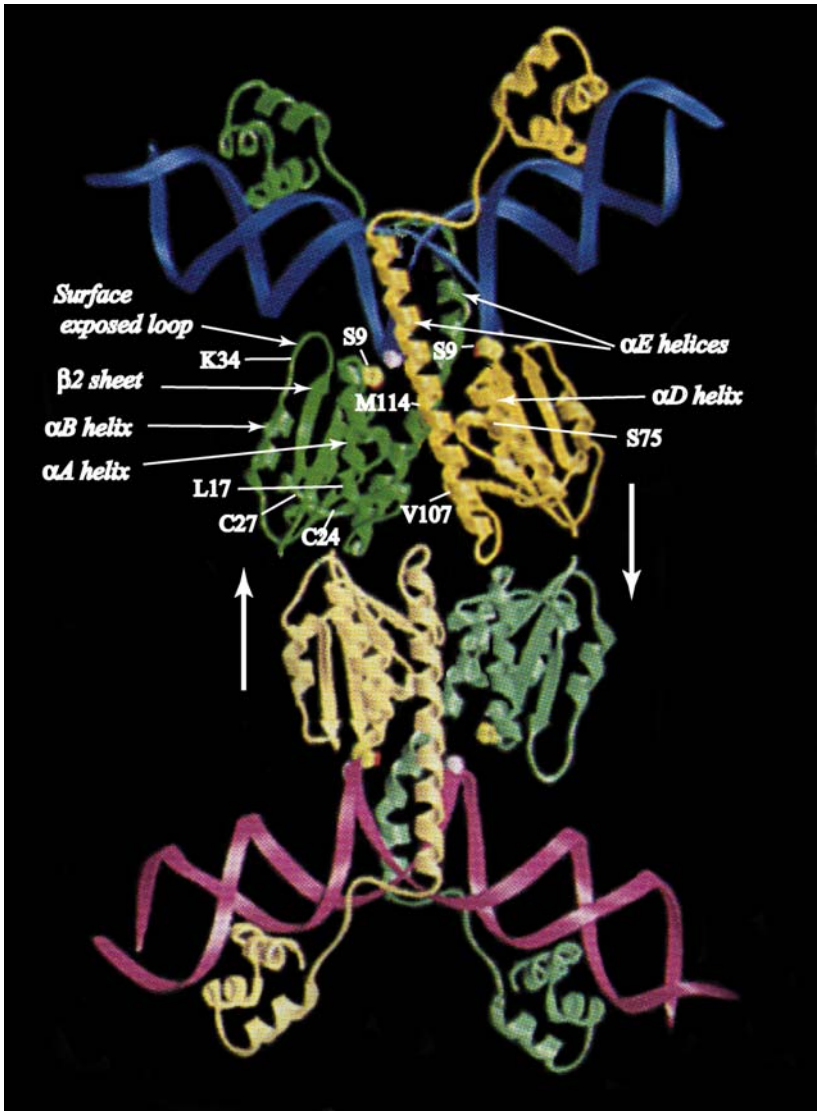


Figure 3.1. Model for the Gin tetramer arrangement, showing the interacting surfaces in synapse.

One Gin monomer is shown in green and yellow ribbon diagrams and complexed with blue DNA; the other dimer is in lighter colours and complexed with red DNA.
The protein secondary structure elements forming the dimer interaction surfaces and the location of the active site residue serine 9 (S9) and studied amino acids are indicated.

Gin dimers are interacting via their N-terminal domains. During synaptic complex formation the catalytic domains of both dimers are probably closely approaching each other and interact via the large surface delimited by loops, β-sheets and α-helices (direction of movement is indicated by arrows). During synapse the yellow monomers are interacting with each other via large surfaces comprising the domains identified in this and previous studies. FIS (not shown on the picture) is proposed to destabilise the dimerisation surface of Gin and facilitate the conformational rearrangement of monomers within the tetramer.

3.3 Why is the ISXc5G10 chimera inversion deficient?

The amino acid sequence of ISXc5 resolvase shows extensive homology with members of the DNA invertase family (Liu *et al.,* 1998). The N-terminal 140 amino acids show 57% identity with the Gin invertase. There are also another two regions in ISXc5 resolvase with high degree of identity to invertases. The first one - from amino acids 56 to 70, with an identity of 73%, possibly the region involved in catalysis and important for tertiary structure. The second one – from amino acids 101 to 138, exhibits a 60% identity, is responsible for the DNA minor groove recognition and includes the αE helix with residues involved in dimer interaction (Hughes *et al.,* 1990; Yang & Steitz, 1995; reviewed in Grindley, 1994).

The generated chimeric protein ISXc5G10 contains the large N-terminal catalytic domain of Gin, including most of the long αE helix that constitutes the dimer interface (residues 1 to 123; Liu *et al.,* 1998; corresponds to residues 1 to 126 in γδ resolvase; Yang & Steitz, 1995). The fusion joined the end of the αE helix of Gin to the flexible arm region and the DNA-binding domain of ISXc5 resolvase (residues 124 to 205; corresponds to residues 127 to 183 in γδ resolvase). It appears that the distinct nature of this latter domain is sufficient to switch the directionality of reaction from inversion to resolution.

Schneider *et al.* (2000) have shown that a substrate containing an isolated pair of ISXc5 *res* subsites I and the recombinational enhancer can be utilised by Gin for catalysing the FIS-dependent inversion reaction *in vivo* and *in vitro*, whereas G10 chimera and resolvase are inactive. It turned out that the orientation of the *res* subsite I was not important for Gin catalysed inversion, perhaps due to the symmetry of the two central AT base pairs in the core sequence of subsite I where strand exchange most likely occurs (Moskowitz *et al.* 1991) and, to the the sequence of *res* subsite I which represents an almost perfect palindrome. However, Gin cannot catalyse inversion with full *res* sites oriented either as inverted or direct repeats, because of the binding to both subsites I and III in *res.* These subsites exhibit about 53% identity with the *gix* site (Schneider *et al.,* 2000) and when present on the same substrate can interfere with each other or compete for the interaction with FIS.

Most known helix-turn-helix proteins create significant DNA bends (Dodd & Egan, 1990; Brennan & Matthews, 1989), and the appearance of strong enhancement of DNase I cleavage at the around the binding site indicates that the DNA is strongly distorted upon

binding. In the DNase I footprinting assay we observed substantial differences in binding properties of Gin when compared to G10 chimera and ISXc5 resolvase, the two latter proteins producing regularly spaced strong DNase I hypersensitivities on binding to *res* subsites I. These differences in the DNA binding make it unlikely that Gin and ISXc5G10 proteins bound at their cognate sites could interact via their DNA-binding domains (which also show a poor amino acid sequence homology). On the other hand, the G10 and ISXc5 have the same resolvase activity and their DNA binding properties appear similar in the DNase I footprinting assay. However, G10 chimera was able to interact with Gin, whereas ISXc5 resolvase was not, indicating that interactions of the proteins involved the identical catalytic domains of Gin and G10.

The results of this work support the view that each step of the Gin catalysed inversion reaction requires defined interactions between Gin molecules. These interactions are responsible for certain conformational changes which in turn could facilitate the following step of the recombination reaction. Every qualitative alteration of contacts between Gin molecules could thus block the reaction specifically. Obviously the quality of these contacts decides on whether FIS and the enhancer are needed for the reaction or not.

There could be several reasons for the fact that no chimera mutants were found that were able to catalyse inversion. First of all, the concentration of the G10 mutant proteins in the cell may be critical for the formation of the tetramer complex at the *res* subsites I. We measured the expression level of mutant proteins systematically and the concentrations were similar to wt Gin, but it is possible that in the case of G10 chimera mutants the proteins need to be expressed at a higher level. Second, it could be that most mutations in the DNA binding domain of G10 impaired binding to the *res* subsite, thus preventing the assembly of a higher order complex required for recombination. Third, the changes in the DNA-binding domain may influence the conformation of the catalytic domain that makes the assembly of an inversion-proficient synapse impossible. Finally, the presence of a DNA-binding domain of ISXc5 resolvase in the G10 chimera leads to the formation of a tetramer with different features. One reason of failure to find any inversion proficient chimeras could be that the DNA-binding domain affects the orientation of the catalytic domain. Even extensive exchanges of entire parts of the DNA-binding domain of chimera and Gin, making the DNA-binding domain of chimera almost similar to Gin, did not make the chimera active, suggesting that the interactions between these domains and the catalytic domain are very different. Although our linker mutants with 10, 14 and 16 amino acid insertions between the DNA binding and catalytic domains were inactive, it is still possible

that nature of the flexible region connecting these domains that is involved in the distinct ability of invertases and resolvases to fulfil their particular functions. Therefore, in order to change interactions of the domains, the random mutagenesis of the whole chimera's gene and more systematic linker analysis would be important. If this approach will allow identification of inversion-proficient mutants it can illuminate the molecular basis, underlying the distinctions between resolvases and invertases.

Importantly, the chimera has lost the ability to be stimulated by FIS to catalyse inversion, although it contains the catalytic domain of Gin. If FIS contacts the DNA binding domain, the G10 chimera contains this domain from a resolvase and therefore, critical residues normally required for an interaction between FIS and invertases may be lacking. However, Merickel *et al.* (1998) proposed that it is a region within the dimer interface, which is contacted by FIS; this is also consistent with our observations that FIS S14P can stimulate the inversion-deficient Gin H106T for recombination. The G10 chimera contains the dimer interface from Gin (positions 102-124 according to Yang & Steitz, 1995) and is catalytically active as a resolvase. It is possible that the global organisation of this region is critical for the communication between FIS and Gin, and in G10 chimera this region is altered significantly, as judged from the inability of Gin and G10 to form heterodimers (Figure 2.32). It seems that synapsis results in a G10 tetramer that is structurally so different from that generated by wild-type Gin, that FIS cannot make appropriate contacts. One possibility is that the G10 forms the complex similar to the wt resolvase, but since the accessory *res* subsites II and III are lacking, there is no activation. Another possibility is that FIS determines the structure of the complex by bending the DNA and in this case directly repeated *res* subsites I will be aligned in an anti-parallel orientation so that the G10 chimera would not be able to recombine.

When the efficiency of tetramer formation by Gin and by G10 were compared, it appeared that the tetramer formed by G10 itself is more stable. The observation that the G10 chimera is able to form a more stable tetramer that wt Gin supports the notion that the stability of the tetramer is correlated with the propensity to catalyse inversion. Mutants of Gin that showed better tetramerisation activity were all inversion-deficient. Thus it is possible that the tetramer formed by chimera is similar to Gin, but its stability is much higher. If FIS affects cleavage by inducing conformational changes in the tetramer of Gin, in the case that the complex is too stable FIS would not be able to fulfil its function. In the case of FIS S14P its higher stimulatory activity could change the configuration of the tetramer formed by Gin H106T and activate it for the inversion. To confirm this notion the study of the

effect of FIS S14P on other inversion deficient Gin mutants with showed high tetramerisation activity would be necessary.

In the case of resolvases the stability of the tetramer is perhaps not so crucial. For the resolution formation of a large nucleoprotein complex containing three tetramers (12 protomers) is necessary, making it roughly three times size of an invertasome. There could be other mechanistic problems, and their solution is perhaps not solely dependent on the stability of a single tetramer. The fact that it was possible to crystallise resolvase, but not invertase, suggests that the complexes formed by these proteins have different dynamics. The invertasomes are very unstable (unpublished data).

From this data we postulate a new rate-limiting step in DNA inversion reaction, and this is the stability of the tetramer. The mechanistic task that has to be solved by the synaptic complex is to destabilise the tetramer after the formation of the invertasome, and it is probably this step at which FIS is acting: if the tetramer is too stable, this will preclude inversion. Perhaps it will be impossible to get any chimeric mutants able to catalyse inversion if the stability of the tetramer will remain like that in resolvases. Thus, one future approach would be to screen for the mutations which destabilise the tetramer. It is noteworthy that by performing in this study the mutagenesis of the DNA-binding domain of chimera alone, we did not primarily affect the stability of the tetramer, but rather the stability of DNA-protein interactions.

According to the Smith & Thorpe (2002) there are three components in the recombinase monomer revealing a modular organisation: the catalytic domain, the DNA-binding domain and the flexible region between them (represented by C-terminus of the αE-helix). According to Yang and Steitz (1995), the only plausible barrier to rotation of the catalytic and DNA-binding domains with respect to each other is provided by the carboxy-terminal portion (residues 122-136) of the two αE-helices, which connect the globular catalytic domain to the extended strand (residues 137-146) and the 3-helix bundle (residues 147-183), that constitute each a DNA binding domain. However, the C-terminal half of the E-helix appears to be structurally unstable; it is unfolded in the absence of DNA (for example, in crystals of the N-terminal domain, Sanderson *et al.,* 1990), and even in the presence of site I it adopts two different conformations with a kink (at residue 127) in one subunit (Yang & Steitz, 1995). Together with the demonstrated ability of a resolvase dimer to bind to sites with a variety of spacing between the DNA recognition sequences (Leschziner *et al.,* 2003), this suggests that there may be a variety of conformations for the resolvase

polypeptide chain from 121-146, and few energetic barriers to a rotation of the DNA (and the DNA binding domain) relative to the catalytic domain. Indeed, the particular position observed in the resolvase-site I cocrystal structure may represent just one of several energetically equivalent conformations, and at least one cocrystal exhibits a distinctly different configuration of the DNA relative to the catalytic domain.

The reverse chimera's mutants generated during our studies contained all these regions and were able to bind DNA, but still did not show any recombination activity, probably being affected already at the level of dimer formation. If the catalytic domain acts only as a "motor", and DNA-binding domain serves only for the recognition of recombination sites, then at least some of the RC chimeric proteins generated in this study should still have been able to catalyse inversion reaction. Furthermore, Schneider *et al.* (2000) suggested that charged residues in Gin (Gln29, Arg51 and Gln53) provide the required inter-dimer interface once the chimeric dimers are bound to *res*. These residues correspond to those of γδ resolvase (Arg2, Arg32, Lys54 and Glu56), which form the so-called 2,3' interaction surface between γδ resolvase dimers and play an essential role in building a recombinogenic synaptosome and resolution (Sanderson *et al.,* 1990; Hughes *et al.,* 1990, 1993; Murley & Grindley, 1998). In our RC proteins the N-terminal domain was from resolvase and this did not make proteins resolution proficient. This observation is consistent with our notion that there is a structural coordination between the catalytic and DNA-binding domains, which leads to the proper recombinasome organisation and recombinational activity.

We therefore propose that the structural self-coordination of the monomer subunits is the main feature of the higher order protein-protein interactions that can lead to the productive recombination.

3.4 Outlook

This study raises many new questions that await further clarification. These concern both the nature of Gin-FIS interactions, and the nature of Gin-Gin interactions in the synapse. For example, is the substitution of serine by proline at position 14 of FIS altering the conformation and/or flexibility of the N-terminus of FIS? And which amino acids in the dimerisation domain of Gin and in the N-terminus of FIS are involved in the interaction? According to our hypothesis the stability of Gin-Gin interactions in the tetramer can

interfere with recombination reaction. Is it possible to correlate the activating potential of FIS S14P with Gin tetramerisation ability? Can a destabilisation of the G10 tetramer by mutation confer FIS-dependence and an inversion proficient phenotype? Can the variation of the length of the linker domain between the DNA-binding and catalytic domains facilitate the conversion of a resolvase to an invertase?

4. MATERIALS AND METHODS

4.1 Chemicals

Name		Name	Manufacturer
Acrylamide	Roth	Lithiumacetate	Merck
Acetic Acid	Merck	Magnesiumchloride	Merck
Agar	Sigma	Magnesiumsulphate	Merck
Agarose	SeaKem	Manganchloride	Merck
Ammoniumacetate	Merck	β-Mercaptoethanol	Sigma
Ammoniumpersulphate	Merck	Methanol	Roth
Ammoniumsulphate	Baker	Paraffin liquid	Merck
Ampicillin	Sigma	Peptone	Difco
Bacto Agar	Difco	Phenol	Roth
Bisacrylamide	BioRad	Polyethylenglycol (PEG 4000)	Fluka
Bromphenolblue	Merck		
Calciumchloride	Merck	Potassium permanganate	Merck
Chloramphenicol	Sigma	Hydrochloric acid	Merck
Chloroform	Merck	Sodiumacetate	
Coomassie Brilliant Blue	Serva	Sodiumcarbonate	Merck
Desoxyribonucleotid-triphosphates	Pharmacia	Sodiumchloride	Roth
		Sodiumdihydrogen-phosphate	Roth
1,2:3,4-Diepoxybutane (DEB)	Serva		
		Sodiumdodecylsulphate (SDS)	Roth
Dimethylsulfoxide (DMSO)	Fluka		
		Sodium-EDTA	Serva
Dimethylsulfate (DMSF)	Sigma	Sodiumhydroxide	Riedel-de Haën
Dithiothreitol (DTT)	Boehringer		
Ethanol	Merck	Sodiumsulphate	Merck
Ethidiumbromide	Sigma	st-DNA	Sigma
Ethylenglycol	Sigma	TEMED	BioRad
Formaldehyde	Merck	Tetracycline	Sigma

Galactose	Serva	Tris-Base	Sigma
Glucose	Merck	Tris-HCl	Sigma
Glycerol (87%)	Merck	Triton X-100	BioRad
Imidazole	Roth	Trypton	Difco
IPTG	Gerbu	Tween-20	Serva
Isopropanol	Merck	X-Gal	Sigma
Lithiumchloride	Merck	Xylencyanol	BioRad
Magnesiumchloride	Merck	Yeast Extract	Difco

4.2 Enzymes, proteins and reagents

Name	Manufacturer
Albumin (BSA)	New England Biolabs (NEB)
Alkaline phosphatase (CIP)	New England Biolabs
DNase I	Boehringer
E. coli DNA polymerase I (Klenow fragment)	Boehringer
Lysozyme	Boehringer
Pfu Turbo DNA polymerase	Stratagene
Proteinase K	Boehringer, Merck
Restriction enzymes	New England Biolabs, Boehringer, Pharmacia
Ribonuclease A	Boehringer
T4 DNA ligase	New England Biolabs
T4 DNA polymerase	New England Biolabs
T4 polynucleotide kinase	New England Biolabs
Taq DNA polymerase	Boehringer

Purified *E. coli* FIS proteins as well as DNA invertases Gin, Gin K34E, H106T, M114V and K34E/M114V were used from preparations done by A. Deufel and P. Merker.

Mutant proteins FIS S14P; Gin L17C, S75C, V107C, L17C/V107C, S75C/V107C, C24S, C27A, C24S/C27A, K34E/M100C, K34E/V107C; ISXc5 resolvase; ISXc5G10 chimera and ISXc5G10 mutants K34E, K34E/M114V, V107C, M114V, N1, N2, N3, N4, N5, N6,

Chim-135, Chim-145, Chim-155, Ch L162N/Q198R and reciprocal chimera RC were purified with His-tags during the present work.

Polyclonal anti-Gin antibodies were kindly provided by Nora Goosen. Monoclonal anti-His mouse antibodies were purchased from Dianova (Calbiochem). FIS rabbit polyclonal antibody was a kind gift of Christian Koch.

γ-^{32}P-labelled deoxyribonucleotides were purchased from Perkin Elmer.

4.3 Molecular markers

4.3.1 DNA-length molecular standards for agarose gel electrophoresis

λ DNA (MBI Fermentas) digested with *Pst*I was used as DNA length standard.

λ-DNA *Pst*I-fragments:	11509 bp	1700 bp	216 bp
	5077 bp	1159 bp	211 bp
	4749 bp	1093 bp	200 bp
	4507 bp	805 bp	164 bp
	2838 bp	514 bp	150 bp
	2560 bp	468 bp	94 bp
	2459 bp	458 bp	87 bp
	2443 bp	339 bp	72 bp
	2130 bp	264 bp	15 bp
	1936 bp	249 bp	

4.3.2 Protein molecular weight standards

The apparent molecular weight of proteins in SDS-polyacrylamide gel electrophoresis was determined according to P7708 Prestained Broad Range protein molecular weight marker (6.5 – 175 kDa) from New England Biolabs.
Protein molecular weight calculations according to the amino aid sequence were done using the programs at http://bioreference.net/proteinmw.htm

4.4 General buffers and solutions

AE-Buffer	50 mM Na-Acetate, pH 5.3 10 mM Na_2-EDTA in H_2O_{dd}
AE-PC	50% AE-Phenol 50% Chlorophorm
DNA loading buffer	0.25% bromphenol blue 1x TAE 50% sucrose
Inversion buffer 10x	200 mM Tris-HCl, pH 7.6 100 mM $MgCl_2$ 5 mM DTT in H_2O_{dd}
Lysozyme solution	10 mg/ml lysozyme 10 mM Tris-Cl, pH 8.0 in H_2O_{dd}
RNase A solution	10 mg/ml RNase A 15 mM NaCl 10 mM Tris-Cl, pH 8.0 in H_2O_{dd}, 15 min boiled
Sample buffer	25% Ficoll 400 0.25% Orange G in H_2O_{dd}
TAE (50x)	2 M Tris-Acetat 100 mM Na_2-EDTA in H_2O_{dd}

TBE (5x)	500 mM Tris-Borat, pH 7.9 10 mM Na_2-EDTA in H_2O_{dd}
TE (10x)	100 mM Tris-Cl, pH 7.9 10 mM Na_2-EDTA in H_2O_{dd}

4.5 Media

YT-Media (Sambrook *et al.,* 1989)	8 g Trypton 5 g Yeast Extract 5 g NaCl with H_2O ad 1 l
dYT-Media (Sambrook *et al.,* 1989)	16 g Trypton 10 g Yeast Extract 5 g NaCl with H_2O ad 1 l
dYT-Glycerol (Sambrook *et al.,* 1989)	16 g Trypton 10 g Yeast Extract 5 g NaCl 800 ml 87% Glycerin with H_2O ad 1 l
YT-Plates (Sambrook *et al.,* 1989)	8 g Trypton 5 g Yeast Extract 5 g NaCl 15 g Agar with H_2O ad 1 l

All the media were sterilised by autoclaving.

Appropriate antibiotics were added where necessary from stock solutions after autoclaving and cooling the media down (~ 60^0C) ad end concentrations:

ampicillin	100 μg/ml
chloramphenicol	25 μg/ml
kanamycin	40 μg/ml
tetracycline	25 μg/ml

Antibiotic stock solutions:

ampicillin	100 mg/ml (in H_2O_{dd})
chloramphenicol	25 mg/ml (in 50% ethanol)
kanamycin	100 mg/ml (in 50% ethanol)
tetracycline	25 mg/ml (in 50% ethanol)

For the blue/white selection and protein induction X-Gal and IPTG were added up to the final concentrations (f.c.):
0,1% (v/v) 100 mM IPTG (f.c. 10 μM)
0,33% (v/v) 2% X-Gal (f.c. 0,01%)

4.6 *Escherichia coli* strains

The nomenclature of the *E. coli* strains is according to Demerec *et al.* (1966).

Strain	Corresponding marker	Reference
DH5α (*E. coli* strain K12 derivate)	F' *end*A1 *hsd*R *hsd*M, *sup*E44 *thi*-1 *gyr*A1 *dlac*ZΔM15 λ⁻ *rel*1 *gyr*A96 *rel*A1 Δ(*lac*ZYA-*arg* F) U 169 *rec*A1	Hanahan, 1983
CSH50 (*E. coli* K12 derivate)	*ara Δ*(*lac pro*) *rpsL thi str*A	Miller, 1972

CSH50*fis*::Kan (*fis*$^-$ derivate of CSH50)	*fis*$^-$	Koch *et al.*, 1988
AD1 CSH50*fis*::Kan::I^q *oxyR::lacZ* inv (inversion test strain)	*fis*$^-$ *lacI*^{q} *lacZinv*	Deufel *et al.*, 1997
WK6 (λcI$^+$)	WK6, lysogenised with λcI$^+$	Klippel *et al.*, 1988a
XL1–Blue MRF' Kan	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F´*proAB lacI*^{q}*ZΔM15* Tn5(Kan^r)]	Stratagene
BL21(DE3)	F$^-$ *dcm ompT hsdS*(r_b^- m_b^-) *gal* λ(DE3)	Stratagene
BL21(DE3)pLysS	F$^-$ *dcm ompT hsdS*(r_b^- m_b^-) *gal* λ(DE3)[pLysS Cm^r]	Stratagene

The *E. coli* K12 strain CSH50 is *pin*$^-$ (Kamp & Kahmann, 1981) and was used for the propagation of inversion test plasmids.

Strains BL21(DE3) and BL21(DE3)pLysS are all-purpose strain for high-level protein expression and easy induction. The BL21(DE3)pLysS provide tighter control of toxic proteins expression. pLysS – pACYC-based plasmid carrying T7 lysozyme gene derivative. Strains used in combination with plasmids containing T7 promoter constructs (*e.g.* pET vectors). T7 polymerase is induced from *lacUV5* promoter with IPTG. Strains were used for the overproduction of Gin, FIS and mutant proteins.

The XL1-Blue MRF´ Kan strain is a kanamycin-resistant (Kan^r) derivative of Stratagene's XL1-Blue strain useful for PCR cloning using vectors harbouring chloramphenicol- or tetracycline-resistance genes. Strain was used for the site-directed mutagenesis constructions on the pMD3E which is Tc^r.

4.7 Oligonucleotides

All oligonucleotides were synthesised by Metabion (Martinsried) or MWG BIOTECH (Ebersberg) companies.

Name	Nucleotide sequence (5'→3')
Gin-Sty-5'	GCTACCTAGGAGAGTGCTGATTGGCTATGTAAG
Gin-KpnI	CCGGTACCTTAATTGATTCGATCGTCG
pGin PK	CAACTGCAGGGTACCATTGATTCGATCGTCGTTTTC
pGin EN	GGAATTCCATATGGTGCTGATTGGCTATGTAAG
Gin M100C	CGTGGAAGAAAAAACGCCCACATGGAGATGACGTATC
Gin C27A	TTGTTCGGCTCCTGCACAAACAAGAGC
3'C24S gin	TTGTTCACATCCTGCAGAAACAAGAGC
C24S/C27A gin	TTGTTCGGCTCCTGCAGAAACAAGAGC
Gin S75C (rev)	CTACGAGACAAATCAAATGTTTC
Gin L17C (rev)	GTTGACAGTCTGTATTCTGGTC
MV114	GTGCCCTGGCTGAAGTGGAACGAGAAC
MV114rev	GTTCTCGTTCCACTTCAGCCAGGGCAC
Gin123-BsiW	GAGCGTACGATGGCTGGACTTG
chimera-PK	GGGGTACCCACGGAGACGTTTATCGG
Chim-Kpn-HindIII	GGGGTACCCCAAAACAGCCAAGCTTTCACTA
Chimera-END	CAGCCAAGCTTTCACTACACG
MV-chim-5'	CCTGGGCGAGGTGGAGCGCGAGC
MV-chim-rev	GCTCGCGCTCCACCTCGCCCAGG
chim162N	GCGAGCGCAATCTGTCGGTCG
chim162Nrev	CGACCGACAGATTGCGCTCGC
chim198R	CAATCGGCGCGGTCGCCGATAAACG
chim198Rrev	CGTTTATCGGCGACCGCGCCGATTG
pMD-chim/start	TGAAGAAGGGCAGCATTCAAAGC
pMD-chim/end	CTGACACCCTCATCAGTGCC
ISXc5-123rev-BsiW	CTGCGTACGCTCGCGAATTAG
ISXc5-Sty5'	GCTACCTAGGAGAATGAAAATCGGCTATGCGCGCG
Resolv-KpnI	CCGGTACCCCTCACTACACGGAGACGTTTATCG

Resolv-NdeI	GCCATATGAAAATCGGCTATGCGCGCG
Resolv-PK(pET)	CCGGTACCCACGGAGACGTTTATCGGCGAC
RG-120ILeu	GCGCGAGCTAATTATCGAGCGTACGATGG
RG-120Ileu-rev	CCATCGTACGCTCGATAATTAGCTCGCGC

4.8 Plasmids

4.8.1 Plasmids used in the present work

Name	Selection marker	Reference	Description
pAK3	Ap^r	Mertens *et al.*, 1988	substrate for inversion test: contains two 25 bp inverted *gix* sites that allow maximum recombination and the enhancer sequence
pMD3E*gin*	Tc^r	Deufel *et al.*, 1997	vector carries the *gin* gene under control of the λ_{pL} promoter; expression can be regulated by the thermosensitive λCI^{857} repressor mutant presented in vector
pUHE25-2ΔCm*fis*	Ap^r	Deufel *et al.*, 1997	carries a *fis* gene under control of the *trc* promoter; vector contains the *lacI*^{q} gene as well that allows for induction of FIS expression by IPTG
pBR322	Ap^r, Tc^r	MBI Fermentas	
pET22b(+)	Ap^r	Novagen	T7 expression vector (for the C-terminal His-tag constructions); was used for recombination proteins overproduction

pET15b	Ap^r	Novagen	T7 expression vector (for the N-terminal His-tag constructions); was used for the FIS overproduction
ptyrTLacZ	Ap^r	Auner *et al.*, 2003	LacZ fusion under *tyr*T promotor
ptyrTLacZΔ61D	Ap^r	Auner *et al.*, 2003	LacZ fusion under *tyr*TD promotor
pCWt11	Tc^r	Liu *et al.*, 1998	contains 2 full ISXc5 *res* sites (standard substrate for resolution)
pACIE	Tc^r	Liu *et al.*, 1998	contains recombination enhancer and 2 ISXc5 *res* subsites I as inverted repeats
pTnpR991 ISXc5ΔC	Ap^r	Schneider *et al.*, 2000	contains ISXc5 resolvase with the 100 amino acid deletion in the C-terminal extension
pFSG10Tr (pTrc99A derivate)	Ap^r	Schneider *et al.*, 2000	contains gene of the ISXc5G10 chimera (fusion of Gin and ISXc5ΔC resolvase)

4.8.2 Plasmids constructed in the present work

Name	Selection Marker	Description
pMD3E*g10* (pMD3E*g10**)	Tc^r	in the pMD3gin the *gin* gene was substituted by gene of ISXc5G10 chimera or * - its mutants
pMD3E*ISXc5ΔC*	Tc^r	in the pMD3gin the *gin* gene was substituted by gene of ISXc5ΔC resolvase

pMD3E*rc*	Tc^r	in the pMD3gin the *gin* gene was substituted by a gene of reverse chimera - RC (ISXc5/Gin fusion)
pE3 (ptyrT derivate)	Ap^r	recombination substrate; contains 2 ISXc5 *res* subsites I as inverted repeats flanking *lacZ* gene presented in "off" orientation
p17i (ptyrTD derivate)	Ap^r	recombination substrate with 2 inverted ISXc5 *res* subsites I, one of which is inserted into the "off" oriented *lacZ* gene
pFres-dir (pBR322 derivate)	Ap^r	contains 2 ISXc5 full *res* sites as direct repeats
pFres-inv (pBR322 derivate)	Ap^r	contains 2 ISXc5 full *res* sites as inverted repeats

The identity of all constructs was confirmed by sequencing.

4.8.2.1 Description of the plasmids construction

pMD3E*g10*

Gene of the G10 chimera was amplified form the pFSG10Tr (Schneider *et al.,* 2000) with the oligonucleotides Gin-Sty-5' and Chim-Kpn-HindIII (see 4.7). The resulted PCR product was digested with *Avr*II and *Kpn*I restriction enzymes and cloned into the corresponding sites of pMD3E*gin* plasmid replacing the *gin* gene.

pMD3E*g10* – mutants

a) N mutants

For generating substitutions in the C-terminal end of the g10 chimera using the homologous region of gin, the megaprimer method (4.10.6.1.2) was used. In the first PCR the Gin-KpnI oligonucleotides (4.7) were used as a 3' in combination with the oligonucleotides listed below for each construction:

N1	5'-GCTGTACAGCTACCTGCGGG**CGAAACGAGCGCATATAG**-3'
N2-aH	5'-CGCAGAAACTGCACTTGTCCCTG**TCAACTCTGTATAAAAAACACC**-3'
N3	5'-CTGCCATTGCGCAGAAACTG**GATGTGGCCCTGTCAACTCTG**-3'
N4-aG	5'-CGAGACCCTATACCGCGAGC**GCAAGCAGGTTGCATTGATC**-3'
N5	5'-CGCTGGCGGCCGAGACC**CAAGGAATCCCCCGCAAGC**-3'
N6-aF	5'-GGCGACCCAAGGGCCTGAC**CAAAGCGGAATGGGAGCAG**-3'

Oligonucleotide sequence which anneals in *gin* is indicated in bold, in *g10* chimera – underlined.

PCR products were used as megaprimers (see 4.10.6.1.2) for the second PCR with Gin-Sty-5' oligonucleotides. The resulting PCRII products were digested with *Avr*II and *Kpn*I restriction enzymes and cloned into the corresponding sites of pMD3E*gin* plasmid replacing the *gin* gene.

b) Chim-135, Chim-145, Chim-155

For the generation of the G10 mutants where N-terminal gin part was extended towards C-terminus, the oligos Gin-Sty-5' were used as 5' in combination with one of the following oligos for each construction:

gin-chimera-133	5'-**CACCGACCCTGCCACG**ATTTCTGGCGGCAGCAAG-3'
gin-chimera-143	5'-**CGGCCTGCGGCGACAGTTT**AGGTGGTCGCCCACC-3'
gin-chimera-153	5'-**GTATAGGGTCTCGGCCGCC**CCGGCCTGCTCCCATTC-3'

Oligonucleotide sequence which anneals in *gin* is indicated in bold, in *g10* chimera – underlined sequence.

PCR products were used as megaprimers for the second PCR with Gin-KpnI oligonucleotides. The resulting PCRII products were cloned into the pMD3E vector as described above.

pMD3E*ISXc5ΔC*

Gene of the ISXc5ΔC resolvase was amplified form the pTnpR991 ISXc5ΔC (Schneider *et al.,* 2000) with the oligonucleotides ISXc5-Sty5' and Resolv-KpnI (4.7). The resulting PCR

product was digested with *Avr*II and *Kpn*I restriction enzymes and cloned into the corresponding sites of pMD3E*gin* plasmid replacing the *gin* gene.

pMD3E*rc*

DNA sequences corresponding to the amino acid positions 122 and 123 of the Gin was modified using site-directed mutagenesis method (chapter 4.10.6.1.1) into the *Bsi*WI restriction site, which did not change the amino acid sequence of the protein. For this procedure the following oligonucleotides were used:

gin-BsiWI-1	5'-GAGAACTAATTATCGAG**CGTACG**ATGGCTGGACTTGC-3'
gin-BsiWI-2	5'-GCAAGTCCAGCCAT**CGTACG**CTCGATAATTAGTTCTC-3'

gin original sequence: CGA ACG → modification: CG**T** ACG

Part of the resolvase gene was amplified from the pMD3E*ISXc5ΔC* with ISXc5-Sty5' and ISXc5-123rev-BsiW oligonucleotides (listed in 4.7). The PCR product was digested with *Avr*II and *Bsi*WI restriction enzymes and cloned into the corresponding sites of pMD3E*gin* plasmid replacing the N-terminal part of the *gin* gene.

pMD3E*rc*-100, pMD3E*rc*-111

In the mutants RC 100 and RC 111 the Gin part was extended (residues 100 to 192 and 111 to 192, respectively) using megaprimer method (4.10.6.1.2). In the first PCR the Gin-KpnI oligos were used as a 3' in combination with the oligos listed below for each construction:

Rg100	5'-<u>CCCATCGACACCACCAGCGCCCAAATGG</u>**GGCGTTTTTTCTTCCA CGTTATG**-3'
Rg111	5'-<u>GTGTTCAACCTGTTCGCCACGCTGCTGG</u>**CTGAAATGGAACGAGA ACTAATTATCG**-3'

Oligonucleotide sequence which anneals in *gin* is indicated in bold, in *rc* reverse chimera – underlined sequence.

PCR products were used as megaprimers for the second PCR with ISXc5-Sty5' (see 4.7) oligonucleotides. The resulting PCRII products were cloned into the pMD3E vector as described for pMD3E*ISXc5ΔC*.

Linker insertions into G10 chimera and RC reverse chimera.

DNA sequences corresponding to the amino acid positions 122 and 123 of the G10 chimera were modified using site-directed mutagenesis method (chapter 4.10.6.1.1) to the *Bsi*WI restriction site, which did not change the amino acid sequence of the proteins.

oligonucleotides:

chimera-BsiWI-1	5'-GCTGATTATCGAG**CGTACG**CAGGCCGGGCTGACGG-3'
chimera-BsiWI-2	5'-CCGTCAGCCCGGCCTG**CGTACG**CTCGATAATCAGC-3'

g10 chimera sequence: CGC ACC → modification: CG**T** AC**G**

pMD3E*rc* was constructed with BsiWI restriction site (described above).

Linker fragments of different length (containing sequences coding for 10, 14 and 16 amino acid peptides) were obtained by hybridisation of the complementary oligonucleotides listed below, flanked with the sequences for *Bsi*WI restriction enzyme (underlined):

Linker 10	5'-GAC<u>CGTACG</u>GGATCCGGATCCGGATCCGGATCC<u>CGTACG</u>GTC-3'
Linker 14	5'-GAC<u>CGTACG</u>GGATCCGGAGGATCCGGAGGATCCGGAACGTCC<u>CGTACG</u>AAG-3'
Linker 16	5'-GAC<u>CGTACG</u>GGATCCGGAGGATCCGGAGGATCCGGAGGATCCGGAACGTCC<u>CGTACG</u>AAG-3'

(These oligonucleotides were used as top strands; oligonucleotides used for the bottom strands were complementary and are not indicated. Sequences of the linker fragments were similar to those used by Akopian *et al*. (2003)).

After annealing and *Bsi*WI digestion linker fragments were inserted into the *Bsi*WI restriction sites of the genes of G10 chimera and RC reverse chimera in pMD3E. All constructs were verified by sequencing.

pE3

After annealing of the oligonucleotides carrying the sequence of the ISXc5 *res* subsite I:

res-blunt1	5'-ATCAGAGTTCATAAAAACGATCGTTTTATTGAACCGTT-3'
res-blunt2	5'-AACGGTTCAATAAAACGATCGTTTTTATGAACTCTGAT-3'

the 38 bp DNA fragments were inserted into the ptyrTLacZ as inverted repeats in *Bsp*MII and *Bst*BI restriction sites flanking the *lacZ* gene. The orientation of sites was identified by sequence analysis and the plasmid containing *res* sites as inverted repeats was taken for the further construction.

The enhancer element was amplified from pAK3 with *Pfu* DNA polymerase using the following oligonucleotides:

pAK-enhancer-5'	5'-CGGAGCACTGTCCGACCGC-3'
ENH-start	5'-GATCCAGAGTGCTGATTGGC-3'

The resulting 270 bp fragment was phosphorylated and inserted into the *Eco*RI restriction site (blunted with T4 DNA polymerase) of the construct.

p17i

DNA fragment containing ISXc5 *res* subsite I was obtained by the self-annealing of the following oligonucleotides:

res-BamHI-1	5'-GGGGATCCATCAGAGTTCATAAAAACGATCGTTTTATTG AACCGTTGGGATCCGG-3'
res-BamHI- 2	5'-CCGGATCCCAACGGTTCAATAAAACGATCGTTTTTATGA ACTCTGATGGATCCCC-3'

The fragment was digested with *Bam*HI restriction enzyme and cloned inside of the *lacZ* gene to the *Bam*HI restriction site of the ptyrTLacZΔ61D vector. The obtained constructs were transformed into the CSH50 $CaCl_2$ competent cells following by growing overnight at 37^oC on the YT-Ap-X-Gal plates. All of the colonies were blue as a result of the β-galactosidase expression indicating that the *lacZ* gene was not disturbed by inserting of the short DNA fragment with *res* subsite I. The orientation of the *res* subsite was verified by sequencing. The obtained plasmid was digested with *Bst*BI/*Tth*111I restriction enzyme and the second *res* subsite I carrying DNA fragment was inserted, obtained by the self-annealing of the following oligonucleotides:

res-BB-Tth-1	5'-TTTCGAACAACGGTTCAATAAAACGATCGTTTTTATGA ACTCTGATGACCCAGTCACG-3'
res-BB-Tth-2	5'-CGTGACTGGGTCATCAGAGTTCATAAAAACGATCGTTT TATTGAACCGTTGTTCGAAA-3'

Constructs were transformed into the CSH50 cells and grown on the YT-Ap-X-Gal plates. All obtained colonies were blue; plasmid DNA was purified and constructs were analysed by sequencing.

To position the part of the *lacZ* gene between the *res* subsites into the "off" orientation, an *in vitro* inversion reaction was performed on the supercoiled substrate with the wt Gin in the presence of wt FIS. Obtained plasmids were transformed into the CSH50 cells and grown on the YT-Ap-X-Gal plates. Colonies containing the constructs where part of the *lacZ* gene had been inverted were white. Plasmid DNA was isolated and analysed by sequencing.

The inversion enhancer was introduced as described for the pE3 construction.

pFres

DNA fragments containing ISXc5 full *res* sites were obtained from the pCWt11 (Liu *et al.*, 1998) by *Ear*I and *Blp*I digestion and blunt ending with T4 DNA polymerase and inserted into the *Hind*III and *Pvu*II sites of pBR322 plasmid. Tetracycline gene in pBR322 was destroyed by deletion of the *Eco*RV-*Nru*I fragment.

(In the pFres-inv ISXc5 *res* site in *Pvu*II was inserted as an inverted repeat with respect to the one in *Hind*III site; in pFres-dir – as a direct repeat).

Constructions of the expression plasmids:

a) pET22b-recombination proteins

For the construction of the expression plasmid for the Gin mutants, their genes were amplified from the pMD3E*gin** using oligonucleotides pGin EN and pGin PK (listed in 4.7), digested with *Kpn*I and *Nde*I and cloned into the corresponding restriction sites of the pET22b*gin* plasmid replacing the *gin* gene.

Genes of ISXc5G10 chimera and G10 mutants were amplified from the pMD3E using oligonucleotides pGin EN and chimera-PK (see 4.7) and after digestion with *Kpn*I and *Nde*I were inserted into the corresponding restriction sites of the pET22b.

pET22b-ISXc5ΔC resolvase was constructed by the same procedure, using oligonucleotides Resolv-NdeI and Resolv-PK(pET) (see 4.7).

b) pET15b-*fis*

pET15b (Novagen) T7 expression vector was used for the N-terminal 6xHis-tag constructions.

For the overproduction of the FIS protein, the *fis* gene was amplified from pUHE25-2ΔCm*fis* with the following oligonucleotides:

FIS-R1-Nde	5'-GGAATTC**CATATG**TTCGAACAACGCGTAAATTC-3'
FIS-Bam-Kpn	5'-GC**GGATCC**GGTACCGTTCATGCCGTATTTTTTC-3'

(the sequence which anneals in *fis* is underlined, restriction sites for *Nde*I and *Bam*HI are marked in bold).

The obtained PCR fragment was digested with *Nde*I/*Bam*HI and cloned into the corresponding sites of pET15b vector.

4.9 Methods of microbiology and genetics

4.9.1 Determination of *E. coli* cell density

Density of cells in a liquid cultural medium was measured photometrically by the Lambda Bio UV-Spectrophotometer (Perkin Elmer) at 600 nm. An optical density of 1 ml of an appropriate pure culture medium at OD_{600} was used as a zero standard. To ensure measuring in linear range, cultures with OD_{600} less than 0.7 were used. In 1 ml of the growing bacterial culture (OD_{600} is around 0.7) amount of cells is approximately corresponds to 1×10^9, depending on the *E. coli* strain and its growth stage.

4.9.2 Cultivation of *E. coli* strains

Bacterial cultures typically were growing at 37^{o}C (the only exceptions were those cell cultures containing vectors for overexpression of recombination proteins, which were growing at 28^{o}C) in the liquid cultural medium shaking at 200 rpm or on the agar-containing medium under aerobic conditions.
Glycerol cultures were prepared using the overnight cell cultures by adding dYT-Glycerol medium to the concentration 1:1 and stored at –80^{o}C.

4.9.3 Transformation of *E. coli* with plasmid DNA

4.9.3.1 $CaCl_2$ transformation

Preparation of *E. coli* $CaCl_2$-competent cells and $CaCl_2$ transformation was performed as described by Cohen *et al.* (1972).
Test transformations with the standard plasmid (pUC18) usually resulted in transformation efficiency over than 1 x 10^6 cells per 1 μg DNA.

4.9.3.2 Electrotransformation by electroporation

Preparation of *E. coli* electroporation-competent cells and electrotransformation was performed according to the protocol for the Electroporation device (BioRad).
Test transformations with the standard plasmid (pUC18) usually resulted in transformation efficiency over than 1x10^9 cells per 1 μg DNA.

After transformation bacteria were plated on YT-agar medium supplemented with the appropriate antibiotics and incubated overnight at 37°C (protein expression vectors pMD3E were growing at 28^{o}C).

4.10 Methods of molecular biology

4.10.1 DNA manipulations

Plasmid DNA was isolated by the standard alkaline lysis method (Birnboim, 1983) or using QIAprep miniprep kit (Qiagen). Supercoiled DNA was isolated using Qiagen Plasmid Maxi Kit.
DNA fragments were purified from low melting agarose gels using JETSORB Gel Extraction Kit (GENOMED) or QIAquick Gel Extraction Kit (Qiagen).

Unless otherwise indicated standard molecular biology methods like cleavage of DNA with restriction endonucleases, DNA dephosphorylation, blunt-ending and ligation, DNA ethanol/isopropanol precipitation, agarose gel electrophoresis were performed in accordance to Sambrook *et al.* (1989), or in accordance to protocols developed by manufacturers.

4.10.1.2 Measurement of the DNA concentration

DNA concentration was measured photometrically at the wavelength (λ) 260 nm using the following formula (Sambrook *et al.,* 1989):
for double stranded DNA: $C = A_{260}$ x 50 μg/ml
for single stranded DNA: $C = A_{260}$ x 40 μg/ml

where C - is DNA concentration in μg/ml; A_{260} - absorption at λ = 260 nm.
Measurements were done on BioPhotometer (Eppendorf) devise.

4.10.2 Preparation of the short DNA fragments

1) 160 bp UAS fragment
The 160 bp DNA fragment containing the upstream activating sequence (UAS) region of the *tyr*T promoter containing 3 FIS binding sites (Lazarus & Travers, 1993) was obtained by PCR using the ptyrTLacZ plasmid template with the following oligonucleotides:

UAS RI-5'	5'-GAATTCCTTTGTTTACGGTAATCG -3'
UAS-3'	5'-AAGCGGGGCGCATCATATCA-3'

2) 156 bp res fragment

The 156 bp DNA fragment containing the 34 bp ISXc5 *res* subsite I was obtained by PCR using the pE3 template with the following oligonucleotides:

res-ptyrT-R	5'-GACCTGACCGCAGAAC-3'
res-185-L	5'-GCGTTGGCAAACAGAG-3'

3) 156 bp gix fragment

The 156 bp DNA fragment containing the 34 bp *gix* L site was obtained by PCR using the pAK3 template with the following oligonucleotides:

TVL-153	5'-AATAGGCGTATCACGAGGCCC-3'
TVR-153	5'-AGAACCTGCGTGCAATCCATC-3'

4) 156 bp biotinylated gix fragment

The 156 bp DNA fragment containing the 34 bp *gix* L site was obtained by PCR using the pAK3 template with the TVR-153 oligonucleotide (mentioned above) and 5'-biotinylated oligonucleotide gixTVL156-bio:

gixTVL156-bio	Biotin-5'-GCGTATCACGAGGCCCTTTCGTC-3'

5) 44 bp gix fragment

The 44 bp DNA fragment containing the 34 bp *gix* site was obtained by hybridisation (described in the chapter 4.10.3) of the following complementary oligonucleotides:

gix-C40	5'-GGGATCCCATTATCCAAAACCTCGGTTTACAGGAAACGGTCGAC-3'
gix-C41	5'-GTCGACCGTTTCCTGTAAACCGAGGTTTTGGATAATGGGATCCC-3'

6) 44 bp res fragment

The 44 bp DNA fragment containing the 34 bp ISXc5 *res* subsite I was obtained by hybridisation (4.10.3) of the following complementary oligonucleotides:

res-BspEI-1	5'-GGATCAGAGTTCATAAAAACGATCGTTTTATTGAACCGTTCCGG-3'
res-BspEI-2	5'-CCGGAACGGTTCAATAAAACGATCGTTTTTATGAACTCTGATCC-3'

4.10.3 Hybridisation of complementary oligonucleotides

Two complementary oligonucleotides were hybridised together under the following conditions:
each oligonucleotide – 5 pmol
NaCl – 50-100 mM
TE buffer (pH 8) ad 50µl
Reaction was carried out at 95^{o} for 1 min, followed by 80^{o} for 2 min, cooled down slowly to 60^{o}C and kept for 30 min - 1hr. Salt was removed using the MicroSpin^{TM} G50 columns (Amersham Biosciences).

4.10.4 Radioactive labelling of the DNA fragments

5'-end-labelled fragments were prepared by PCR with one of the primers labelled by γ-^{32}P-dATP using the T4 polynucleotide kinase (PNK).

Reaction volume – 25 µl:

8 pMol	dephosphorylated linear DNA
20 µCi	γ-^{32}P-dATP
2.5 µl	10x PNK buffer (NEB)
5 U	T4 polynucleotide kinase (NEB)
ad 25 µl	H_2O_{dd}

The reaction was carried out at 37^{o}C for 30 min and terminated at 65^{o}C for 20 min. Purification was performed using G10 columns or through native polyacrylamide gel.

Alternatively, restriction fragments were 3'-labelled using Klenow or 5'-labelled using T4 polynucleotide kinase (Sambrook *et al*., 1989).

4.10.5 Extraction of the DNA fragments from the polyacrylamide gel

Purification of the radiolabelled DNA fragments was performed through a native polyacrylamide gel (PAA) gel (percentage of which and migration time were calculated according to the size of the fragments) ("Crash and Shake" method, Sambrook *et al.,* 1989). Samples containing DNA fragments were mixed with the sample buffer (1µl of the 10x buffer to 10µl of the sample) and loaded on the PAA gel. λ DNA digested with *Pst*I and labelled with γ-^{32}P-dATP was used as a length standard. After migration the gel was exposed to the X-ray film for app. 30 sec to identify the positions of DNA fragments to be extracted.
Alternatively, ethidium bromide (EtBr) method of DNA visualisation by UV light (302 nm) was used (Lim & Hunt, 1994).
Gel slices containing the DNA fragments were incubated overnight at 37^{o}C in the elution buffer. To the liquid phase containing DNA 2.5 volumes of 100% C_2H_5OH was added and the DNA was precipitated by centrifugation at 18000 rpm for 30 min. Pellet was washed with 70% C_2H_5OH, precipitated by centrifugation at 18000 rpm for 15 min and resuspended in H_2O_{dd} after drying at RT.

Elution buffer	0.5 M NaAc
	0.2% SDS
	10 mM EDTA

4.10.6 PCR analysis

Standard PCR amplification was performed in 50 µl reaction mix containing 0.1 µg of purified DNA, 50 pmol of each oligonucleotide, 0.1 mM of each of 4 dNTP's and 2U *Taq* polymerase (Boehringer) in 1x *Taq* reaction buffer (Boehringer) according to following program: 1 cycle of 94°C denaturation for 3 min; 30 cycles at [94°C (30 sec), 54°C (1 min), and 72°C (2 min)] and a final elongation cycle of 10 min at 72°C.
As PCR devices the MWG-hybaid (omniGene) and the PTC-100^{TM} Programmable Thermal Controller (MJ Research Inc.) were used.

4.10.6.1 PCR mutagenesis

4.10.6.1.1 Introduction of site-specific mutations using QuikChange XL system (Stratagene)

For the introduction of the site-specific mutations the pMD3E plasmids were used carrying genes of the recombinase proteins.

The introduction of the site-specific mutations into the *gin*, *ISXc5* resolvase and *ISXc5G10* chimera genes were done by the Site Directed Mutagenesis method developed by Stratagene.
Briefly, the kit protocol utilises a supercoiled, double stranded DNA plasmid and two complementary synthetic oligonucleotide primers containing a desired mutation. The oligonucleotide primers extend during temperature cycling by means of the high-fidelity *Pfu* Turbo™ DNA polymerase (Stratagene). The product is then treated with *Dpn*I endonuclease, which cuts fully- or hemi-methylated 5'-GATC-3' sequences in duplex DNA, resulting in the selective digestion of the template DNA. The *in vitro* synthesised and nicked plasmid DNA including the desired mutation is then transformed into *E. coli*.

For the transformation of the pMD3E carrying genes with introduced mutations the XL1–Blue MRF' Kan competent cells (Stratagene) (these are the analogous to the competent cells proposed in QuikChange®XL Site-Directed Mutagenesis Kit XL10-Gold) were used which allow to propagate the Tc^r pMD3E plasmids.

a) Reaction mix:
50 ng template DNA
5 µl of 10x Pfu Turbo reaction buffer
125 ng of oligonucleotide primer #1 (100 ng/µl)
125 ng of oligonucleotide primer #2 (100 ng/µl)
1 µl of dNTP mix (10mM)
double-distilled water (H_2O_{dd}) to a final volume of 50 µl
2.5 U PfuTurbo DNA polymerase

Cycling parameters for the QuikChange® XL method:

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	1 minute/kb of plasmid length
3	1	68°C	7 minutes

b) 1 µl of the *Dpn*I restriction enzyme (10 U/µl) was added directly to each amplification reaction and incubated at 37°C for 1 hr to digest the parental (i.e., the nonmutated, methylated supercoiled dsDNA).

c) Electrotransformation of 2 µl of the *Dpn*I-treated DNA into XL1–Blue MRF´ Kan electrocompetent cells.

Cells were incubated for 30 min in 1 ml of dYT medium and 250 µl of each sample were plated out on YT-Tc agar plates. Plates were incubated at 28^{o}C overnight. Amount of colonies after each transformation was app. 100, and 5 were checked for mutations by sequencing.

4.10.6.1.2 "Megaprimer" method

This method was used for substitution constructions, during generation of gene fusions and for introduction of site-specific mutations according to the protocols described by Landt et al. (1990) and Kuipers et al. (1991). This procedure requires just one mutagenic primer and two universal primers, which may contain convenient restriction sites for cloning. In this method two subsequent amplification rounds are used, the first with the mutagenic oligonucleotide and the antiparallel universal primer and the second one using the purified first fragment as a megaprimer together with the second universal primer, and subsequent digestion and cloning of the fragment.

For the fusion constructions first PCR was done with primer consisting from two parts, one of which (15 and more bp) anneals in the amplified gene, another (15 and more bp) – in the gene to be fused with in combination with N- or C-terminal primers, which anneal at the end of gene. PCR products, containing not annealed overhangs, after purification through agarose gel were used for the amplification in the second PCR reaction as megaprimers (Figure 4.1).

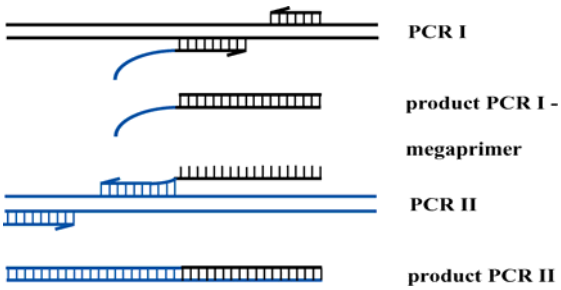


Figure 4.1. General scheme of the PCR megaprimer method.

Using this method the following mutants were generated:
Gin: L17C, C24S, C27A, C24S/C27A, S75C, M100C
G10 chimera: Chim-135, Chim-145, Chim-155, N1, N2, N3, N4, N5, N6
RC reverse chimera: Rg100, Rg111

4.10.6.1.3 Random mutagenesis

For obtaining a high level of mutagenesis two methods were combined: an error-prone PCR method, where the error rate of *Taq* DNA polymerase is further increased by employing PCR reaction buffers containing Mn^{2+} and unbalanced dNTP concentrations in the presence of dITP (Spee *et al.,* 1993; Vartanian *et al.,* 1996; Shafikhani *et al.,* 1997) and a Mutazyme® DNA polymerase (PCR mutagenesis kit, Stratagene). Combination of mutational spectrums of both *Taq* DNA polymerase and Mutazyme® DNA polymerase introduces a more uniform mutational spectrum in which mutations at As and Ts occur at the same frequency as Gs and Cs. (Mutazyme is more likely to mutate G's and C's and generate GC→AT transitions over AT→GC transitions, and *Taq* under error prone conditions is more likely to mutate A's and T's and introduce AT→GC transitions over GC→AT transitions.)
With this method mutation rates of 1–16 mutations per kb can be achieved.
Error-prone PCR conditions: one of the 4 dNTPs is present in limiting amounts in each of 4 separate PCR reactions (in the case of the limiting dNTP misincorporation of one of the other dNTPs is favoured; mutagenesis rate: 8.3 mutations/10^4 bp (Spee *et al.,* 1993). Misincorporation is stimulated when dITP is present, in the next cycle this would result in the incorporation of any of the three natural nucleotides left, as a complement to dITP. Mutagenesis rate: 27.1 mutations/10^4 bp (Spee *et al.,* 1993).

Reaction mix (4 samples for each dNTP mix):

10 ng of a template plasmid

10 µl of 10x Error-prone PCR buffer

10 µl DMSO (for GC rich template regions)

3 µl of 10 mM $MnCl_2$

50 pmol of each oligo, surrounding region to be mutagenised

dNTP mix: 4 µl for each sample: A (20G/2A/20T/20C + 138 H_2O)
T (20G/20A/2T/20C + 138 H_2O)
G (2G/20A/20T/20C + 138 H_2O)
C (20G/20A/20T/2C + 138 H_2O)

200 µM dITP

2 U of *Taq* polymerase

2 U of Mutazyme® DNA polymerase

H_2O_{dd} ad 100 µl

10x Error-prone PCR buffer	100 mM	Tris-HCl, pH 8.3
	500 mM	KCl
	70 mM	$MgCl_2$
	0.1% (w/v)	gelatine

The $MnCl_2$ concentration can be changed to alter the mutagenesis rate according to needs. The above protocol calls for 0.3 mM $MnCl_2$ which is giving an approximate 5 point mutations/kb. (The solution colour has to be clear; brown colouring means it oxidised to Mn^{3+} which will kill the polymerase).

The PCR stock was split into 10 µl aliquots and reactions were run separate; after completion of the reaction they were mixed together. (The separation of PCR master mix into different aliquots prevents the over-representation of single mutations in the library. This is because if a mutation occurs in the first rounds of PCR, then this mutation will be present in most or all of the subsequent products, thereby reducing the diversity of the library).

Cycling parameters:

Segment	Cycles	Temperature	Time
1	1	95°C	2 min
2	30	95°C	30 sec
		60°C	1 min
		72°C	1 min
3	1	72°C	10 min

To increase mutation frequencies up to 20 mutations per kb, sequential PCRs were performed, in which a small aliquot of the first PCR reaction was re-amplified in a second PCR reaction.

PCR products were pooled together and purified trough agarose gel (1% TBE) using Qiagen gel purification kit.

The mutated PCR products were then cloned into an expression vector and the resulting mutant library was screened for changes in protein activity.

4.10.6.1.3.1 Mutagenesis of the *fis* gene

Under error-prone PCR condition the *fis* gene was amplified from pUHE25-2ΔCm*fis* template using following oligonucleotides:

FMRg	5'-GGTGACAGATCTATGTTCGAAC-3'
FM-32	5'-CATCACAAGCTTAAAAAAGGCGCTTCCCC-3'

FMRg primes on the 5'-end of *fis* and contains sequence for the *Bgl*II restriction enzyme and FM-32 primes on the 3'-end of *fis* and contains sequence for the *Hind*III restriction enzyme.

The resulting PCR products were pooled, digested with *Bgl*II/*Hind*III and cloned into the corresponding sites of the pUHE25-2ΔCm*fis* replacing the *fis* wt gene. Ligates were transformed into CSH50*fis*::Kan by electroporation and grown overnight at 37^{o}C on the YT-ampicillin plates. Plasmid DNA from 10 colonies was isolated and analysed by sequencing for verifying the mutation level. All other colonies were pooled together, plasmid DNA was isolated and stored at -20^{o}C.

4.10.6.1.3.2 Mutagenesis of the C-terminal part of the ISXc5G10 chimera

Under error-prone PCR condition the C-terminal part of the gene of the ISXc5G10 chimera was amplified from pMD3E*g10* template using following oligonucleotides:

chimera 117-123 5'-GAGCTGATTATCGAGCGCACC-3'
and Chim-Kpn-HindIII (see 4.7)

Afterwards a second PCR round was performed using the PCR I products as a 5'-end primer (as in megaprimer method, 4.10.6.1.2) with a 5'-end primer Gin-Sty-5' (see 4.7).
PCR fragments were pooled and cloned into *Avr*I/*Kpn*I sites of pMD3E*gin* replacing the *gin* gene. Pool of plasmids was transformed into the WK6 (λcI^+) cells by electroporation. Cells were grown overnight at 28°C on the YT-tetracycline plates. The resulted colonies were pooled and plasmid DNA isolated. Plasmids DNAs from a total of 10 independent clones were isolated and analysed by sequencing, using pMD-chim/start and pMD-chim/end oligonucleotides (see 4.7).

4.10.7 Sequence analysis

Genome sequences were obtained from *E. coli* data banks. Analysis of protein and gene homologies was performed using:
BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/)
and ClustalW on EMBL (http://www2.ebi.ac.uk.clustalw/).

Plasmid sequence and homology analyses were performed using DNA-Strider 1.2 and SequenceNavigator programs.

Plasmid sequencing was performed by MPI for Züchtungsforschung (Cologne) or by automatic sequencing (see below).

4.10.7.1 Automatic sequencing

Sequencing was performed on the Perkin Elmer ABI_{Prism} 377 DNA Sequencer. For the "Thermo-cycling" 20 µl of the mixture containing 0.6-1.2 µg ds-DNA, 10 pmol oligos and

8 µl "Premix" (ApliTag® ABI_{Prism}, which contains Dye-terminator buffer and *Taq* polymerase) was used. Annealing temperature (in the range of 40-60°C) was calculated according to the oligonucleotide sequence and PCR was performed in MJResearch PTC100 device. As following, 10 µl 5M NH_4OAc and 125 µl 100% C_2H_5OH were added to the reaction mix, and DNA was precipitated by centrifugation at 14000rpm for 30 min and resuspended in 4 µl loading buffer (5:1(v/v) formamid:0.5M EDTA, pH 8).
Sequence analyses were done using the Sequence-Navigator program.

4.11 *In vivo* recombination assays

4.11.1 *In vivo* inversion

To analyse inversion *in vivo E. coli* tester strain AD1 (CSH50*fis*::Kan::I^q *oxyR*::*lacZ*inv) (Deufel *et al.,* 1997) was used. In AD1 the *lacZ* gene flanked by inversely oriented *gix* sites is placed in "off" orientation with respect to the P_{Cm} promoter. A productive inversion event places the *lacZ* gene in "on" orientation allowing expression of β-galactosidase from the P_{Cm} promoter (Deufel *et al.,* 1997).
The appropriate expression vectors for FIS (pUHE25-2ΔCm*fis* or *fis* mutants) and for recombination proteins Gin, G10 chimera, RC reverse chimera or their mutants (pMD3E) were subsequently introduced to the tester cells. Expression of FIS is under control of the *trc* promoter. Vector contains the $lacI^q$ gene that allows for induction of gene expression by IPTG. Expression of recombination proteins is under control of the λ_{pL} promoter present in pMD3E (Deufel *et al.,* 1997). Expression can be regulated by the thermosensitive λCI^{857} repressor mutant presented in vector.
Resulting transformants harbouring both plasmids were grown in the presence of appropriate antibiotics at 28°C on X-Gal plates containing 10 µM IPTG for 12 hr (overnight). Recombinational protein expression was repressed during growth of the cells at 28°C. Shifting the culture to 42°C for 2 hr inactivated the repressor and rapidly led to high levels of protein synthesis. Returning the induced cells to 28°C repressed further recombinational protein transcription while allowing recombination to continue. The use of short induction times of usually just a few minute's duration synchronised recombination. The recombinational activity was evaluated by the intensity of the blue colour of colonies.

Colonies remain white when no inversion occurs, whereas a productive inversion gives rise to blue colonies.
When the pool of mutagenised plasmids was used for inversion analysis, plasmid DNA carrying the gene of interest was isolated from single colonies and the phenotype was verified by retransformation of isolated plasmid; the isolated DNA was further sequenced.

Inversion on the ISXc5 *res* subsite I containing p17i substrate was tested in the CSH50 cells under conditions described above.
Additionally, in some cases, inversion of p17i was analysed by restriction with *Aat*II, which normally results in two fragments of 5332 and 983 bp; two additional fragments of 4252 and 2063 bp are generated when inversion has occurred. *Aat*II restriction site is unique in all pMD3E espression vectors, so the resulting linearised fragment did not interfere with the analysis.

4.11.1.1 Selection of the Gin H106T activating FIS mutant

Selection was performed in the *E. coli* inversion tester strain AD1 transformed with plasmid pMD3*gin*H106T carrying *gin* H106T under the control of the temperature sensitive λ_{PL} promoter. The pool of plasmids containing the mutagenised *fis* gene (4.10.6.1.3.1) under the control of an IPTG-inducible promoter (pUHE*fis**) was transformed into this tester system by electroporation. The effect of *fis* mutants was analysed by growing the transformants in the presence of appropriate antibiotics under conditions described above. The recombinational activity was evaluated by the intensity of the blue colour of colonies (as described above). Plasmid DNA from the blue colonies was isolated and retransformed into the tester system for the phenotype verification. When the phenotype was confirmed by the retransformation, the plasmid DNA used for retransformation was sequenced.

4.11.1.2 Selection of the G10 chimera mutants with an inversion-proficient phenotype

Pool of the pMD3E carrying genes of the G10 chimera mutants with a mutagenised C-terminal part were transformed to CSH50 with the test plasmid p17i by electroporation. Cells were grown on the X-Gal plates containing 10 μM IPTG and appropriate antibiotics. After growing under conditions described above, blue colonies indicative of an inversion-

proficient phenotype were collected and plasmid DNA from each colony was isolated. The mutant plasmids were tested a second time for their inversion phenotype by transformation to CSH50 with p17i. In the case of appearance of the blue phenotype, the mutant pMD3E DNA was sequenced.

4.11.2 *In vivo* resolution

In vivo resolution was tested in *E. coli* strain CSH50 on the pFres resolution substrate containing ISXc5 full *res* sites as directed (pFres-dir) or inverted (pFres-inv) repeats.
The appropriate expression vectors (pMD3E) were subsequently introduced to cells harbouring substrates and selection was done on YT-agar plates containing the appropriate antibiotics for plasmid selection under conditions described for inversion test.
Resulting transformation colonies harbouring both plasmids were inoculated into dYT and grown under antibiotic selection to an OD_{600} of 0.3–0.4 at 28^{o}C, with following shift to 42^{o}C for 2 hr and plasmid DNA was isolated. The purified DNA was digested with *Xmn*I, the nicked DNA was separated on a 1% agarose gel in TBE buffer, visualised and photographed under UV light. Restriction of pFres-dir (pFres-inv) results in two fragments of 5015 and 2403 bp; two additional fragments of 3853 bp and 3565 bp are generated when resolution has occurred.
*Xmn*I restriction site is unique in all pMD3E, so the resulting linearised fragment of ~ 6000 bp was migrating over the 5015 bp fragment of the substrate DNA and thus did not interfere with the analysis.

4.12 *In vitro* inversion assay

Inversion reactions were performed in 20 μl at 37^{o}C for 30 min, and the mixture contained 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, DTT (up to 0.5 mM), 1 μg of supercoiled DNA, 100 ng of Gin, and 50 ng of FIS. Reactions were terminated by heating at 80^{o}C for 10 min.
When performed on pAK3 *gix*-containing substrate, the reaction products were analysed by restriction with *Pst*I in the same buffer, followed by phenol/chloroform extraction and electrophoresis through a 2% agarose gel using 0.5x TBE as electrophoresis buffer. After electrophoresis the DNA was stained with EtBr and visualised under UV light.

Restriction of pAK3 results in three fragments of 4206, 1004 and 920 bp. Because of the asymmetric location of the restriction sites with respect to the recombination sites, two additional fragments of 3961 and 1249 bp are generated when inversion has occurred.

When performed on pE3 ISXc5 *res* subsite I containing substrate the reaction products were analysed by restriction with *Eco*RV, followed by electrophoresis through a 1% agarose gel. Restriction of pE3 results in two fragments of 5498 and 1257 bp. In the case of productive inversion two additional fragments of 4682 and 2073 bp are generated.

On the p17i substrate, which contains ISXc5 *res* subsite I - *lacZ* fusion, inversion products were analysed by restriction with *Aat*II followed by electrophoresis through a 1% agarose gel. Restriction of p17i results in two fragments of 5332 and 983 bp; two additional fragments of 4252 and 2063 bp are generated when inversion has occurred.

To measure recombination efficiency, the plasmid DNA was digested with restriction enzymes that distinguished the orientation of the invertible segment and subjected to agarose gel electrophoresis. Quantification was performed using ImageQuant software (Molecular Dynamics).

4.13 Biochemical methods

4.13.1 Preparations of protein extracts

4.13.1.1 Crude extract of FIS

Crude extract of FIS was prepared using CSH50*fis*::Kan cells containing pUHE25-2ΔCm*fis*. Expression of FIS is under control of the *trc* promoter. Vector contains the *lacI*^{q} gene that allows for induction of gene expression by IPTG.
Due to the heat stability of FIS it was possible to apply the following protocol for the quick FIS extract preparation.
1) 2 ml of overnight culture dilute in 50 ml (1:25) of dYT + Ap
2) grow at 37ºC until OD_{600} = 0.4 – 0.7
3) add 200µl IPTG (0.1M), incubate 1hr at 37ºC
4) centrifuge for 10 min at 4000rpm

5) resuspend pellet in 700µl of B50 buffer

6) sonification

7) 5' at 95ºC, cool down

8) centrifuge for 15' at 22000rpm, 4ºC

9) add glycerol (end concentration – 20%) to the supernatant

10) store at -20ºC

B50 buffer 25 mM Tris-HCl, pH 7.5
50 mM NaCl
1 mM EDTA
10% Glycerol
0.2 mM Pefabloc
2 mM DTT

4.13.1.2 Overproduction and purification of proteins with His-tags

Proteins were expressed using the T7 expression system pET22b (for the overproduction of recombinase proteins and their mutants) and pET15b (for the overproduction of FIS and FIS mutants). *E. coli* BL21(DE3)cells were transformed with the plasmids and selected at 37^{o}C on YT plates containing 100 µg/ml ampicillin. A single colony was picked into 100 ml of dYT medium containing 100 µg/ml ampicillin, and grown at 37^{o}C until a density of approximately OD_{600} = 0.3 - 0.5 was reached, and the protein expression was induced by IPTG at a final concentration of 0.2 mM. Growth was induced for a further 2 hr and the cell pellet was obtained by centrifugation at 4^{o}C at 6000 rpm for 10 min. Pellet was washed with 10 ml of cold binding buffer, centrifuged again and kept at –20^{o}C for 1 hr or overnight. As following, pellet was resuspended in 4 ml of binding buffer, cells were lysed by a 2-3 French press steps and the lysates were centrifuged at 4^{o}C for 60 min at 22000 rpm. Purification of the 6xHis-tagged proteins from the cleared lysates was performed on nickel-sepharose matrix columns (Novagen) according to the Novagen protocol with some modifications.

Ni-sepharose material was prepared for use as following:

- washed 2x with 2 volumes of H_2O_{dd}
- charged with 5 volumes of 50 mM $NiSO_4$

- washed 2x with 4 volumes of binding buffer

After every step material was centrifuged for 1min at 1000 rpm.

Lysates were applied to the columns and the proteins were bond to the material during flow through. Upon binding washing with 5 volumes of binding buffer and 10 volumes of washing buffer was performed, and proteins were dissociated from the material with elution buffer. The protein samples in the eluates were concentrated and dialysed against GVP buffer using dialysis membrane (Pierce) with a 10 kDa molecular weight cut-off and stored at -80^{o}C.

Binding buffer	5 mM imidazole	GVP buffer	0.1 mM ED TA
	500 mM NaCl		0.1 mM DTT
	20 mM Tris-HCl, pH 7.9		0.5 M NaCl
0.2 mM proteinase-inhibitor (Boehringer) was added to the buffer before use			0.1% Triton X-100
			20 mM Tris-HCl, pH 7.5
			50% glycerol
Washing buffer	50 – 200 mM imidazole*		0.2 mM Pefabloc
	500 mM NaCl		
	20 mM Tris-HCl, pH 7.9		
Elution buffer	500 mM-1 M imidazole*		
	500 mM NaCl		
	20 mM Tris-HCl, pH 7.9		

*Imidazole concentrations used were adjusted depending on the particular protein under purification.

4.13.2 Determination of protein concentration

Protein concentrations were determined using a Micro BCA Protein Reagent Assay Kit (Pierce, USA). Relative concentrations of proteins were estimated either by comparing Coomassie stained samples (4.13.3.1.1) with standards after SDS-PAGE or by the Bradford dye-binding assay (Bradford, 1976).

4.13.3 Protein gel electrophoresis

4.13.3.1 Denaturing SDS – polyacrylamide protein gel electrophoresis

Separation of the proteins by molecular weight was performed by SDS-containing polyacrylamide (PAA) gel electrophoresis according to Laemmli *et al.* (1970) in the minigel vertical apparatus (Biorad).

Solution	Separating gel (end concentration)	Stacking gel (end concentration)
1 M Tris-HCl, pH 8.8	375 mM	-
1 M Tris-HCl, pH 6.8	-	125 mM
acrylamide	15 - 18% (w/v)	4% (w/v)
bisacrylamide	0.4% (w/v)	0.08% (w/v)
10% SDS	0.1% (w/v)	0.1%
H_2O	ad desired volume	ad desired volume

0.05% (w/v) APS and 0.0025% (v/v) TEMED were used for the gel polymerisation.

Before loading on the gel samples were mixed with 1 volume of 2x "cracking buffer" and incubated at 95^{o}C for 2 min in Thermomixer (Eppendorf). Electrophoresis was performed at 200 V for 1 hr in protein gel buffer.

Protein gel buffer	25 mM	Tris-HCl, pH 8.5
	200 mM	glycine
	1% (w/v)	SDS
2x Cracking buffer	125 mM	Tris-HCl, pH 6.8
	4% (w/v)	SDS
	20% (v/v)	glycerol
	10% (w/v)	β-mercaptoethanol
	0.002% (w/v)	Bromophenol Blue

4.13.3.1.1 Staining of PAA gels with Coomassie Brilliant Blue

The protein gel was stained for 1 hr in a staining solution which was prewarmed to ~50°C to accelerate the process. Afterwards the gels were destained for 1 hr in destaining solution (prewarmed to ~50°C).

Staining solution	225 ml	H_2O
	225 ml	methanol
	50 ml	acetic acid
	1.25 g	Coomassie Brilliant Blue R 250 (Serva)

filtered through 1 mm Whatman filter.

Destaining solution	450 ml	H_2O
	450 ml	methanol
	100 ml	acetic acid

4.13.3.2 Gel retardation assay

Binding of the proteins to the DNA fragments was studied in native PAA gels (Fried & Crothers, 1981) by comparison of the electrophoretic mobility of the protein-DNA complexes with that of the free DNA.

The standard reaction mix (20 µl) contains:

- 100 cps (2 to 5 nM) of γ-P^{32}-ATP end-labelled DNA fragment
- different concentration of proteins (as indicated for each experiment) in 2 µl GVP buffer
- 2 µl of a 10x binding buffer

10x Binding buffer	10 mM	EDTA
	250 mM	Tris-HCl, pH 7.9
	500 mM	NaCl
	20 mM	DTT
	0.1 mg/ml	stDNA
	5 mg/ml	BSA

After incubation for 20 min at 37°C the reaction was stopped by adding 3 µl of DNA loading buffer and the samples were loaded on the native 4-8 % PAA gel (gel size: 180x200x1.5 mm) running at 100V. Gel-electrophoresis was performed in 0.25x TBE as the electrophoresis buffer. Gels were dried and the complexes were visualised either by autoradiography or by phosphorimaging using the Strom 860 PhosphorImager (Molecular Dynamics).
Quantification was performed using the ImageQuant software (Molecular Dynamics).

Native PAA gel	4-8% (w/v)	acrylamide
	0.16% (w/v)	bisacrylamide
	0.1% (w/v)	APS
	0.05% (v/v)	TEMED
	0.25x	TBE

4.13.4 DNase I protection assay

For DNase I footprinting analysis the 156 bp *res*- or *gix*-containing DNA fragments obtained by PCR amplification with γ-P^{32}-dATP-labelled oligonucleotides which were complementary to the top or the bottom DNA strand were used. After purification through 6% polyacrylamide gel (4.10.5), a constant amount the radiolabelled DNA was incubated with increasing amounts of proteins, as indicated for each experiment, under the conditions used for DNA band-shift analysis. DNA was analysed by electrophoresis through 6% (w/v) polyacrylamide sequencing gel.
Corresponding G-ladder sequence (Maxam & Gilbert, 1980) was prepared according to the protocol from Sambrook et al. (1989).

Sequencing gel	6% (w/v)	acrylamide
	0.3% (w/v)	bisacrylamide
	8 M	urea
	0.1% (v/v)	TEMED
	0.4%	APS
	in 0.5x TBE buffer	

4.13.5 Tetramerisation assay

For the formation of the tetramers proteins were incubated under conditions described for the gel retardation assay (4.2.3.3.2.) with the mix of app.:

8.5 fmol of γ-P^{32}-dATP-labelled 156 bp DNA fragment
126 fmol of "cold" 44 bp DNA fragment

To increase dimer/tetramer formation the DDT/DEB were added to the reaction mix up to the final concentration: DEB - 50 mM and DTT - 10 mM. Incubation time was 1 min.

Gel electrophoresis was performed on 6% PAA as described for the gel-retardation assay (4.13.3.2.). Gels were dried and the complexes were visualised either by autoradiography or by phosphorimaging using the Strom 860 PhosphorImager (Molecular Dynamics). Quantification of the dimer/tetramer ratio was performed using the ImageQuant software (Molecular Dynamics).

4.13.6 Protein cross-linking

All cross-linking experiments were performed in a total volume of 12 µl with 370 nM Gin. The cross-linking reactions on the cysteine residues (0 Å) were performed by diluting Gin in a TMN buffer containing 20 mM Tris (pH 7.6), 10 mM $MgCl_2$ and 300 mM NaCl without DTT. The mixture was incubated for 5 min at 37^{o}C after which 4 µl protein sample buffer (50 mM Tris (pH 6.8), 1% SDS, 5% glycerol, and 0.0025% bromphenol blue) without β-mercaptoethanol was added. The samples were boiled for 5 min before loading on gel. The DEB (4 Å) protein cross-linking reactions were carried out in TNM buffer with or without 10 mM DTT. The samples were preincubated for 5 min at 37^{o}C after which DEB in 50 mM triethanolamine (pH 7.6) was added to the final concentration 50 mM and the incubation was continued for additional 5 min. The cross-linking reaction was stopped by adding 200 mM Tris (pH 7.6), incubation was continued for 2 min at 37^{o}C, loading buffer with or without 8% v/v β-mercaptoethanol was added and the samples were boiled for 5 min before loading on gel.

4.13.7 Transfer of proteins onto nitrocellulose membranes: semi-dry blotting system

The semi-dry transfer of proteins from the acrylamide gel onto a nitrocellulose PVDF membrane (Immobilon P, 0.45 μm, Millipore) was done with an electroblotting apparatus CarboGlas (Schleicher & Schüll). On the bottom (anode part) of the apparatus two filter papers (Whatman GB004) soaked with anode buffer A1 were placed and covered with another filter paper soaked with anode buffer A2 and then with a nitrocellulose membrane preincubated for 10 min with the anode buffer A2. The PAA gel was placed upon the nitrocellulose membrane and covered with three filter papers soaked in cathode buffer K. Transfer was done at 4°C at 0.8 mA/cm^2 for 1 hr. Afterwards the membrane was reversibly stained with Ponceau S (Sigma).

Anode buffer A1	300 mM Tris, pH 10.4; 10% Methanol
Anode buffer A2	25 mM Tris, pH 10.4; 10% Methanol
Cathode buffer K	25 mM Tris; 40 mM ε-aminocaproic acid; pH 9.4; 20% Methanol

4.13.7.1 Staining membranes with Ponceau S

For detection of proteins on the membrane after transfer manipulations, the membranes were incubated in Ponceau S solution for 15 min at room temperature under constant agitation. The membranes were rinsed in water and the position of proteins and molecular weight standard were marked.

Ponceau S solution	0.2% (w/v)	Ponceau S
	1.0%	acetic acid

4.13.8 Immunodetection of proteins in Western blot using horseradish peroxidase-conjugated antibodies

After electrophoretic transfer of proteins, nitrocellulose membranes were blocked with 5% milk/TBST for 1 hr at room temperature and then incubated with the first antibodies (Gin rabbit polyclonal antibodies or mouse His-tag specific antibodies) diluted to the desired

concentration in 5% milk/TBST for 2 hr at room temperature or overnight at 4°C. After 3x washing for 10 min in 1% milk/TBST the membrane was incubated with the secondary antibody (anti-rabbit or anti-mouse IgG peroxidase-conjugated antibodies, Sigma) diluted in 1% milk/TBST at RT for 30 min. Afterwards the membrane was washed 3x for 15 min with 1% milk/TBST and once with TBS. Detection was performed with the ECL Plus Detection Kit (Amersham) using the manufacturer protocol, or in a mixture of solutions 1 and 2 (1:1) by incubation for 1 min. Then the filters were exposed with X-ray films (Hyperfilm; Amersham). Autoradiograms were digitised with the NIH-IMAGE software and quantified.
To verify the data all experiments were repeated at least three times.

TBST buffer
50 mM Tris-Cl, pH 7.5
150 mM NaCl
0,1% Tween 20

TBS buffer
50 mM Tris-Cl, pH 7.5
150 mM NaCl

Developing solutions:

Solution 1
2.5 mM luminol (in DSMO)
0.4 mM p-coumaric acid (in DMSO)
0.1 M Tris-HCl, pH 8.5

Solution 2
5.4 mM H_2O_2
0.1 M Tris-HCl, pH 8.5

4.13.9 Beads-experiment

Biotinylated 156 bp PCR fragments containing *gix* site (4.10.2) were immobilised to the magnetic streptavidin-covered beads (Dynal®) according to the protocol of the manufacturer. Binding of Gin protein to the DNA fragment was performed in the 1x binding and washing (B&W) buffer at 37°C for 10 min followed by 3 washing steps with 100 µl of 1x B&W buffer. As a result complexes were obtained where one Gin dimer was bound to the *gix* site of each fragment. Then G10 chimera and ISXc5 resolvase proteins were added each bound to the 44 bp DNA fragment carrying ISXc5 *res* subsite I. After incubation at 37°C for 30 min all liquid supernatants were removed and beads were washed

3 times with 50 µl of 1x B&W buffer in order to remove all unbound proteins (in the case of G10 and ISXc5 the supernatants were kept for the comparison of the amounts of unbound proteins). In the next step biotin-streptavidin interactions on the beads were destroyed by incubation of the samples at the 95^{o}C for 5 min in the buffer containing SDS and β-Mercaptoethanol and the beads were separated from the reaction mix by short centrifugation. Aliquots of supernatants were loaded on the denaturing PAA gel and analysed by Western blot using the His-tag-specific antibodies.

2x Binding and washing (B&W) buffer	10 mM Tris-HCl pH 7.5 1 mM EDTA 2 M NaCl

5. SUMMARY

The Gin inversion system of bacteriophage Mu requires the formation of a synaptic complex of unique topology, where the two Gin dimers bound at the recombination *gix* sites are interacting to form an enzymatically active tetramer, which then catalyses the site-specific recombination reaction. After the assembly of the synaptic complex the DNA strand cleavage is activated by the DNA-bending protein FIS bound at the recombinational enhancer sequence. During reaction the complex undergoes conformational changes resulting in a site-specific inversion of a DNA segment in the phage Mu genome.

In this thesis the protein interactions in the synaptic complex were analysed. First, the question on the interactions between FIS and Gin during formation of the synaptic complex was addressed. In a genetic test system a mutant *fis*S14P has been selected that can rescue the recombination-deficient phenotype of the mutant Gin H106T. FIS S14P was shown to activate the Gin H106T mutant *in vivo* but not *in vitro*. The possible reasons are the differences in the *in vivo* and *in vitro* conditions, and the observed altered DNA bending ability of the FIS S14P mutant. The position of the mutation S14P in the "β-hairpin arm" of the FIS N-terminus suggests it could directly interact with the hydrophobic dimerisation interface of Gin around the position H106.

Next, the predictions of the preliminary model of the Gin invertasome organisation have been verified and the catalytic domains of Gin were demonstrated indeed to be involved in tetramer formation. To do this, specific mutations at the proposed synaptic interfaces were introduced and biochemical studies of different mutants of Gin invertase affected in their ability to promote synapsis were performed. It was possible to show that in addition to the already identified surfaces of the Gin dimer-dimer interactions, comprising of the αE helix and the flexible loop between the β2 sheet and the αB helix of Gin, also the αD helix and the loop between αA helix and β2 sheet are involved in the stabilisation of the Gin tetramer. Cysteine substitutions placed on these surfaces could be efficiently cross-linked in the tetramer in the presence of DNA and FIS, indicating their close proximity in the synapse.

Furthermore, Gin mutants with either increased or decreased tetramerisation abilities were isolated and characterised, and the effects of these mutations on recombination were studied. These data led to the notion that the tetramer structure should be flexible, since all mutations that stabilise the complex cause inversion deficiency. In turn, the complexes

formed by the hyperactive mutants seem to have high conformational flexibility, although at the expense of the loss of specificity. Notably, introduction of substitutions that stabilise the Gin tetramer also lead to suppression of hyperactive features.
A chimeric recombinase protein, containing the N-terminal catalytic domain from Gin and the DNA-binding domain of ISXc5 resolvase, was found to form a more stable tetramer complex, than Gin. The chimera ISXc5G10 is inversion deficient, but can still catalyse resolution. Again, these observations support the notion that the stabilisation of the tetramer can strongly impair the ability to catalyse inversion, but may have less effect on the resolution activity. The DNA-binding domain of ISXc5G10 chimera was mutagenised to obtain a protein with an inversion proficient phenotype, but no mutants of this type could be found, perhaps because in the chimera not only the DNA binding domain, but the gross organisation of the protein is different.

Thus, according to the obtained data the Gin dimers bound to the recombination sites are interacting with each other via catalytic domains and recombination involves gross reorganisations of contact surfaces. The obtained results allowed to clearly distinguish between the two previously proposed mechanistically different models of recombination (the "subunit exchange" and "static subunits" models), and favour the “subunit exchange" model. Such a model serves as a useful working hypothesis for future experiments dedicated to the detailed understanding of the mechanism of recombination reaction catalysis by members of the serine recombinase family.

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