Gin Invertase of Bacteriophage Mu Is a Dimer in Solution, with the Domain for Dimerization in the N-Terminal Part of the Protein†

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ABSTRACT: The Gin protein of bacteriophage Mu mediates recombination between two inverted repeat sequences. Gin binds as a dimer to each of these recombination sites. We show that Gin is a dimer in solution also, and that the dimerization is probably stabilized by hydrophobic interactions between the subunits. The subunits of the dimer could efficiently be cross-linked with the 4-Å cross-linker diepoxybutane. Spontaneous oxidation of Cys(24) and/or Cys(27) also resulted in intersubunit cross-linking. One or both cysteine residues are located at the interface of the Gin dimer, which maps the dimerization domain in the N-terminal part of the protein. 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 form a stable protein−DNA complex. In the protein−DNA complex, however, oxidation of cysteine residues still seems to be possible, indicating that the N-terminal parts of two Gin subunits are also in close proximity when bound to DNA.

DNA inversion reactions are site-specific recombination reactions used by different organisms to allow alternate expression of different sets of genes [for a recent review, see Van de Putte and Goosen (1992)]. The inversion system of bacteriophage Mu, which results in a switch of host range specificity (Van de Putte *et al.*, 1980), belongs to a family of closely related systems including the phase-variation system of *Salmonella typhimurium* (Zieg & Simon, 1980), the host range switch of phage P1 (Iida *et al.*, 1982), the inversion system of the defective *Escherichia coli* prophage e14 (Plasterk *et al.*, 1983; Van de Putte *et al.*, 1984), and the inversion system of the defective *E. coli* phage p15B (Sandmeier *et al.*, 1992).

The inversion reaction of bacteriophage Mu is catalyzed by the 21.7-kDa μ-encoded invertase Gin and is stimulated by the host protein Fis (Koch & Kahmann, 1986; Kanaar *et al.*, 1986). In the first step of the inversion reaction the Gin protein binds as a dimer to the two inversely oriented 34-bp recombination sites which flank the invertible G-segment (Mertens *et al.*, 1988). In the next step a complex is formed between two Gin dimers bound to the inverted repeats and a third DNA sequence, the enhancer, to which the host factor Fis is bound (Kahmann *et al.*, 1985; Bruist *et al.*, 1987). In the active synaptic complex each monomer of Gin introduces a single-stranded nick and remains covalently attached to the 5′-phosphate of the DNA (Klippel *et al.*, 1988), resulting in double-stranded staggered breaks at both recombination sites. Finally, possibly through the exchange of Gin subunits, the actual DNA inversion occurs, followed by religation of the DNA.

The topological changes of the DNA caused by the inversion reaction are well studied (Kanaar *et al.*, 1988, 1989, 1990), but little is known about the interactions between the proteins involved in the inversion reaction. In the synaptic complex two different Gin−Gin contacts can be expected: one involved in formation of the Gin dimer, which binds to each of the recombination sites, and one involved in formation of the tetramer, which brings the two recombination sites together. In this paper we have studied the dimerization of Gin. With gel filtration and protein cross-linking we show that Gin is a dimer in solution and that the cysteine residues at position 24 and/or 27 are located at the dimer interface.

MATERIALS AND METHODS

Chemicals. Diepoxybutane (DEB)[1] was obtained from Aldrich Chemie; iodoacetamide (IAA) and 2-nitro-5-thiocyanobenzoic acid (NTCB) were purchased from Sigma; *N*-chlorosuccinimide (NCS) was obtained from Merck.

Bacterial Strains and Plasmids. The strain used for overproduction of Gin is PP3090. This strain was constructed by introduction of the *fis::kan* mutation from MC1000-*fis767* (Johnson *et al.*, 1988) in BL21::DE3 [*E. coli* B, *ompT*, r_b^-,m_b^-] (Studier & Moffatt, 1986) by P1 transduction. The Gin overproducing plasmid pRKs9 (kindly provided by R. Kanaar) contains the *gin* gene in pET11a (Studier *et al.*, 1990) under control of the T7 promoter. In this construct an *Nde*I site was introduced at the translation start site of the *gin* gene, thereby changing the first codon of the gene from a GTG to an ATG. Plasmid pGP261 used for the retardation assay is a pBR derivative with one Gin recombination site on a 650-bp *Eco*RI fragment. Plasmid pGP273 (Kanaar, 1988) containing the two Gin recombination sites separated by 1.6 kb is used as substrate for the inversion assay.

Purification of Gin. PP3090 with pRKs9 was grown to OD_{700} 0.35. Gin expression was induced with 1 mM IPTG,

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[1] Abbreviations: DEB, diepoxybutane; IAA, iodoacetamide; NTCB, 2-nitro-5-thiocyanobenzoic acid; NCS, *N*-chlorosuccinimide.

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and the cells were grown for an additional 2–3 h. Lysis of the cells and purification of Gin was done by following the procedure described by Kanaar *et al.* (1988). The Gin protein was stored at −80 °C in 20 mM Tris (pH 7.6), 10 mM $MgCl_2$, 500 mM NaCl, 2 mM dithiothreitol (DTT), and 50% glycerol.

Protein Gel Electrophoresis and Western Blotting. Protein samples were analyzed on a 15% SDS–polyacrylamide gel (Laemmli, 1970) or a 15% tricine–SDS–polyacrylamide gel (Schäger & von Jagow, 1987). For Western blotting the proteins were electrotransferred to Hybond C (Amersham Life Science) following the procedure of the manufacturer (LKB). The Gin protein was visualized with Gin antibodies using the ECL system (Amersham Life Science).

Gel Filtration Chromatography. A Superose 12 column with a bed volume of 20 mL was equilibrated with a buffer containing 20 mM Tris (pH 7.6), 10 mM $MgCl_2$, and 500 mM NaCl, with or without 1 mM DTT. The column was calibrated using the following markers: dextran blue (M_r 2000 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 45 kDa), carbonic anhydrase (M_r 29 kDa), and cytochrome *c* (M_r 12.4 kDa). To determine the molecular weight of Gin in solution, 25 μg of Gin in the equilibration buffer was loaded on the column and fractions were tested after elution of the column for the presence of Gin by gel electrophoresis followed by Western blot analysis.

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 buffer containing 20 mM Tris (pH 7.6), 10 mM $MgCl_2$, and 300 mM NaCl (TMN) without DTT. The mixture was incubated for 30 min to 1 h at 37 °C, after which 4 μL of protein sample buffer [50 mM Tris (pH 6.8), 1% SDS, 5% glycerol, and 0.0025% bromophenol blue] without β-mercaptoethanol was added. The samples were boiled for 5 min before they were loaded on the gel. The DEB (4 Å) protein cross-linking reactions were carried out in TMN buffer with or without 2 mM DTT. The samples were preincubated for 5 min at 37 °C, after which 70 mM DEB in 50 mM triethanolamine (pH 7.6) was added, and the incubation was continued for an additional 30 min. The cross-linking reaction was stopped by adding 200 mM Tris (pH 7.6). Incubation was continued for 2 min at 37 °C; protein sample buffer with or without 8%, v/v, β-mercaptoethanol was added; and the samples were boiled for 5 min before they were loaded on the gel.

Reaction of the Cysteine Residues with IAA. After unfolding of Gin in TMN with 2 mM DTT and 6 M urea for 40 min at room temperature, the cysteine residues were blocked by the addition of 10 mM IAA and incubation for 45 min at room temperature. After the blocking reaction the incubation mixture was diluted 30-fold in TMN buffer without DTT and urea and incubated at 37 °C for 30 min to 1 h to allow refolding of the protein.

Cleavage at the Cysteine Residues. Cleavage at the cysteine residues was performed by incubation of 200 ng of protein for 16 h in the dark at 37 °C in a total volume of 10 μL in 100 mM $NaBO_3$ (pH 9) with or without 8 M urea and 10 mM NTCB (Jacobson *et al.*, 1973). After incubation the samples were diluted 1:1 in protein sample buffer with β-mercaptoethanol and loaded on a 15% tricine gel, which was followed by Western blot analysis.

Cleavage at Tryptophan Residues. The cleavage of the protein at tryptophan residues was performed essentially as described by Lischwe and Ochs (1982). The (cross-linked) protein was run in the first dimension on a 15% tricine gel. The lanes containing the protein (monomer and dimer) were excised from the gel and incubated under vigorous shaking with H_2O/acetic acid/urea in the ratio of 1:1:1 twice for 10 min, followed by incubation with 15 mM *N*-chlorosuccinimide in the same solution for 2 × 15 min. After these incubations the lanes were washed two times in H_2O for 10 min followed by equilibration in protein sample buffer with or without β-mercaptoethanol for 10 min. The NCS-treated lanes were run in the second dimension on a 15% tricine gel, which was followed by Western blot analysis.

Gel Retardation Assay and Analysis of the Protein in the Protein–DNA Complex. Plasmid pGP261 was digested with *Eco*RI, providing a 650-bp DNA fragment with one recombination site of Gin. Before retardation the protein was oxidized with 10 mM H_2O_2 for 30 min at room temperature or reduced with 10 mM DTT for 30 min at 4 °C. Binding reaction mixtures (18 μL) consisted of 0.7 pmol of DNA and the indicated amounts of protein in 20 mM Tris (pH 7.6), 10 mM $MgCl_2$, and 100 mM NaCl with or without 10 mM DTT. The reaction mixtures were incubated for 20 min at room temperature and subsequently loaded onto a 5% polyacrylamide gel, which was run at 10 mA at room temperature. The gels were stained with ethidium bromide. The retarded bands were excised, incubated in protein sample buffer without β-mercaptoethanol under vigorous shaking for about 15 min, and loaded on a second-dimension 15% tricine gel. This gel was subsequently analyzed by Western blot analysis.

In Vitro Inversion Assay. Inversion reactions were performed in 30 μL at 37 °C for 1 h and contained 20 mM Tris•HCl (pH 7.6), 10 mM $MgCl_2$, 130 mM NaCl, 200 ng of supercoiled pGP273, 100 ng of Gin, and 40 ng of Fis. Reactions were terminated by heating at 65 °C for 10 min. The reaction products were analyzed by restriction with *Bam*HI in the same buffer, followed by phenol/chloroform extraction and electrophoresis through a 1% agarose gel. Restriction of pGP273 results in two fragments of 3400 and 2400 bp. Due to the asymmetric location of the restriction sites with respect to the recombination sites, two additional fragments (3200 and 2600 bp) are generated when inversion has occurred.

Computer Analysis of Hydrophobic and Solvent-Exposed Regions in Gin. Sequence analysis, e.g., hydrophobicity plots and surface probability plots, was done with the program Profilegraph 1.3. Hydrophobicity values were calculated according to the scales of Kyte and Doolittle (1982). The surface probability in the folded state was calculated with the algorithm of Rose (Rose *et al.*, 1985).

RESULTS

Gin Is a Dimer in Solution. To determine the molecular weight of the Gin protein in solution, a gel filtration experiment was performed. The protein was loaded on a Superose 12 gel filtration column, in a buffer containing 500 mM NaCl and 2 mM DTT. After elution of the column the fractions were tested for the presence of the protein using gel electrophoresis followed by Western blot analysis. The results from the gel filtration experiment are depicted in

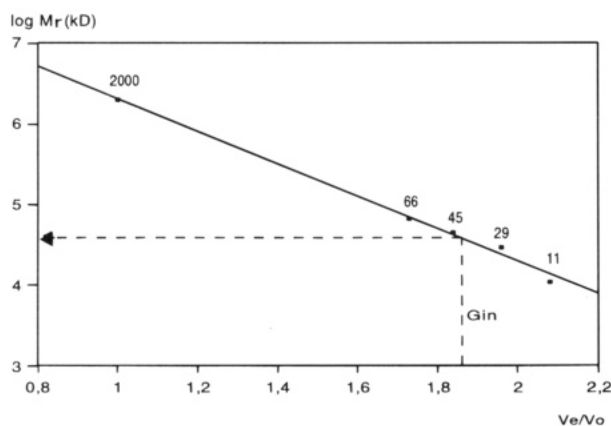

FIGURE 1: Gel filtration of Gin. The molecular weight (M_r) is plotted *versus* the ratio of the elution volume (V_e) to the void volume (V_0). Twenty-five micrograms of Gin was loaded on a Superose 12 column in TMD buffer containing 500 mM NaCl, and 0.5-mL fractions were collected. Western blot analysis of 50 μL of each fraction indicates a peak of approximately M_r 40 000. The positions of markers of dextran blue (M_r 2000 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 45 kDa), and carbonic anhydrase (M_r 29 kDa) and that of eluted Gin are indicated.

Figure 1. Gin is eluted as a single peak with a V_e/V_0 value of 1.86 corresponding to a molecular mass of about 40 kDa. Because the molecular mass of Gin calculated from the amino acid sequence is 21.7 kDa (Plasterk *et al.*, 1983a), we conclude from the results of the gel filtration experiment that under the conditions used Gin is a dimer in solution. Similar results were obtained when Gin was loaded in a buffer without DTT (data not shown).

Cross-Linking of Gin in Solution. To get more information on the domain(s) of Gin involved in dimerization, cross-linking experiments were performed. Because of the high number of lysines equally distributed throughout the Gin protein, cross-linking agents that react with the amino group of lysines were used. Treatment with 10- or 12-Å cross-linkers resulted in formation not only of dimeric proteins but also of multimeric forms (results not shown), indicating that nonspecific cross-links were also formed. Incubation with the 4-Å cross-linker diepoxybutane (DEB), however, resulted in a specific cross-linked product migrating in the gel at the size of the expected dimer (Figure 2A). When the amount of DEB was varied between 0 and 100 mM, the optimum for the amount of cross-linked protein was found at a concentration of 50 mM DEB (Figure 2B). At higher concentrations of DEB all the available lysines have apparently already reacted individually with DEB before cross-linking can occur. To test the specificity of the cross-linking reaction, we varied the concentration of Gin from 230 to 1150 nM using different reaction volumes but keeping the total amount of Gin constant. The amount of cross-linked protein appeared to be independent of the concentration of Gin (results not shown). This indicates that the cross-links are formed within the Gin dimer and not between dimers. Next we varied the NaCl concentration of the incubation mixture from 50 to 500 mM (Figure 2C). The results show that under low-salt conditions the protein can be cross-linked, but to a lesser extent than under high-salt conditions, probably meaning that the cross-linked dimer is stabilized by hydrophobic interactions.

In conclusion, the cross-linking experiments show that at the Gin dimer interface there are at least two lysines at a distance of about 4 Å that can be specifically cross-linked and that the dimerization seems to be stabilized by high-salt concentrations.

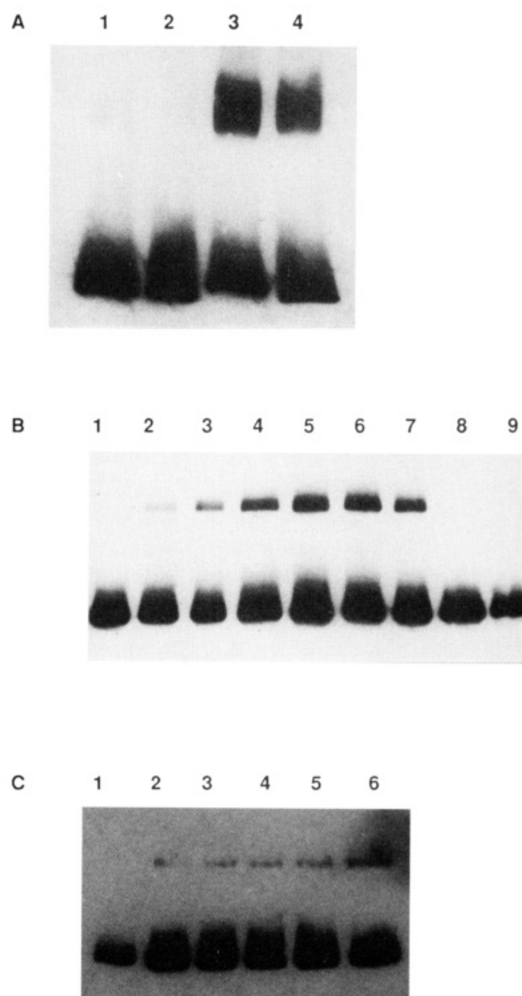

FIGURE 2: DEB cross-linking of Gin. Every lane contains 100 ng of Gin, and the samples were loaded in protein sample buffer without β-mercaptoethanol. (A) Lanes 1 and 2, Gin incubated in TMN with 2 mM DTT; lane 3, Gin in TMN with 2 mM DTT cross-linked with 50 mM DEB; lane 4, Gin in TMN without DTT cross-linked with 50 mM DEB. The sample in lane 1 was loaded without boiling; the samples of lanes 2−4 were boiled for 5 min before loading. (B) Gin cross-linked in TMN with different concentrations of DEB. Lane 1, 2 mM DTT, no DEB; lane 2, no DTT, no DEB; lane 3, 20 mM DEB; lane 4, 30 mM DEB; lane 5, 40 mM DEB; lane 6, 50 mM DEB; lane 7, 60 mM DEB; lane 8, 70 mM DEB; lane 9, 100 mM DEB. The samples in lanes 3−9 all contained 2 mM DTT during the cross-linking reaction. (C) Gin cross-linked with 50 mM DEB in TMD buffer with different concentrations of salt. Lane 1, 50 mM NaCl; lane 2, 100 mM NaCl; lane 3, 200 mM NaCl; lane 4, 300 mM NaCl; lane 5, 400 mM NaCl; lane 6, 500 mM NaCl.

Disulfide Bond Formation between Gin Monomers. Surprisingly, when DEB and DTT were omitted from the reaction mixture also, a product could be detected that migrated at the same position as the DEB cross-linked product (Figure 3A, lane 2). This product is not observed when DTT is present in the incubation mixture (Figure 3A, lane 1) or when the samples are boiled in the presence of β-mercaptoethanol before loading on a gel (result not shown). Therefore this dimeric product is most likely formed by oxidation of cysteine residues. In Gin only two cysteine residues are present both of which are located in the N-terminal domain: one at position 24 and one at position

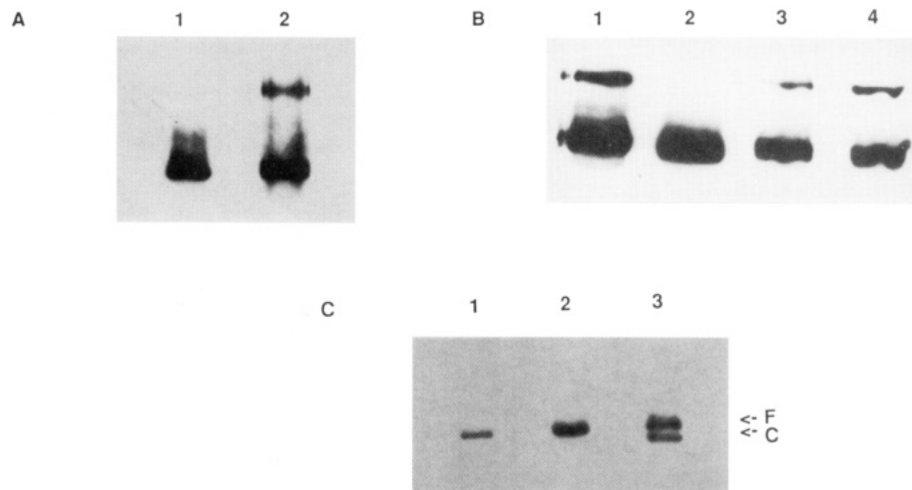

FIGURE 3: Cysteine residues in Gin are located at the dimer interface. (A) Oxidation of Gin. Lane 1, 100 ng of Gin in TMN with 2 mM DTT; lane 2, 100 ng of Gin in TMN without 2 mM DTT. The samples in both lanes were loaded in protein sample buffer without β-mercaptoethanol. (B) Reaction of the cysteine residues with IAA. Incubation of Gin with IAA was done with or without prior unfolding of the protein. The completeness of the reaction with IAA was tested by oxidizing the protein as described in Materials and Methods. Lane 1, unfolding of the protein in 6 M urea followed by refolding and oxidation; lane 2, unfolding of the protein followed by incubation with IAA, refolding, and oxidation; lane 3, oxidation of the protein without prior unfolding or IAA treatment; lane 4, reaction of the protein with IAA without prior unfolding followed by oxidation. (C) Cleavage of Gin at cysteine residues in $NaBO_3$ (pH 9) buffer with NTCB. Lane 1, untreated Gin; lane 2, Gin treated with NTCB in $NaBO_3$ buffer without 8 M urea; lane 3, Gin treated with NTCB in $NaBO_3$ buffer with 8 M urea. The arrows indicate the positions of the full-length protein (F) and the cleaved product (C).

27. The apparent close proximity of one or both of these cysteine residues in the Gin dimer, leading to the formation of a zero-length cross-link between the subunits, strongly suggests that the dimerization domain is located in this N-terminal part of the protein. If indeed the cysteine residues are located at the dimer interface, this could imply that dimerization is shielding these residues from the solution. We tested the accessibility of the cysteine residues in the reduced protein by looking at their ability to react with iodoacetamide (IAA) and with 2-nitro-5-thiocyanobenzoic acid (NTCB). Reaction of the cysteine residues with IAA should block these residues for oxidation. However, after treatment of Gin with IAA the cysteine residues could still be oxidized to form the dimeric product to the same extent as without IAA treatment (Figure 3B, lanes 3 and 4). When the Gin protein was unfolded in 6 M urea before addition of IAA, followed by a refolding of the protein, no oxidation was observed (Figure 3B, lane 2). This indicates that the cysteine residues can only react with IAA if they are made accessible by unfolding the protein. When as a control the Gin protein was unfolded and refolded without the incubation with IAA, oxidation did occur (Figure 3B, lane 1), showing that after the urea treatment the dimer can be reconstituted. A different method to test the accessibility of the cysteine residues in Gin is reaction of the protein with NTCB. NTCB causes cleavage of proteins at cysteine residues (Jacobson *et al.*, 1973). As can be seen from Figure 3C, lanes 2 and 3, cleavage of Gin with NTCB was only possible after unfolding of the protein in 8 M urea. From these experiments we can conclude that the cysteine residues are not exposed in a native Gin dimer. This implies that the observed zero-length cross-link is formed within the dimer and not between dimers and that the cysteine residues that are cross-linked must be located very close to each other at the interface of this dimer. To test whether the activity of the protein was influenced by reaction of the cysteine residues of Gin with IAA, an *in vitro* inversion assay was performed with the IAA-treated protein (Figure 4). Renaturation of Gin after unfolding in urea without IAA treatment resulted in the reconstitution of an active protein that promotes inversion (Figure 4, lane 3). However, reaction of the unfolded protein with IAA followed by refolding resulted in an inactive Gin incapable of the inversion reaction (Figure 4, lane 4). Apparently the coupling of the IAA to the cysteine residues inactivates the Gin protein.

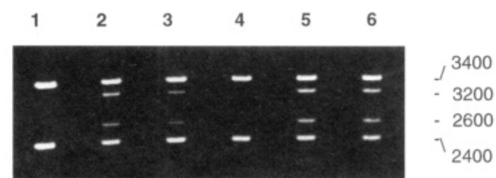

FIGURE 4: *In vitro* inversion assay showing the effect of blocking of the cysteine residues with IAA. pGP273 was incubated with Gin (with or without prior unfolding in 6 M urea and/or reaction with IAA) and Fis. The resulting ethidium bromide-stained gel of the *Bam*HI-digested DNA is shown. Without inversion, two fragments of 3400 and 2400 bp are present. When inversion has occurred, two additional fragments of 3200 and 2600 bp are generated. Lane 1, no Gin; lane 2, untreated Gin; lane 3, Gin after unfolding in urea and refolding; lane 4, Gin after unfolding in urea, blocking with IAA, and refolding; lane 5, Gin without unfolding in urea; lane 6, Gin without unfolding in urea and without blocking with IAA.

Mapping of the Domain in Gin Involved in Cross-Linking with DEB. To test which domain of Gin is involved in the formation of the DEB cross-link, the cross-linked dimer was treated with *N*-chlorosuccinimide (NCS), which cleaves the protein at tryptophan residues (Lischwe & Ochs, 1982). After cleavage, the resulting protein fragments were analyzed. Since each subunit of Gin has two tryptophan residues (positions 61 and 148), treatment of the non-cross-linked protein with NCS is expected to result in three completely cleaved fragments (45, 61, and 87 amino acids), two partially cleaved fragments (132 and 148 amino acids), and the noncleaved Gin (Figure 5A). The results of such an experiment reveal all of these protein fragments except the 61 amino acid fragment. This is probably due to the absence

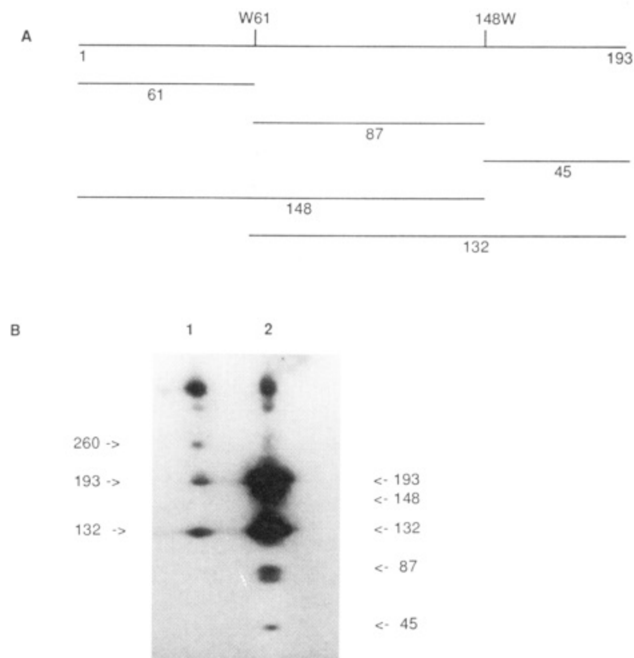

FIGURE 5: Treatment of Gin with *N*-chlorosuccinimide. (A) A linear representation of the 193 amino acid Gin protein. The positions of the tryptophan residues are indicated. The fragments of different amino acid lengths that can be expected when the protein is treated with *N*-chlorosuccinimide are indicated. (B) Lane 1, DEB-cross-linked dimer of Gin treated with NCS; lane 2, monomer of Gin treated with NCS. Only fragments resulting from complete and partial cleavage of Gin with NCS on tryptophan residues are indicated.

of antigenic determinants in this part of the protein. In addition to the expected fragments, products of about 78 and 140 amino acids also can be detected. These products probably result from the low reactivity of NCS with histidine and or tyrosine residues (Ramachandran & Witkop, 1976) present in Gin. When the DEB cross-linked dimer was treated with NCS (Figure 5B, lane 1), the 45 and 87 amino acid fragments are not detectable because of the lower quantity of the dimeric protein. A small amount of a 193 amino acid protein is present, due to partial degradation of the dimer to monomer as a result of the low pH during NCS treatment. The 132 amino acid fragment in the NCS-treated dimer is present, however, in relatively larger amounts with respect to the 193 amino acid protein (lane 1) than in the NCS-cleaved monomer (lane 2), indicating that this fragment is cleaved from the dimer. This implies that no cross-links are formed in the 132 amino acid fragment but that cross-linking has occurred between lysines in the first 61 amino acid of Gin. The additional dimer-specific fragment of about 260 amino acids could then arise from the dimer of 386 amino acids from which one part of 132 amino acids has been cleaved. When the dimer resulting from the oxidation of cysteine residues was also analyzed with NCS, the same two specific products of about 260 and 132 amino acids could be detected (result not shown).

DNA Binding Properties of Oxidized and Reduced Gin. We tested whether the disulfide-bonded Gin dimer was able to bind to a recombination site with gel retardation experiments. Before the retardation, equal amounts of Gin were oxidized with 10 mM H_2O_2 or reduced with 10 mM DTT. The extent of oxidation was determined by loading part of the treated protein on a protein gel followed by Western blot analysis. As can be seen from Figure 6A, lane 2, about 30–50% of the protein is oxidized after the H_2O_2 treatment, while there are hardly any covalent dimers in the reduced protein sample (Figure 6A, lane 1). After the oxidation or reduction of the protein, gel retardation reactions were performed (Figure 6B). Next the retarded DNA fragments were excised from the gel, and the oxidation state of the protein was analyzed on a protein gel. From the results shown in Figure 6C two important observations can be made. First, disulfide bonded protein can be detected in both protein–DNA complexes, although initially hardly any covalent dimers were present in the reduced protein fraction. Second, the percentage of oxidized protein bound to the DNA hardly differs regardless of whether oxidized (lane 1) or reduced (lane 2) protein was used for the retardation. From this last observation we can conclude that there is apparently a preference for the reduced form of the protein to bind to the DNA. The fact that covalent dimers can be isolated from a protein–DNA complex even when the protein used was initially reduced, however, suggests that cross-linking can occur after binding to the DNA. When reduced Gin protein in solution was incubated in protein sample buffer under the

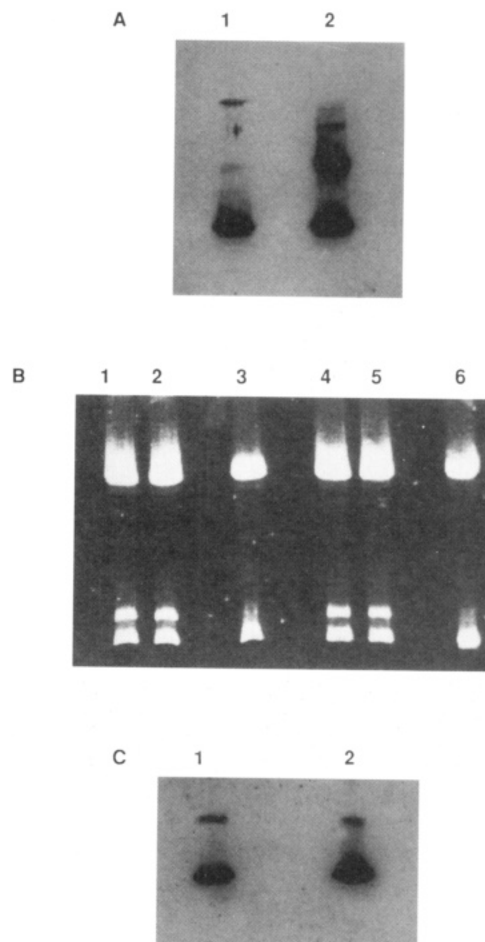

FIGURE 6: Gel retardation experiment with oxidized and reduced Gin. (A) Oxidized and reduced protein before DNA binding. Lane 1, Gin in TM buffer with 500 mM NaCl reduced with 10 mM DTT; lane 2, Gin in TM buffer with 500 mM NaCl oxidized with 10 mM H_2O_2. (B) Gel retardation experiment with oxidized and reduced protein. Lanes 1 and 2, DNA with reduced protein; lanes 3 and 6, DNA without protein; lanes 4 and 5, DNA with oxidized protein. All incubations were in TM buffer with (lanes 1 + 2) or without (lanes 4 + 5) 10 mM DTT and 100 mM NaCl for 20 min at room temperature. (C) DNA-bound protein. Lane 1, protein from the retarded band of the oxidized fraction; lane 2, protein from the retarded band of the reduced fraction.

same conditions as the gel slice with the protein–DNA complex, no covalent dimers could be detected (not shown), which makes it unlikely that the cross-links are formed after excision of the gel slice. We therefore want to propose that two different types of cysteine cross-links can be formed, one that is formed in the Gin dimer in solution and one that is formed on the DNA. This would imply that the structure of the Gin dimer in solution is different from that of the Gin dimer bound to the DNA, but that in both cases the N-terminal parts of the two Gin subunits are in close proximity.

DISCUSSION

In this paper we present evidence that Gin behaves as a dimer in solution. The results of the gel filtration experiment show that Gin, under the conditions used, elutes at the position of a 40-kDa protein. Moreover, dimer-specific cross-links in Gin could be made by keeping the protein under nonreducing conditions, resulting in disulfide bond formation between cysteine residues present in the protein, or by treatment with the 4-Å cross-linker DEB. With both cross-linking methods a product could be detected migrating at the size of the expected dimer on the protein gel. The specificity of the cysteine cross-linking became especially clear when we studied the accessibility of the cysteine residues. Treatment of Gin with IAA or NTCB showed that the cysteines are not solvent exposed. Still they could be oxidized spontaneously, leading to cross-linked dimers. This strongly suggests that the cysteine residues are shielded from the solution because they are situated at the interface of the Gin dimer.

Our observation that Gin behaves as a dimer in solution during the gel filtration experiment is in agreement with the results found earlier with gel filtration experiments under comparable conditions for the homologous Hin invertase (Glasgow *et al.*, 1989) and the rather homologous $\gamma\delta$ resolvase (Liu *et al.*, 1993). For Hin almost the same value of V_0/V_e is found as in our experiments with Gin. It has been published earlier by Mertens *et al.* (1986), however, that Gin is a monomer in solution. This conclusion was made from both gel filtration and glycerol gradient experiments. The only way we can explain these contradictory results is by the different conditions used in their experiments compared to the conditions used in our experiments. In the gel filtration experiment described by Mertens *et al.* (1986) a low concentration of salt (50 mM) was used. Since our DEB-cross-linking experiments suggest that the Gin dimer is stabilized by high-salt concentrations, one might expect to find monomers under low-salt conditions. However, when we tried to repeat our gel filtration in low salt (50 or 125 mM NaCl), the protein no longer eluted from the column. This is most likely due to the formation of aggregates since it has been shown that Gin, like the Hin and resolvase proteins, precipitates at low-salt concentrations (Reed, 1981; Krasnow & Cozzarelli, 1983). The glycerol gradient used by Mertens *et al.* (1986) showed that active Gin protein sediments at the position of the monomer at both low- and high-salt concentrations. Possibly the glycerol or the Triton that is present in these gradients disturbs the hydrophobic interactions of the Gin dimer. Interestingly, the glycerol gradient used by the authors cited above also revealed that a fraction of the Gin protein sedimented at the position of the dimer. This fraction, which was shown to be inactive in inversion, probably contains the disulfide-bonded dimers.

The dimer contacts of Gin are at least partly located in the N-terminus of Gin since the only cysteine residues present in the protein are at positions 24 and 27. Also, cleavage of the DEB-cross-linked protein with NCS indicated that the DEB cross-links are made in the N-terminal part (i.e., the first 61 amino acids) of the protein. Candidates for residues involved in the DEB cross-linking are the lysines at positions 34, 46, 50, and 54. In our experiments we never find more than 50% DEB-cross-linked dimers. This could be due to the close proximity of these lysines in the monomer. Possibly the lysines are in a more favorable position for intra-subunit cross-linking and subsequently no longer available for inter-subunit cross-linking.

In crystals of the homologous $\gamma\delta$ resolvase two different types of dimers could be detected, the so-called 1-2 dimer, which has the dimer interface in the C-terminal helix of the catalytic domain, and the so-called 2-3 dimer, with the domain for dimerization in the more N-terminal part of the protein (Sanderson *et al.*, 1990). Biochemical experiments showed that the domain for dimerization of $\gamma\delta$ resolvase both in solution and after binding to the DNA is at the 1-2 dimer interface, whereas the region of the 2-3 dimer is thought to be responsible for the inter-dimer interactions (Hughes *et al.*, 1990, 1993). The domain that is responsible for the dimerization of Gin in solution does not correspond to either of the two regions in $\gamma\delta$ resolvase since the cysteine residues in both the 1-2 and the 2-3 dimer would be too far apart to form a zero-length cross-link. When we compare the protein region of Gin containing the cysteine residues with the corresponding region of the other invertases and resolvases (Figure 7), the homology within the families of invertases or resolvases is very high, but the homology between the families is rather low (Figure 7A). Also when we compare the hydrophobicity and surface probability of Gin and $\gamma\delta$ resolvase (Figure 7B,C), there is a striking difference between the proteins in this particular region. For $\gamma\delta$ resolvase the residues in the region between residues 20 and 30 are predicted to be predominantly solvent exposed (Figure 7B), while in Gin the corresponding region is more hydrophobic and shows a strong dip in the surface probability graph. This is in accordance with our experiments that show that the dimerization is stabilized by hydrophobic interactions and the observation that the cysteine residues are shielded from the solution and therefore not accessible for reaction with IAA or NTCB.

Although the dimerization domains of Gin and $\gamma\delta$ resolvase in solution seem to be different, this does not imply that they are also different on the DNA. Biochemical experiments have indicated that $\gamma\delta$ resolvase also binds to the DNA as a 1-2 dimer (Hughes *et al.*, 1993). Extrapolation of the data obtained from the structure of the DNA binding domain of Hin complexed with DNA (Feng *et al.*, 1994) puts the C-terminal α-helices of the catalytic domains in close proximity on the DNA. It is therefore very likely that Gin, when bound to the DNA, forms 1-2 dimers similar to those of resolvase, which would imply that the dimer as it is in solution is different from the dimer as it binds to the DNA. Still, our gel retardation experiments showed that disulfide-bonded Gin could be isolated from a separated Gin–DNA complex. The amount of covalent dimer, however, did not differ, regardless of whether an oxidized or a reduced fraction

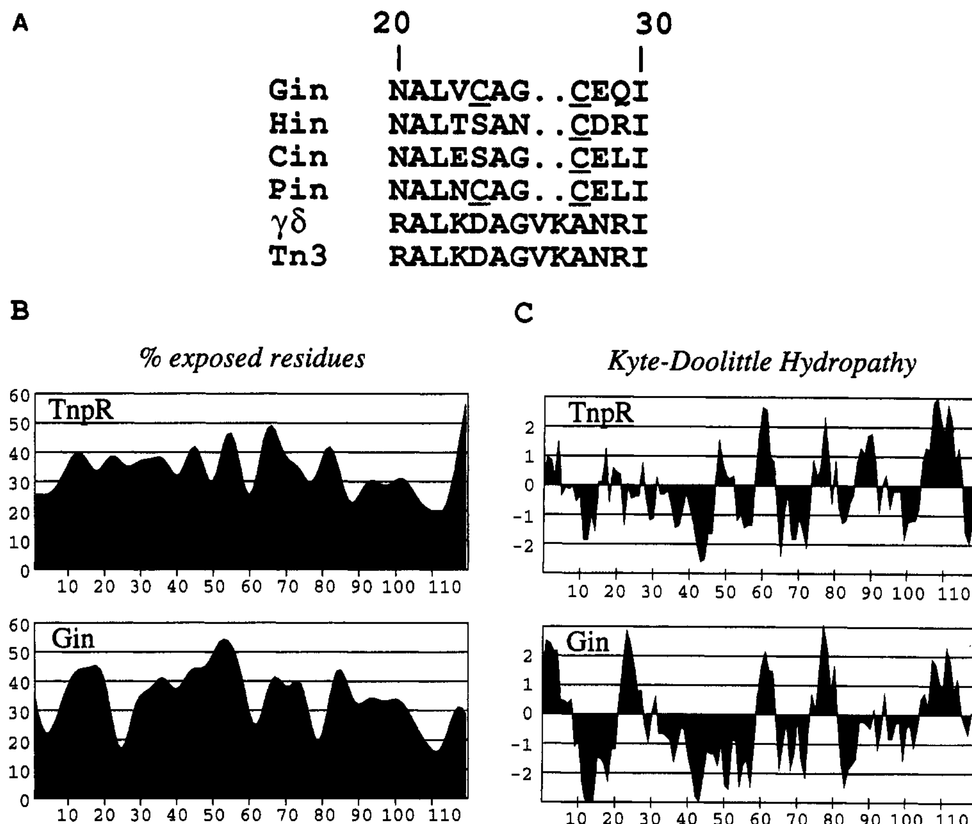

FIGURE 7: Comparison of $\gamma\delta$ resolvase and Gin. Sequences of Gin and $\gamma\delta$ resolvase are aligned in panel A, and only the first 120 amino acids are shown in panels (B) and (C). (A) Sequences of the family of invertases compared with the sequences of the family of resolvases in the region close to the cysteine residues in Gin from amino acid 20 to 30. (B) Surface probability plots. The surface probability in the folded state was calculated with the algorithm of Rose (Rose *et al.*, 1985). (C) Hydrophobicity plots. The hydrophobicity and hydropathy values were calculated according to the scales of Kyte and Doolittle (1982).

of the Gin protein was used for the DNA binding. This indicates that there is a preference for binding of a noncovalent dimer. The subunits of the dimer probably have to reorient slightly upon binding to the DNA, and such motions might be constrained in the covalent dimer. Similar observations were reported with covalent dimers of the λ phage repressor (Benevides *et al.*, 1991). The covalent dimers of this latter protein, which were formed by introduction of a cysteine residue in the dimerization domain of the subunits, showed a 10-fold reduced binding to the operator as compared to the noncovalent form.

Although disulfide bonds inhibit DNA binding of the Gin dimer, the presence of cross-linked dimers in the protein–DNA complexes suggests that cysteine cross-linking can occur after the protein has bound to the DNA. Since there are two cysteine residues in each Gin monomer, three different disulfide bonds can be formed theoretically: Cys(24)–Cys(24), Cys(24)–Cys(27), and Cys(27)–Cys(27). Therefore, the cross-link that is formed in solution could be different from the cross-link formed when Gin is bound to the DNA. Possibly the reorientation of the Gin subunits upon DNA binding places another combination of cysteines in a favorable position for disulfide bond formation. Both in solution and on the DNA, however, the Gin–Gin interactions are in the same N-terminal domain of the protein.

If the dimerization of Gin on the DNA is comparable to that of resolvase but if at the same time in the protein–DNA complex the N-terminal regions of two Gin subunits are in close proximity, this could imply that Gin initially binds not as a dimer but as a tetramer to the DNA. The subunit interaction within the Gin dimer that we observe in solution might then reflect the inter-dimer interactions in the synaptic complex, which are slightly changed upon formation of the 1-2 dimeric contacts. To test the possibility of tetrameric binding, stoichiometric analysis of Gin–DNA complexes would be required, but this, however, is hampered by the presence of inactive protein molecules in Gin preparations (Kanaar *et al.*, 1986; Mertens *et al.*, 1986).

The homologous invertases Hin and Cin contain a serine residue at the position corresponding to Cys(24) in Gin, showing that a cysteine residue at this position is not important for inversion activity. Cys(27), however, seems important for inversion, since it is conserved in all invertases. A mutation in this residue, C27Y, results in a Gin protein that still normally binds to the recombination sites but is inversion deficient (Spaeny-Dekking *et al.*, 1995). Reaction of the cysteines in the unfolded Gin protein with IAA also completely blocks the activity of the protein. Although we cannot exclude that the coupled IAA interferes with the refolding of the protein, the effect might also be more direct by inactivating Cys(27). Nevertheless, we regard it as unlikely that the function of this cysteine in the inversion reaction is to form disulfide bonds, since in the cytoplasm of *E. coli* cells cysteines are maintained in a reduced state by the action of thioredoxin reductase (Derman *et al.*, 1993).

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