Protein Engineering vol.9 no.2 pp.231–238, 1996

Destabilizing interactions between the partners of a bifunctional fusion protein

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Hybrid MalE–GVP is a bifunctional protein *in vitro* since it binds maltose as protein MalE of *Escherichia coli* and since it is dimeric and specifically binds single-stranded DNA as protein GVP of phage M13. The oxidation rate of a unique cysteine residue was used to compare the stabilities of GVP in its free and hybrid forms, under conditions where MalE was either folded or unfolded by a denaturing agent. The results showed that both the covalent link and tertiary non-covalent interactions between MalE and GVP destabilized GVP in MalE–GVP. To test whether GVP had identical structures in its free and hybrid forms, mutations were used as local conformational probes. The effects of these mutations on the capabilities of MalE–GVP to dimerize and to bind single-stranded DNA were assayed *in vitro*. They were compatible with the effects of the same mutations on the global activity of free GVP *in vivo* and with the effects that could be predicted from the known data on free GVP, in particular its crystal structure. Thus, one partner of a hybrid protein can be destabilized by the other partner while maintaining its structural and functional characteristics.

Keywords: hybrid protein/M13 gene *V* protein/maltose-binding protein/protein dimer/stability

Introduction

Fusion proteins (also called hybrid proteins) are widely used in fundamental research and for biotechnological applications, in particular to stabilize proteins, purify them, increase their solubility or immobilize them (Uhlen *et al.*, 1992), and to create new proteins from existing domains or modules. The hybrid combines the main properties of both parental polypeptides in many cases but the interactions between the two partners of a hybrid have been studied very rarely in detail. However, this detailed knowledge is essential for several of the above applications.

Our objective was to study the influence of one partner on the structural and functional properties of the other partner in a hybrid protein. We chose the maltose-binding protein (MalE) of *Escherichia coli* and the single-stranded DNA binding protein (GVP) that is encoded by gene *V* of bacteriophage M13 for this study, because many structural, functional and mutagenesis data are available on these two proteins. In previous work, we have shown that a hybrid (MalE–GVP) between MalE and GVP is exported into the bacterial periplasm and can be purified by affinity chromatography on crosslinked amylose, as MalE; it is homodimeric and binds single-stranded but not double-stranded DNA as GVP (Blondel and Bedouelle, 1990). In the present work, we used the fact that MalE does not contain any cysteine residue and that GVP contains only one, which is buried within the structure, to analyse the influence of MalE on the stability of GVP in hybrid MalE–GVP. We also used mutations of GVP as probes to compare the local structures of its free and hybrid forms.

MalE is involved in the transport of maltose and maltodextrins across the bacterial envelope, and it binds these sugars with a dissociation constant around 1 μM. MalE is initially synthesized with an N-terminal signal peptide which is cleaved during its export to the periplasm (Bedouelle *et al.*, 1980). The mature form of MalE has 370 amino acid residues (Duplay *et al.*, 1984). The three-dimensional structure of MalE has been determined at high resolution by X-ray crystallography (Sharff *et al.*, 1992). The folding of MalE and its relation with export have been studied in detail (Liu *et al.*, 1988). MalE is widely used as a vector for the export and purification of foreign polypeptides and proteins (Bedouelle and Duplay, 1988; Guan *et al.*, 1988; Blondel and Bedouelle, 1990, 1991; Brégégère *et al.*, 1994).

GVP controls the propagation of the filamentous bacteriophages M13, f1 and fd at several levels. It binds cooperatively and stoichiometrically to the single-stranded intermediate during the replication of the phage DNA and, in this way, inhibits the synthesis of the complementary strand. It also binds specifically to the messenger RNAs of several phage genes at the level of their leader sequences, and acts in this way as a translational repressor (for reviews, see Folkers *et al.*, 1994; Folmer *et al.*, 1994; Guan *et al.*, 1994; Skinner *et al.*, 1994; Terwilliger *et al.*, 1994).

The amino acid sequence of GVP has 87 residues (Van Wezenbeek *et al.*, 1980). Experiments on sedimentation through sucrose gradients, sedimentation at equilibrium and chemical crosslinking have shown that GVP exists as a dimer in solution (Oey and Knippers, 1972; Rasched and Pohl, 1974; Pretorius *et al.*, 1975; Cavalieri *et al.*, 1976; Pörschke and Rauh, 1983). The three-dimensional structure of GVP has been determined by NMR spectroscopy and by X-ray crystallography (Folkers *et al.*, 1994; Guan *et al.*, 1994; Skinner *et al.*, 1994). The structures show that two monomers of GVP are intimately associated about a dyad axis and mostly composed of β-structures. The interactions between GVP and nucleic acids have been studied by a large variety of techniques, including chemical modification, fluorescence and NMR spectroscopy, electron microscopy and mutagenesis (for reviews, see Folmer *et al.*, 1994; Guan *et al.*, 1994; Terwilliger *et al.*, 1994). The nucleic acid binding domain is mainly located on two β-loops, each coming from a different monomer: the DNA-binding loop (residues 15–30) and the dyad loop (residues 67–79) (Folkers *et al.*, 1993).

GVP carries a unique cysteine residue, Cys33, which is fully buried in the folded protein and located far from the subunit interface. The chemical reactivity of Cys33 and the circular dichroisms at 211 and 229 nm were used to measure

the fraction of unfolded GVP molecules in experiments of reversible denaturation with guanidinium hydrochloride (GuHCl). The denaturation profiles, obtained from these different measures, superpose and show that Cys33 becomes accessible when the protein loses its secondary structure (Liang and Terwilliger, 1991).

Materials and methods

Strains, media and buffers

Strains JM101 (Yanisch-Perron *et al.*, 1985), RZ1032 (Kunkel *et al.*, 1987), PD28 (Duplay *et al.*, 1987) and HB2200 (Bedouelle and Duplay, 1988), plasmids pDR540 (Russell and Bennett, 1982), pEMBL9$^+$ (Dente and Cortese, 1987), pPD1 (Duplay *et al.*, 1984), pAB1 and pAB2, phages M13AB2 (Blondel and Bedouelle, 1990) and M13KO7 (Vieira and Messing, 1987) and the media used (Miller, 1972) have been described. The media contained 100 μg/ml ampicillin and, where indicated, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). M63 buffer is M63 medium without added sugar. Buffer A is 50 mM Tris–HCl (pH 7.5)–2.5 mM 2-mercaptoethanol; buffer B is 1 mM EDTA in buffer A; buffer C is 50 mM NaCl–1 mM maltose in buffer A; buffer D is 50 mM Tris–HCl (pH 7.5)–50 mM NaCl.

Construction of vectors expressing MalE–GVP and its derivatives

Plasmid pAB2 carries the *malE–gV* hybrid gene, which codes for hybrid MalE–GVP, under control of promoter *malEp*, which is inactive in strain HB2200. Plasmid pAB10 carried gene *V*, which codes for GVP, under control of promoter *Ptac*, which is repressed in strain JM101. Mutations were introduced into GVP and MalE–GVP at the genetic level. The genetic constructions were made in conditions where these two proteins were not produced. We initially created the codon changes in gene *V* by oligonucleotide site-directed mutagenesis of phagemid pAB6, which carried gene *V* without any promoter, using a published method (Kunkel *et al.*, 1987). Subsequently, we transferred the mutated genes *V* from pAB6 into the expression vectors pAB10 and pAB2 (Figure 1). The mutagenic oligonucleotides had the following sequences: GTC CTC TGA CAA AGT TG for mutation F68D, CGG TTC CCT TGA CAT TGA CCG TCT G for mutation M77D and CTC GTT CTG GTG GTG AGC AGC TTT GT for deletion Δ1. We checked the entire DNA sequence of the mutant genes *V*. The linking sequence between *malE* and gene *V* was lengthened by introducing the palindromic oligonucleotide 5′-GATCGAG-GCCTC -3′ into the BamHI site of plasmid pAB2.

Toxicity experiments

The toxicity of free GVP and its mutant derivatives for the producing cells was measured as follows. An isolated colony of strain JM101, grown on minimal glucose medium and containing the wild-type plasmid pAB10 or a mutant derivative, was suspended in 1 ml of M63 buffer; 200 μl samples of serial dilutions were spread on plates of the same medium, supplemented or not with IPTG. The colonies were counted after 48 h of growth at 37°C and the ratio of the colony counts after growth without and with IPTG was calculated. The average value and the standard deviation from three independent experiments are given.

Protein purification

Protein MalE, the wild-type hybrid MalE–GVP(wt) and its mutant derivatives were produced from strains PD28(pPD1), PD28(pAB2) and its mutant derivatives, and they were purified by affinity chromatography on crosslinked amylose as described (Blondel and Bedouelle, 1990). Analysis of the purified fractions by SDS–polyacrylamide gel electrophoresis showed that 80–90% of the molecules were full-length hybrid chains and that the remainder had an apparent molecular mass around 42 kDa and thus corresponded to a hybrid between MalE and the 10 N-terminal residues of GVP. These incomplete molecules did not interfere with our analysis of the hybrids because they did not contain residue Cys33 of GVP, or its DNA-binding site or its interface of dimerization, and because they could be easily identified by gel electrophoresis. MalE–GVP(wt) could be further purified by affinity chromatography on denatured-DNA cellulose, as described (Blondel and Bedouelle, 1990), but its mutant derivatives could not because they did not bind DNA. We performed most experiments both with fully purified (>95% homogeneous) and incompletely purified preparations of MalE–GVP(wt), and obtained identical results. Free GVP was purified as described (Liang and Terwilliger, 1991) and was >95% pure as judged from gel electrophoresis.

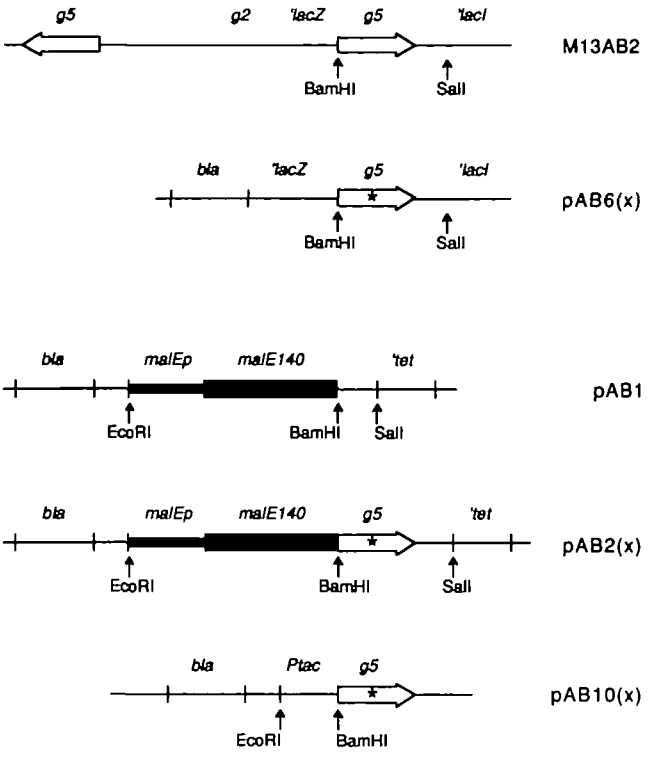

Fig. 1. Constructions of the *malE–gV* hybrid gene and of its mutant derivatives. Phagemid pAB6 was constructed by ligation of the short *BamHI–SalI* restriction fragment, coming from phage M13AB2 and containing gene *V*, between the corresponding sites of phagemid pEMBL9$^+$. pAB6 carries gene *V* without its promoter in an orientation opposite to that of promoter *lacZp*. The mutations of gene *V* were constructed by oligonucleotide site-directed mutagenesis of pAB6. Plasmid pAB2 and its mutant derivatives were constructed by ligation of the short *BamHI–SalI* fragment containing gene *V* and coming from M13AB2 or the mutant derivatives of pAB6, between the corresponding sites of plasmid pAB1. pAB2 and its derivatives carry *malE-gV* under control of promoter *malEp* and code for MalE–GVP. Plasmid pAB10 and its mutant derivatives were constructed by ligation of the *EcoRI–BamHI* fragment of plasmid pDR540 that carries promoter *Ptac*, between the corresponding sites of pAB2 and its derivatives. pAB10 and its derivatives carry gene *V* under control of *Ptac* and code for free GVP. The presence of mutations in the derivatives of pAB2, pAB6 and pAB10 is indicated by the asterisks.

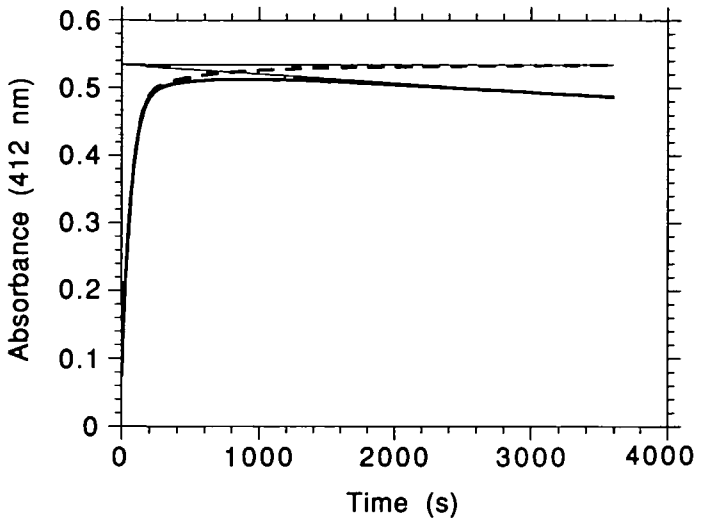

Fig. 2. Analysis of the oxidation kinetics. MalE–GVP(wt) (37.8 μM initial concentration) was oxidized with DTNB as described in Materials and methods, and the variation of $A_{412\ nm}$ with time was followed for 3600 s. The recorded data, $A_{412\ nm}(t)$, are represented with a bold solid line. A linear regression was performed on the data from the last 500 s and gave the thin oblique line with equation $0.534 - 1.313\times10^{-5}t$. The corrected data are represented with a bold dotted line $[A_{412\ nm}(t) + 1.313\times10^{-5}t)$ The new asymptote is given by the thin horizontal line which has an ordinate equal to 0.534. The corrected data from the first 500 s were fitted to an exponential with errors on the amplitude and the relaxation time equal to 0.3% and 0.5%, respectively, in this particular experiment.

Concentrations of the protein fractions

We measured the concentrations in proteins by three different methods. We used the Bio-Rad reagent with bovine serum albumin as a standard. We used $A_{280\ nm}$ with the following values as extinction coefficients (l/g.cm): 1.47 for MalE; 0.73 for GVP; 1.32 for MalE–GVP (Blondel and Bedouelle, 1990). We titrated the unique cysteine of GVP and MalE–GVP with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959) in the presence of 5.6 M GuHCl to unfold the proteins fully. We followed this titration by measuring $A_{412\ nm}$ and taking 13 600 l/mol.cm as the extinction coefficient of nitrothiobenzoate. MalE gave no signal in these experiments of cysteine titration. The values of the concentrations that we obtained with the three methods were in good agreement.

The solutions of protein were concentrated and their buffers were changed by precipitation with ammonium sulphate in 50 mM Tris–HCl (pH 7.5) at 40% saturation for GVP and at 100% saturation for MalE and its hybrid derivatives.

Cysteine oxidation

We used the variation of $A_{412\ nm}$, measured in a double-beam spectrophotometer, to monitor the oxidation of the unique cysteine of MalE–GVP and of GVP by DTNB. The proteins were transferred from their stock buffer into buffer D, which did not contain 2-mercaptoethanol, by precipitation with ammonium sulphate and dialysis against buffer D, as described above. After dilution to the working concentration in the same buffer and addition of 1.25 mM maltose, the protein samples (800 μl) were incubated for 1.5 h at 25°C to enable the monomeric and dimeric forms to reach equilibrium. At the end of this incubation, the oxidation reaction was initiated by addition of 50 μl of a DTNB solution [20 mM DTNB–40 mM Tris–OH (pH 6.5)] in each of the two spectrophotometer cuvettes. The final pH was close to 7.5. We followed the reaction for 10 h for GVP, 1 h for MalE–GVP and 500 s for the experiments performed in totally denaturing conditions (5.6 M GuHCl) at 25°C.

After reaching a maximal value, the absorbance slowly decreased with time, probably owing to reoxidation of reduced DTNB. We adjusted a linear equation to the data corresponding to this second part of the kinetics. The linear term of this equation was subtracted from the experimental absorbance values to give corrected data, and the constant term gave an estimation of the plateau value for these new data (Figure 2). The corrected data could be fitted to a single exponential function. We also determined the plateau value by measuring the variation of absorbance that corresponded to the oxidation of the same preparation of protein in totally denaturing conditions (5.6 M GuHCl). The plateau values obtained by the two methods were in good agreement. The absorbance values that were obtained for MalE–GVP(wt) at early times showed that the dead time of the experiment was about 10 s. In totally denaturing conditions, the plateau was reached within the dead time of the experiment. For free GVP, a precipitate formed during the reaction and interfered with the measurements. In contrast, for hybrid MalE–GVP or in the presence of 1.5 M GuHCl for free GVP, no precipitate formed. When such a precipitate formed or when the reaction was too slow to reach its plateau in a reasonable time, we estimated the relaxation time of the reaction by calculating the ratio between the initial rate and the plateau value, determined in 5.6 M GuHCl.

Centrifugation and gel filtration

The zonal centrifugations were performed in buffer C as described (Blondel and Bedouelle, 1990). Each sample contained bovine liver catalase as a marker. The samples that contained MalE, MalE–GVP(wt) and its three mutant derivatives at the same initial concentration were centrifuged simultaneously in five different tubes. The sedimentation coefficients at 20°C in water ($s_{20,w}$) were calculated from the migration distances (x), measured from the meniscus of the gradients, using the equation $s_{20,w} = k(ax + b)$. Factor k allowed us to correct the position of catalase, which slightly varied from one gradient to another in the same run of centrifugation. We took $k = 1$ for the gradient that contained both MalE ($s_{20,w} = 3.30$ S;

Blondel and Bedouelle, 1990) and catalase (11.10 S; Sober and Harte, 1968).

The gel filtrations were performed in buffer C as described (Blondel and Bedouelle, 1990). The Stokes radii (R_S) were calculated from partition coefficients (K) using a linear relationship. We took $K = (V_t - V_e)/(V_t - V_0)$, where V_t is the elution volume of a small molecule that was present in all the samples, V_e is the elution volume of the protein of interest and V_0 is the void volume of the column. The linear relationship between R_S and K was calibrated as described, using the same standard proteins (Blondel and Bedouelle, 1990).

The errors in $s_{20,w}$ and R_S were evaluated by comparing the published values (Sober and Harte, 1968) with those obtained in our experiments for standard proteins. The initial concentrations of MalE and its derivatives in the samples were 1, 3, 10 or 30 μM. The gradient and elution fractions were analysed by electrophoresis through SDS–polyacrylamide gels. We found that the hybrids were not degraded during the runs.

Results

Oxidation of GVP and MalE–GVP

To compare the stabilities of GVP in its free form and in hybrid MalE–GVP, we looked for a conformational probe that would exist in GVP but not in MalE. We could not use the fluorescence of the aromatic residues because MalE contains both tyrosines and tryptophans whereas GVP contains only tyrosines. We therefore used the oxidation of the unique cysteine of GVP by DTNB since MalE does not contain this type of residue. When free GVP and the wild-type hybrid MalE–GVP(wt) were first unfolded with 5.6 M GuHCl, the cysteine oxidation by DTNB was completed to more than 99% in the dead time of the experiment, about 10 s (see Materials and methods). In the absence of denaturant, the oxidation of MalE–GVP(wt) was a simple exponential function of time (Figure 2). The relaxation time of this function increased with the initial concentration of MalE–GVP(wt). The oxidation was 85 times faster for MalE–GVP(wt) than for free GVP when their concentrations were 5.6 μM (with relaxation times of 46.5 and 3900 s, respectively). Thus, the fusion of GVP with MalE strongly increased its oxidation rate (Table I).

Interactions between the partners of MalE–GVP

MalE and GVP (at 5 μM) are unfolded by GuHCl with concentrations of mid-transition of 1.05 and 2.5 M GuHCl, respectively. At 1.5 M GuHCl, nearly 100% of the MalE molecules are unfolded whereas >98% of the GVP molecules remain folded (Liu *et al.*, 1988; Liang and Terwilliger, 1991). To separate the contribution of the covalent linkage between GVP and MalE from the contribution of tertiary non-covalent interactions between them to the destabilization of GVP, we repeated the oxidation experiments in the presence of 1.5 M GuHCl. The kinetics of cysteine oxidation remained nearly unchanged for free GVP after the addition of 1.5 M GuHCl. In contrast, they were changed and included two phases instead of one for MalE–GVP(wt). The first phase was finished in the dead time of the experiment and contributed to 61% of the reaction amplitude. The relaxation time of the slow phase was 343 s in the presence of denaturant compared with 38.5 s in its absence, for an initial concentration of MalE–GVP(wt) of 3.1 μM (Table I).

Table I. Oxidation by DTNB

Protein	Concentration (μM)	Amplitude (%)	Relaxation time (s)
No addition			
Free GVP	5.6	95	3900
MalE–GVP(wt)	37.8	100	72
	12.7	100	57
	3.8	100	42
	1.3	100	26
MalE–GVP(lk)	3.7	62	53
+ 1.5 M GuHCl			
Free GVP	5.6	86	3490
MalE–GVP(wt)	3.1	39	343

The reactions were performed in the absence (*No addition*) or in the presence (*+ 1.5 M GuHCl*) of denaturant. The initial concentration of protein in the reactions was determined by titration of the unique Cys residue in 5.6 M GuHCl. The kinetics were analysed as described in Materials and methods. The amplitude and the relaxation time of the observed exponential phases are given as a percentage of the total amplitude and in seconds, respectively; the errors were below 4% and 3%, respectively, according to the program (KALEIDAGRAPH) that we used to fit the experimental data to exponentials, except for free GVP. In this case, the relaxation time was calculated as the ratio of the titre in Cys residue to the initial rate of oxidation; the error might be important. The value of the amplitude (100%) for the oxidation of MalE–GVP(wt) in the absence of GuHCl takes the dead time of the experiment into account.

Length of the polypeptide link

The C-terminal residue of the native protein MalE is Lys370 (Duplay *et al.*, 1984). MalE–GVP(wt), which is encoded by plasmid pAB2, carries the sequence NH_2–Arg–Ile–Pro–COOH between residues 369 of MalE and 1 of GVP (Blondel and Bedouelle, 1990). We lengthened this linking polypeptide at the genetic level to decrease potential steric clashes between MalE and GVP in the vicinity of their fusion point (see Materials and methods). The resulting hybrid protein, MalE–GVP(lk), carried the sequence NH_2–Arg–Ile–Glu–Ala–Ser–Ile–Pro–COOH between residues 369 of MalE and 1 of GVP. The kinetics of cysteine oxidation were close for the two hybrids, MalE–GVP(wt) and MalE–GVP(lk) (Table I). Thus, the lengthening of the polypeptide link between MalE and GVP by 4 residues did not increase the stability of MalE–GVP.

Probing the local structure of MalE–GVP with mutations

The experiments on cysteine oxidation showed that GVP was destabilized in hybrid MalE–GVP. This result raised the following questions: have the free and linked forms of GVP the same structure?; and do dimerization and binding to single-stranded DNA occur through the same molecular interactions for MalE–GVP and for free GVP? Several approaches were *a priori* possible to answer these questions, including the direct comparison of the three-dimensional structures or the quantitative comparison of global functional parameters. However, these approaches were either time consuming (crystallography) or hindered by certain properties of MalE–GVP. In particular, the equilibrium constant for the dissociation of the MalE–GVP dimer into monomers is difficult to measure by equilibrium sedimentation because MalE–GVP has a tendency to aggregate (Blondel and Bedouelle, 1990). The constant of association between MalE–GVP and DNA cannot be measured by fluorescence spectroscopy owing to the high content of MalE in Tyr and Trp residues.

We therefore devised an alternative approach. We con-

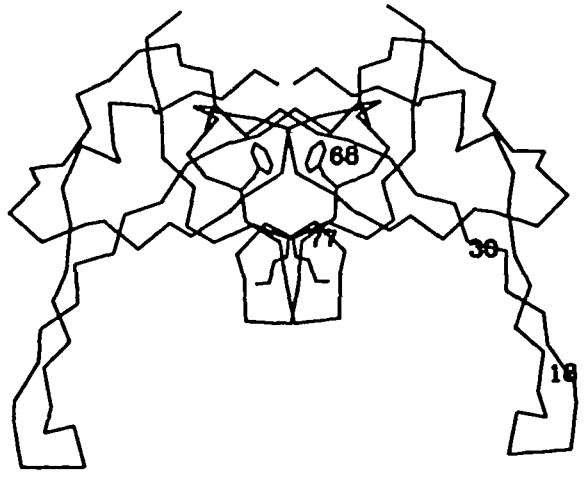

Fig. 3. Positions of the mutations in the structure of GVP. The figure shows the C_α backbone trace of the GVP dimer in the crystal structure (Skinner *et al.*, 1994). The side chains of residues Phe68 and Met77 are represented and the positions of residues 18 and 30 are indicated. Residues 19–29 are replaced by a Gly residue in deletion Δ1.

structed mutations in MalE–GVP and compared their effects, measured semi-quantitatively *in vitro*, with the effects that could be predicted from the data on free GVP, in particular its three-dimensional structure. The mutations were chosen to alter strongly the ability of GVP to dimerize or bind DNA. We replaced residues 19–29 of GVP by a Gly residue to delete the DNA-binding loop (deletion Δ1). In the structure of GVP, residues 19–28 make no contact with the remainder of the molecule and residue Asn29 only contacts the side chain of Phe13. Δ1 could create a loop, Thr14–Thr15–Arg16–Ser17–Gly18–Gly–Glu30, and only weakly perturb the remainder of the molecule (Figure 3). We changed residue Phe68, within the subunit interface, into Asp (mutation F68D). The Cδ1 atoms of Phe68 in one subunit and Ile78 in the other subunit are 3.96 Å apart (Figure 3). The design of these two mutations was initially based on an incorrect structure of GVP (Brayer and McPherson, 1983), but it remained valid with the corrected structure (Folkers *et al.*, 1994; Guan *et al.*, 1994; Skinner *et al.*, 1994). We also studied the change of Met77 into Asp (M77D) in the dyad loop.

In vitro DNA binding

In previous work (Blondel and Bedouelle, 1990), we showed that MalE–GVP(wt) is retained by a column of denatured-DNA cellulose but not by a column of double-stranded DNA cellulose, and that it can be eluted from the column of single-stranded DNA with a salt gradient. We used free MalE, which does not bind DNA, and the Klenow polymerase, which binds double-stranded DNA, as controls in these experiments. In the present work, we compared the bindings of MalE–GVP(wt) and its mutant derivatives to a column of denatured-DNA cellulose, using the same method. We found that MalE–GVP(M77D) was retarded but not retained by the denatured-DNA cellulose: the peak of protein that flowed through the column during its loading was trailing but only traces of protein were eluted from the column by a gradient of NaCl after its washing. MalE–GVP(F68D) and MalE–GVP(Δ1) were neither retained nor retarded by the denatured-DNA cellulose. Thus, the binding of MalE–GVP to single-stranded DNA was abolished by mutations F68D and Δ1, and strongly decreased by M77D.

Table II. Sedimentation coefficient ($s_{20,w}$) and Stokes radius (R_S) of the hybrids

Hybrid	Peak		
	I	II	III
$s_{20,w}$ (S)			
MalE	3.30 (M)	–	–
MalE–GVP(wt)	3.30 (m)	5.05–5.20 (M)	–
MalE–GVP(Δ1)	3.25 (m)	4.95–5.05 (M)	–
MalE–GVP(F68D)	–	3.45–4.80 (M)	≈5.0 (m)
MalE–GVP(M77D)	–	3.75–4.35 (M)	≈5.3 (m)
R_S (nm)			
MalE	2.8 (M)	–	–
MalE–GVP(wt)	3.0 (S)	3.0–4.0 (M)	(m)
MalE–GVP(Δ1)	3.0 (S)	3.0–3.9 (S)	4.6 (M)
MalE–GVP(F68D)	2.9–3.0 (M)	–	4.3 (S)
MalE–GVP(M77D)	2.9–3.1 (M)	–	4.6 (S)

The values of $s_{20,w}$ and R_S, and the errors on these values (±0.05 S and ±0.3 nm) were determined as described in Materials and methods. These values can be compared with those for MalE ($s_{20,w}$ = 3.30 S, R_S = 2.8 nm) and MalE–Lzp ($s_{20,w}$ = 4.95 S, R_S = 4.0–4.4 nm) (Blondel and Bedouelle, 1990, 1991). The sedimentation and gel filtration profiles of MalE–GVP(wt) and its derivatives generally showed three peaks (labelled I to III), more or less separated. The relative importances of these peaks varied with the particular hybrid and its concentration; they are given in parentheses: M, major peak; S, secondary peak; m, minor peak. The initial concentration of hybrid in the sample was 3, 10 or 30 μM in the sedimentation experiments and 1, 3, 10 or 30 μM in the filtration experiments. See Figures 4–7.

Oligomerization of the mutant hybrids

We have previously shown that MalE–GVP(wt) can dimerize, as free GVP. To do so, we measured its sedimentation coefficient ($s_{20,w}$) by zonal centrifugation through a sucrose gradient, its Stokes radius (R_S) by gel filtration, then its molecular mass from the two preceding parameters (Blondel and Bedouelle, 1990). Here, we compared the variations of $s_{20,w}$ and R_S for the wild-type and mutant hybrids in similar experiments, as functions of their concentrations (Table II). We used two controls to identify the monomeric and dimeric species of the mutant hybrids: the native protein MalE, which is monomeric, and hybrid MalE–GVP(wt), which can dimerize. We also compared the results with those previously obtained for hybrid MalE–Lzp, which is dimeric. MalE–Lzp is a hybrid between MalE and the 35 residues of the leucine zipper of yeast protein GCN4 (Blondel and Bedouelle, 1991).

Sedimentation experiments

The sedimentation profiles for MalE–GVP(wt) and MalE–GVP(Δ1) were nearly identical. They showed one peak and a minor shoulder towards the low values of $s_{20,w}$ (Figure 4). The $s_{20,w}$ value of the peak varied little with the concentration of protein in the initial sample (Figure 5) and was slightly higher than the $s_{20,w}$ value of MalE–Lzp (4.95 ± 0.05 S). These results showed that mutation Δ1 did not affect the association properties of the MalE–GVP monomers and indicated that MalE–GVP(wt) and MalE–GVP(Δ1) formed dimers at all the tested concentrations.

The profiles for MalE–GVP(F68D) and MalE–GVP(M77D) showed one peak and a minor shoulder towards the high values

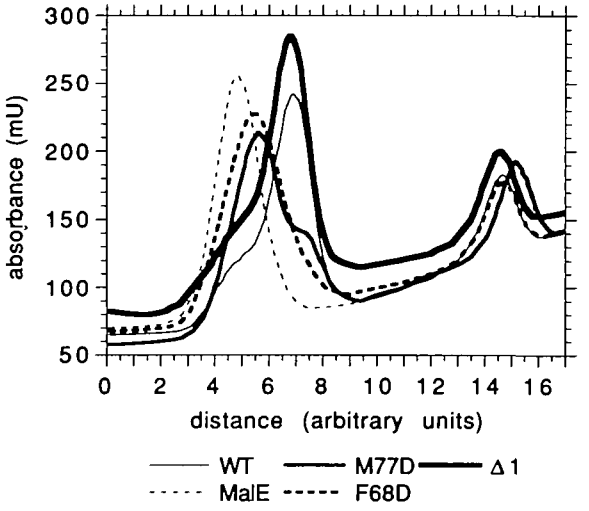

Fig. 4. Sedimentation of the hybrids. The graph gives $A_{280\ nm}$ as a function of the distance of migration from the meniscus of the gradients. The concentration of MalE and of the MalE–GVP derivatives (wild-type, M77D, F68D and Δ1) in the samples was 10 μM. Each protein sample contained catalase, which sedimented around 15 arbitrary units, as a marker.

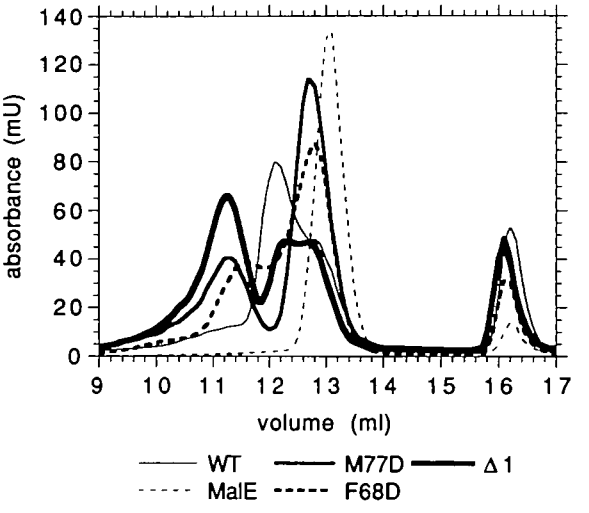

Fig. 6. Gel filtration of the hybrids. The graph gives $A_{280\ nm}$ as a function of the elution volume. The concentration of MalE and of the MalE–GVP derivatives (wild-type, M77D, F68D and Δ1) in the samples was 10 μM. The samples contained a small molecule, which eluted at about 16.1 ml and was used as a marker.

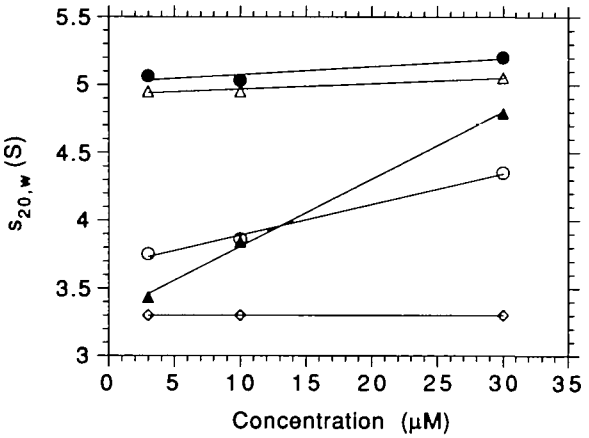

Fig. 5. Variation of the sedimentation coefficient ($s_{20,w}$) of the hybrids with their initial concentration. ◇, Wild-type MalE; ●, MalE–GVP(wt); Δ, MalE–GVP(Δ1); ▲, MalE–GVP(F68D); ○, MalE–GVP(M77D).

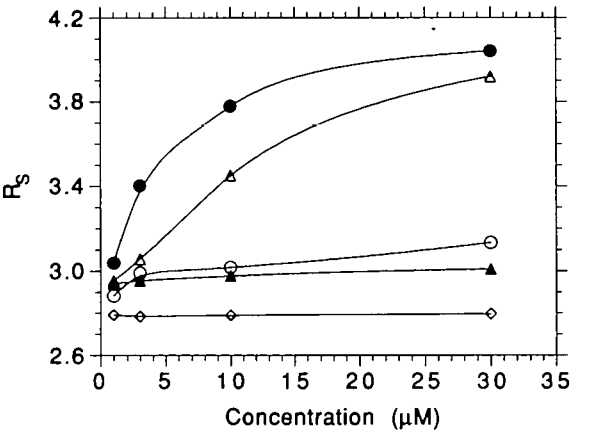

Fig. 7. Variation of the Stokes radius (R_S) of the hybrids with their initial concentration. ◇, Wild-type MalE; ●, MalE–GVP(wt); Δ, MalE–GVP(Δ1); ▲, MalE–GVP(F68D); ○, MalE–GVP(M77D).

of $s_{20,w}$ (Figure 4). The $s_{20,w}$ value of the peak increased with the concentration of protein, and was between those for MalE and MalE–GVP(wt) (Figure 5). This variation of $s_{20,w}$ with concentration showed that the peak corresponded to a mixture of monomeric and dimeric molecules in rapid equilibrium. The results showed that mutations F68D and M77D decreased but did not abolish the dimerization of the MalE–GVP monomers.

Gel filtration experiments

The profiles of gel filtration for MalE–GVP(wt) and MalE–GVP(Δ1) showed three peaks, more or less separated (Figure 6). The R_S value for the median peak increased with increasing concentration of protein (Figure 7) and was between those for MalE (2.8 ± 0.3 nm) and MalE–Lzp (4.4 ± 0.3 nm). The R_S values of the extreme peaks did not vary with concentration and were around 3.0 and 4.6 nm. The peak with a high R_S was major for MalE–GVP(Δ1) and very minor for MalE–GVP(wt). These results showed that the median peak corresponded to a mixture of monomeric and dimeric molecules in rapid equilibrium, and the peak with a low R_S to compact monomeric molecules. The peak with a high R_S could correspond to soluble aggregates or to partially unfolded monomers. Thus, Δ1 still allowed the dimerization of MalE–GVP but apparently favoured its unfolding or aggregation.

The profiles for MalE–GVP(F68D) and MalE–GVP(M77D) showed two peaks whose characteristics were identical with those of the two extreme peaks for MalE–GVP(wt) and MalE–GVP(Δ1) (Figure 6). The major peak had an R_S value around 3.0 nm, close to the MalE one, and thus corresponded to compact monomeric molecules. The results showed that mutations F68D and M77D prevented the reversible dimerization of MalE–GVP.

The profiles of gel filtration showed several peaks whereas those of sedimentation showed one peak and a minor shoulder. To test whether these differences were due to the high concentration of sucrose in the sedimentation experiments, we repeated the filtration experiments in a buffer containing 10%

sucrose, at an initial concentration of 3 μM for all the hybrids and at initial concentrations between 1 and 10 μM for mutant Δ1. We found no effect of sucrose on the profiles of filtration and on the R_S values.

Effects of the mutations on the in vivo activity of free GVP

The wild-type GVP inhibits growth when it is produced at high levels in *E.coli*. This inhibition can be quantified and used to measure the activity of mutant GVPs *in vivo* (Terwilliger *et al.*, 1994). We measured the toxicity of free GVP for *E.coli* when it was produced from plasmid pAB10 and its mutant derivatives, through bacterial plating experiments. This method of measurement is both sensitive and reliable (Bedouelle *et al.*, 1990; Vidal-Cros and Bedouelle, 1992). The efficiency of plating of strain JM101(pAB10) was decreased by a factor $4.6 \pm 0.5 \times 10^4$ when the production of free GVP was induced with IPTG. This factor was only 174 ± 59 when GVP carried mutation M77D, 1.5 ± 0.1 for F68D and 1.0 ± 0.2 for Δ1. Thus, M77D strongly decreased the toxicity and thus the functionality of free GVP *in vivo*, and F68D and Δ1 abolished it.

Discussion

Destabilization of GVP in hybrid MalE–GVP

The oxidation of MalE–GVP(wt) by DTNB was a simple exponential function of time (Figure 2). The absence of a rapid phase, terminated in the dead time of the experiment, indicates that the GVP portion of each hybrid chain was at least partially folded. The relaxation time of the exponential function increased with the concentration of protein (Table I). Several kinetic mechanisms could account for this dependence of the relaxation time on concentration but their analysis is beyond the scope of this work since it would require fast kinetic techniques and our conclusions do not depend on their exact nature.

In the absence of denaturant, the oxidation was much faster for MalE–GVP(wt) than for free GVP (83 times faster at a protein concentration of 5.6 μM). Moreover, in 1.5 M GuHCl, a larger proportion of molecules was oxidized in the dead time of the experiment and thus unfolded for MalE–GVP(wt) than for free GVP (61% vs 14% at similar protein concentrations). Thus, GVP was destabilized in the hybrid. If we consider the molecules of GVP, either free or linked, that were folded in 1.5 M GuHCl, two comparisons can be made. In the presence of denaturant, the oxidation was about 10 times faster for MalE–GVP(wt) than for free GVP. Thus, the covalent bond between the N-terminal end of GVP and the unfolded form of MalE was destabilizing. The oxidation of MalE–GVP(wt) was faster in the absence of denaturant than in its presence (nine times faster at a protein concentration of 3.1 μM). Thus, tertiary non-covalent interactions that depended on the folded state of MalE also contributed to the destabilization of GVP.

A single-chain variant of GVP, in which the C-terminal end of one subunit is covalently linked to the N-terminal end of the other subunit by a short peptide link, has an improved stability (Liang *et al.*, 1993). Therefore, the fact that the N-terminal end of GVP is partially buried in its crystal structure cannot fully explain the destabilizing effect of the covalent link between MalE and GVP (Figure 3) (Folkers *et al.*, 1994). The non-optimal character of the linking polypeptide between MalE and GVP, Ile–Pro in MalE–GVP(wt) and Ile–Glu–Ala–Ser–Ile-Pro in MalE–GVP(lk), could also be destabilizing. Indeed, when the composition of natural proteins in amino acids and the composition of the linking polypeptides between their domains are compared, Ala, Pro and Ser are over-represented in the linking peptides but Ile and Glu are slightly under-represented (Argos, 1990). We found that the lengthening of the linking polypeptide between MalE and GVP by four residues did not improve the stability of MalE–GVP (Table I). Hence it is unlikely that the destabilizing non-covalent interactions that we observed between the folded forms of MalE and GVP were due to steric clashes between these two proteins in the vicinity of their fusion point, or to steric clashes between the two MalE portions of a MalE–GVP dimer.

The kinetics of oxidation for the mutant derivatives of MalE–GVP(wt) were complex and did not enable us to compare the stabilities of the mutant hybrids with that of MalE–GVP(wt) in a simple way.

Comparison of the predicted and observed effects of mutations

Deletion Δ1 does not affect the dimerization interface in the structure of free GVP. In contrast, mutation F68D introduces a charged side chain in this interface. Δ1 did not affect the association of the MalE–GVP monomers in the sedimentation experiments, and it still allowed their reversible dimerization in the filtration experiments. F68D strongly diminished the association of the MalE–GVP monomers in both types of experiments. Thus, the effects of Δ1 and F68D on the dimerization of MalE–GVP were compatible with the predictions that can be made from the structure of GVP. The results indicate that the residues that are removed by Δ1 are not essential for the folding of GVP. They also indicate that F68D does not fully prevent the folding of GVP since MalE–GVP(F68D) was still able to dimerize at high concentration in protein in the sedimentation experiments and adopted a compact monomeric conformation in the filtration experiments.

The experiments on sedimentation and filtration led us to the same conclusions about the effects of mutations Δ1, F68D and M77D on the dimerization of MalE–GVP. The partial differences in behaviour that we observed for a given hybrid in the two types of experiments could come from the distinct physical bases of the underlying techniques. Sedimentation occurs in a homogeneous liquid phase whereas filtration involves a liquid and a solid phase. The unfolding of a molecule decreases its apparent molecular mass in sedimentation, whereas it increases this mass in filtration. In filtration, the unfolded molecules travel in a restricted volume, which increases their local concentration and could favour aggregation at the expense of folding. A folded monomer can enter narrow pores of the resin that do not accept dimers or couples of monomers, which could disfavour its dimerization. These considerations explain why the molecules of MalE–GVP tended to migrate with the extreme values of R_S in filtration and with the intermediate values of $s_{20,w}$ in sedimentation.

Deletion Δ1 removes several residues of GVP that have been implicated in its binding to DNA (see Introduction). We found that Δ1 abolished the binding of MalE–GVP to a column of denatured-DNA cellulose. The DNA binding site is divided between the two subunits of the GVP dimer (see Introduction) and residue Phe68 is buried within the hydrophobic interface between these subunits. We found that mutation F68D prevented the dimerization of MalE–GVP and its binding to a column of denatured-DNA cellulose. Thus, the effects of Δ1 and F68D on the binding of MalE–GVP to single-stranded DNA were consistent with the predictions that can be made from the structural and functional data on free GVP.

Mutations Δ1 and F68D abolished the cellular toxicity of

free GVP and M77D strongly decreased it. These effects of the three mutations on toxicity are compatible with those previously observed for other changes of the same residues. Indeed, point mutations at residues 19–21, 23–25 and 28–29, that are removed by Δ1, and the changes of Phe68 into His, Thr or Tyr abolish the toxicity of free GVP; the changes of Met77 into Cys or Phe strongly decrease it (Terwilliger *et al.*, 1994). Δ1 and F68D abolished the binding of MalE–GVP to single-stranded DNA *in vitro* and M77D strongly diminished it. Thus, the effects of the three mutations on the activity of free GVP *in vivo* and on the binding of MalE–GVP to DNA *in vitro* were correlated. This correlation suggests that the three mutations affected the structure of GVP similarly when it was in a free form or linked to MalE.

Residue Met77 belongs neither to the binding site of GVP to DNA nor to the dimerization interface, even though it is close to both of them (Folkers *et al.*, 1993, 1994; Guan *et al.*, 1994; Skinner *et al.*, 1994). Hence its effects on the properties of MalE–GVP could not be predicted. The available data on free GVP suggest that mutation M77D could decrease the dimerization of MalE–GVP and its binding to DNA indirectly, by introducing a negative charge in the vicinity of the structurally and functionally important residue Lys69, or by modifying the conformation of the Lys69 side chain (Dick *et al.*, 1988; Zabin and Terwilliger, 1991; Folmer *et al.*, 1994; Terwilliger *et al.*, 1994).

Conclusions

Hybrid MalE–GVP(wt) dimerizes and binds single-stranded but not double-stranded DNA, as GVP. It binds amylose and maltose as MalE. Thus, MalE–GVP behaves as a bifunctional fusion protein. Two mutations, Δ1 and F68D, had effects on the dimerization and DNA-binding properties of MalE–GVP *in vitro* that were compatible with their effects on the activity of free GVP *in vivo* and with the structure of GVP. This suggests that MalE–GVP and free GVP dimerized and bound DNA through similar interactions. Thus, the structural and functional properties of the free and linked forms of GVP were close. However, MalE destabilized GVP in their hybrid, owing to the covalent link and tertiary non-covalent interactions between them. Thus, the structural properties of GVP were not fully identical in its two forms and they were affected by the fusion with MalE. MalE could partially stabilize a non-native conformation of GVP, e.g. a monomeric form, in which Cys33 would be more exposed to a chemical attack than in the native dimeric form and which would normally be very minor. This hypothesis is plausible because part of the MalE–GVP molecules behaved as compact monomers in gel filtration, MalE naturally interacts with several proteins of the *E.coli* envelope and the binding site of maltose and maltodextrins at the surface of MalE is hydrophobic.

The properties of hybrids between MalE and other proteins suggest that MalE–GVP is not an exceptional case. For example, fusion with MalE can affect the specificity, affinity or stability of an antibody fragment, positively or negatively according to the particular construction (Brégégère *et al.*, 1994). Our results on MalE–GVP stress that hybrids can be considered as artificial multi-domain proteins and designed to facilitate the study of this class of molecules. MalE is an interesting tool for such studies because numerous data are available on its structure, export and folding (Liu *et al.*, 1988; Sharff *et al.*, 1992).

Acknowledgements

We thank Dr T.C. Terwilliger for the communication of the gene *V* protein coordinates prior to publication.

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Received February 28, 1995; revised November 9, 1995; accepted November 13, 1995