Analyses of the stability and function of three surface mutants (R82C, K69H, and L32R) of the gene V protein from Ff phage by X-ray crystallography

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Abstract

The high-resolution crystal structure of the gene V protein (GVP) from the Ff filamentous phages (M13, f1, fd) has been solved recently for the wild-type and two surface mutant (Y41F and Y41H) proteins, leading to a plausible model for the polymeric GVP-ssDNA complex (Guan Y, Zhang H, Wang AHJ, 1995, *Protein Sci* 4:187–197). The model of the complex shows extensive contacts between neighboring dimer GVPs involving electrostatic interactions between the K69 from one and the D79 and R82 from the next dimer. In addition, hydrophobic interactions between the amino acids L32 and L44 from one and G23 from the next dimer also contribute to the dimer–dimer interactions. Mutations at the L32, K69, and R82 amino acid sites generally destabilize the protein and many of these affect the function of the phage. We have studied the structural effects of three mutant proteins involving those sites, i.e., L32R, K69H, and R82C, by X-ray crystallographic analysis at 2.0 Å resolution. In L32R GVP, the structural perturbation is localized, whereas in K69H and R82C GVPs, some long-range effects are also detected in addition to the local perturbation. We have interpreted the protein stability and the functional properties associated with those mutations in terms of the observed structural perturbations.

Keywords: electrostatics; molecular modeling; protein–DNA interactions; single-stranded DNA-binding protein; X-ray crystallography

The gene V protein from the Ff filamentous bacteriophages (M13, f1, and fd) plays critical roles in the life cycle of those phages (Salstrom & Pratt, 1971). GVP is a dimeric protein of 87 amino acids (Fig. 1) and it binds to DNA in a highly cooperative manner without pronounced sequence specificity (Sang & Gray, 1987; Bulsink et al., 1988; Fulford & Model, 1988). During the rolling circle replication process, GVP binds to the ssDNA cooperatively and, ultimately, a superhelical GVP-DNA complex is formed. The structure of such helical complexes has been studied by a number of biophysical methods, including electron microscopy (Gray, 1989; Olah et al., 1995), neutron scattering (Gray et al., 1982; Olah et al.,

1995), NMR and fluorescence spectroscopies (King & Coleman, 1987, 1988; Dick et al., 1989; Stassen et al., 1992; Folkers et al., 1993). The results of these studies have provided valuable insights regarding the interactions of GVP and ssDNA.

Although a definitive view of the complex will be lacking until the three-dimensional structure of a protein–DNA oligonucleotide complex is solved by X-ray crystallography, the crystal structure of the wt-GVP, determined recently (Skinner et al., 1994), as well as two mutants with changes at the tyrosine 41 (Y41) position (Guan et al., 1994), have made it possible to propose a plausible model of the GVP-DNA complex (Skinner et al., 1994; Guan et al., 1995). In particular, the participation of Y41 in the protein dimer–dimer contacts in the GVP-ssDNA complex (King & Coleman, 1988; Stassen et al., 1992) was explained adequately. A similar model has been proposed independently using the three-dimensional structure of Y41H GVP determined by NMR (Folkers et al., 1994; Folmer et al., 1994). Such models have been supported by Olah et al. (1995) using neutron scattering and modeling.

Our GVP-DNA model (Guan et al., 1995) consists of a left-handed helix with a pitch of ~90 Å and eight GVP dimers per turn of the helix, with an outer diameter of ~80 Å. The protein shell of

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Abbreviations: GVP, gene V protein; wt-GVP, wild-type GVP; L32R, Y41H, Y41F, K69H, and R82C: Leu 32 → Arg 32, Tyr 41 → His 41, Tyr 41 → Phe 41, Lys 69 → His 69, and Arg 82 → Cys 82 mutant GVP; ssDNA, single-stranded DNA; RMSD, RMS deviation; SA, simulated annealing; PEG, polyethylene glycol.

Fig. 1. **A:** Amino acid sequence of GVP (Cuyper et al., 1974) with amino acids that are of interest marked in boxes. **B:** Topology diagram of the secondary structure of the GVP dimer. Two β -hairpins are labeled according to their functions.

the complex has a continuous inner channel in which two antiparallel ssDNA strands form a helical ribbon, with the sugar-phosphate backbones at the middle and the bases pointing away from each other. The bases are stacked with the Phe 73 residue from GVP intercalated between two DNA bases. The optimum binding to a tetranucleotide unit requires the participation of four GVP dimers, which may explain the cooperativity of the GVP binding to DNA.

An important feature of the model of the GVP-ssDNA complex is the extensive protein-protein contact surface ($\sim 800 \text{ \AA}^2$) between neighboring dimers, as shown in Figure 2. Several amino acids in the dyad hairpin region from adjacent dimers abut one another. For example, K69 from one dimer forms a salt bridge with the neighboring D79, which in turn is held in place by the salt bridge interaction with R82 of the same GVP molecule. Another observation is that the hydrophobic L32 amino acid is exposed to solvent. This amino acid is close to the DNA-binding wing of the neighboring dimer in the complex and may play some role in the stability of the protein-DNA complex. Thus, it is clear that many of the surface amino acids of GVP have important functions, either in the protein stability, DNA binding, or protein-DNA complex formation. Mutation of certain surface amino acids may have adverse effects on those functions.

In this work, we have chosen three surface amino acids, L32, K69, and R82, and changed them to amino acids with opposite properties. The hydrophobic L32 is mutated to the positively charged R32, whereas the positively charged R82 is mutated to the hydrophobic C82. The positively charged and flexible K69 is mutated to

a somewhat hydrophobic and less flexible H69. The thermodynamic data of these mutations have been obtained and all three mutants have been found to be destabilized to varying degree by -1.6 , -1.3 , and -1.5 kcal/mol in L32R, K69H, and R82C, respectively (Sandberg & Terwilliger, 1993; Sandberg et al., 1995). We sought to address the molecular basis of the destabilization by determining the structure of those mutants at high resolution and comparing them with the wt-GVP structure.

Results

Refinement of four GVP structures

The refinement of the four GVP crystal structures resulted in good agreement between the observed and calculated structure factors, with the crystallographic R -factors ranging from 18.7% to 22.4%. From 44 to 53 water molecules, most of which are within $3.5 \text{ \AA}$ of a protein atom, were located in the asymmetric unit. The five-stranded β -barrel core has average temperature factors of 23.7, 25.8, 28.1, and $18.7 \text{ \AA}^2$ for the backbone atoms and 28.3, 31.4, 32.6, and $23.1 \text{ \AA}^2$ for the side-chain atoms for wt, L32R, K69H, and R82C GVP, respectively. The loop regions, in particular the DNA-binding hairpin loop (amino acids G18-K24), have high temperature factors, indicating disordered structures.

Overall, the models fit the electron density consistently, as shown by the $(2F_o - F_c)$ electron density maps in Figure 3A,B,C,D,E,F. In the wt-GVP, the three amino acids of interest are seen to fit the density quite well (Fig. 3A,B,C), except the side chain of K69 appears to be disordered near the end. The corresponding amino acids in the mutant structures are also well placed in the density (Fig. 3D,E,F). However, some points are worth noting. In the R82C mutant, the temperature factor associated with the SH group of Cys 82 ($B = 35 \text{ \AA}^2$) is higher than that of the internal cysteine Cys 33 ($B = 13 \text{ \AA}^2$), which may suggest that the side-chain conformation of Cys 82 is somewhat disordered. This is consistent with the surface location of Cys 82.

All three GVP mutants, like the wt-GVP, are largely composed of β -structure, with 63 of the 86 ϕ - ψ angles in the β -sheet region of the Ramachandran plot for each mutant (data not shown). Some unusual side-chain rotamers, compared with other well-refined structures, could be identified by the program O (Jones et al., 1991). Careful inspection of these side chains indicates that they fit the electron density well, suggesting that these amino acids actually adopt unusual rotamer conformations, instead of the results of erroneous refinement. For example, residue L44 in wt-GVP has a χ_1 value of -145° , instead of the "normal" value -110° . By rotating the C_α - C_β bond, L44 is in van der Waals contact with L32 with favorable hydrophobic interactions and avoids a close contact with Y34. For T62 in wt-GVP, it has a χ_1 value of -161° , instead of the normal value of about 55° or -55° . Its O γ 1 forms hydrogen bonds to the V63 backbone nitrogen atom and the I2 backbone oxygen atom. If it adopts the "normal" χ_1 conformation, it can no longer form those hydrogen bonds and its C γ 2 atom will be in close contact with the V63 nitrogen atom, with a distance of only $2.9 \text{ \AA}$ between the two atoms.

Structural effects of the three mutants

The overall structure of the three mutant GVPs in general does not deviate greatly from that of the wt-GVP. A least-squares fit of the mutants with wt-GVP using all backbone atoms of residue 1-86

Fig. 2. Ribbon diagram of two adjacent wt-GVP dimers (green and purple) found in the model of the GVP–ssDNA complex showing the relevant amino acids R82, K69, and L32. Two other important amino acids, D79 and L44, are also shown.

produces an RMSD of only 0.25, 0.44, and 0.67 Å for L32R, K69H, and R82C, respectively (0.63, 1.00, 1.00 Å for all non-hydrogen atoms, including side chains). The structural deviation varies in different regions of the protein, as shown in Figure 4. The DNA-binding wing is disordered, especially at the loop region, and its amino acids are expected to be more variable in conformation. For L32R, there is very little change in the backbone conformation relative to wt-GVP (RMSD = 0.25 Å). Aside from the DNA-binding wings, which are disordered, the largest conformational change occurs at the side chain of K46 and E30, as discussed below. Residue Q12 in the K69H and R82C mutants adopts a different side-chain rotamer conformation from the wt-GVP. However, it is associated with weak electron density and away from the mutation sites. Consequently, the conformational difference in the side chain should not affect our interpretations. For both R82C and K69H, there are two regions of relatively large structural deviation, with one centered around amino acids 48–54 (RMSD for C_α = 1.22 Å [K69H versus wt] and 0.72 Å [R82C versus wt]) and the other around amino acids 70–81 (RMSD for C_α = 0.54 Å [K69H versus wt] and 0.56 Å [R82C versus wt]).

A close-up view of the superposition of the local structure at the site of the mutations is shown in Figure 5. In Figure 5A, it can be seen that the salt bridge between E30 and K46 was disrupted by the introduced positively charged R32. This is presumably the reason why K46 in Figure 4 has a quite large RMSD. In Figure 5B, a shift of the dyad hairpin is observed for K69H. If the C_α of H69 stayed at the same location as that of K69, the imidazole ring of H69 would be in van der Waals clash with the backbone of M77 and I78, which is expected to cause a shift of the dyad hairpin position. In Figure 5C, it can be seen that, for R82C, many of the atoms surrounding the R82 site did not change positions on the right side of the figure. However, a number of the side chains immediately around the R82 side chain have moved. As will be discussed, this is due to the loss of the salt bridges associated with the guanidinium of R82. The local structural perturbations are investigated individually below.

L32R mutant

The mutation of L32 to R32 resulted in a destabilization of the dimeric protein by an unfolding $\Delta\Delta G_0$ of -1.6 kcal/mol (Sandberg

Fig. 3. The $(2F_o - F_c)$ Fourier electron density maps of the regions at the mutated amino acids 32, 69, and 82. **A,B,C:** Wt-GVP. **D,E,F:** R32, H69, and C82 in mutant proteins.

& Terwilliger, 1993). The DNA-binding energy is also increased by 1.70 kcal/mol (Sandberg & Terwilliger, 1993), presumably due to the additional positively charged residue near the site of DNA binding. The protein remains fully functional in phage. The hydrophobic L32 is found on the surface of the β -core near the dimer-dimer contact loop. Closer inspection indicates that it is near another surface hydrophobic amino acid L44. This pair of hydrophobic leucines forms a small hydrophobic patch at a depression on the two corners of the GVP dimer (Fig. 6A). The replacement of L32 with a bulkier and positively charged R32 causes some local structural perturbation. The side chain of R32 is forced to stay away from L44 and Q10, which in turn makes the side chains of K46 and E30 adopt different conformations (Fig. 6B). The salt bridge between K46 and E30 in the wt-GVP is therefore disrupted in L32R. In fact, the bulky R32 makes this region become crowded. This presumably is the reason for decreased stability of the mutant.

K69H mutant

The mutation resulted in a destabilization of the protein by an unfolding $\Delta\Delta G_0$ of -1.3 kcal/mol (Sandberg & Terwilliger, 1993). The protein becomes temperature-sensitive in phage. The positively charged K69 is located near the center of the dyad hairpin

stem. Its side chain is found to be somewhat flexible. Because its amino end of the side chain is near the D79 amino acid, it is reasonable to assume that it is hydrogen bonded to the side chain of D79 (Fig. 6C). The replacement of the positively charged K69 by a slightly bulkier aromatic side chain of H69 did not appear to cause noticeable structural changes. However, the H69 side chain is now positioned close to the other strand of the β -stem near M77 and I78 (Fig. 6D). A van der Waals contact is found between H69 and I78, inducing a movement of the dyad β -hairpin in the mutant structure relative to that in the wt-GVP.

R82C mutant

The mutation resulted in a destabilization of the dimeric protein by an unfolding $\Delta\Delta G_0$ of -1.5 kcal/mol (Sandberg et al., 1995). The DNA-binding energy is decreased significantly by -2.74 kcal/mol, presumably due to the loss of a positively charged residue. The protein becomes temperature-sensitive in phage (Sandberg & Terwilliger, 1993; Sandberg et al., 1995). The positively charged R82 is located near the beginning of the dyad hairpin stem and tucked close to the β -core structure. Its side chain is extensively involved in the salt bridge interactions (Fig. 6E). The guanidinium group is hydrogen bonded to the side chain of D50 on the one side and to the side chain of D79 on the other side. Therefore, R82 is

Fig. 4. RMSD between the wt-GVP and mutant GVP by superpositioning common atoms. Residues 20–24 from the DNA-binding wing are not shown because of their disordered nature of the structures. Dotted lines represents the fitting using only main-chain atoms and the solid lines using side-chain atoms.

probably important in stabilizing the local structure around R82. The replacement of the positively charged R82 by an uncharged C82 causes a significant rearrangement of the local side-chain

conformation (Fig. 6F). No more salt bridges involving R82 with D50 and D79 were observed. Instead, the smaller $\text{CH}_2\text{-SH}$ side chain of C82 fills the space between the side chains of Y56, S66, and H64 and a hole is created at the site where the guanidinium group used to be. Such a structural perturbation is expected to destabilize the structure.

Discussion

GVP from the filamentous phages are excellent systems for studying the interactions between protein and ssDNA. Not only are the high-resolution crystal structures (Guan et al., 1994, 1995; Skinner et al., 1994) of the M13 GVP, the high-resolution NMR structures of the M13 GVP (Folkers et al., 1994) and the Pf3 GVP (Folmer et al., 1995) available, but abundant biophysical and biochemical data also afford us a rich source of information about the ssDNA-binding proteins from Ff phages. The small dimeric GVP is designed efficiently so that many important amino acids are strategically placed. Many positively charged amino acids (including Arg 16, Arg 21, and Arg 80) are located in the deep concave surface, creating a large area of positive potential, whereas the opposite surface is neutral. This charge asymmetry should facilitate the binding of GVP to DNA using its concave surface. However, other surface amino acids not implicated directly in DNA binding seem to have effects on the property of the GVP or the viability of the phage. Although the relation between the mutation of interior hydrophobic amino acids and protein stability has been studied extensively (Skinner & Terwilliger, 1996; Zhang et al., 1996), it is less clear how surface mutations may affect the protein stability. In this paper, we analyzed three such surface mutants, L32R, K69H, and R82C.

The possible biological role of these three amino acids may lie in their involvement in the extensive protein–protein contacts between neighboring dimers in the model of the GVP–ssDNA complex (Folmer et al., 1994; Guan et al., 1995) as shown in Figure 2. K69 from one dimer forms a salt bridge with the neighboring D79, which in turn is held in place by the salt bridge interaction with R82 of the same GVP molecule. Thus, the two positively charged amino acids K69 and R82 are influenced by each other indirectly. It is interesting to note that both mutants involving K69 and R82, i.e., K69H and R82C, are temperature sensitive in phage. Our structural analysis showed that, in the R82C mutant, the salt bridge network involving R82, D50, and D79 is disrupted, which may destabilize the protein structure. Because D79 is also involved with K69 from the neighboring GVP in the dimer–dimer contacts, a change in the conformation of residue 69 may have an effect on the formation of the GVP–ssDNA complex, and thus the viability of the phage at normal temperature. A change of K69 to H69 not only removes its potential to form the salt bridge with D79 of the neighboring GVP, it also causes a small conformational rearrangement of the dyad hairpin. The latter observation may explain the unfolding energy of -1.3 kcal/mol in the K69H mutant. For R82C mutant, the analysis is a little more complicated. A change of R82 to C82 destroyed the R82–D79–K69 salt bridge network, similar to the effects of the K69H mutation. This might be the reason why R82C is less stable than wt-GVP (unfolding energy of -1.5 kcal/mol). At the same time, R80 moves to the hole created by R82C mutation (Fig. 6E) and a new salt bridge between D50 and R80 is formed. In our model of the GVP–ssDNA complex (Guan et al., 1995), R80 is involved in the interaction between ssDNA and GVP (Folkers et al., 1993). So a movement of R80 will influence the

Fig. 5. Superposition of the wt-GVP and mutant GVP near the mutated amino acids. **A:** Wt versus L32R. **B:** Wt versus K69H. **C:** Wt versus R82C. Thick lines represent wt-GVP and thin lines represent the corresponding mutants.

DNA-binding ability of mutant, perhaps explaining the increase of DNA-binding free energy by 2.74 kcal/mol.

It is of interest to note that the hydrophobic L32 is found on the surface of the β -core near the dimer-dimer contact loop. Closer inspection indicates that it is near another surface hydrophobic amino acid, L44, at which position a conserved hydrophobic amino acid (Leu or Ile) is found in the GVP from phages M13, I ϕ 8, and Pf3. This pair of leucines (L32/L44) may have a favorable interaction to produce a hydrophobic site on the surface of the GVP dimer. It is not entirely clear why these two hydrophobic amino acids are required on the surface of the GVP at that location. Our model of the GVP-DNA complex suggests that L44 from one

monomer is in contact with the G23 of the neighboring monomer in the polymer. Mutation of the hydrophobic L32 into a positively charged R32 could upset the hydrophobic environment around L44. Moreover, the bulky R32 side chain is situated in a restricted space (Fig. 6B), resulting in a loss of entropy upon folding (Sandberg et al., 1995). A combination of those factors could explain the unfolding energy of -1.6 kcal/mol for the mutant L32R GVP.

In conclusion, studies of key surface mutant GVPs may allow a fuller understanding of the roles of amino acids on the surface of the proteins. Extensive thermodynamic data are already available from previous mutational analysis (Sandberg & Terwilliger, 1993; Sandberg et al., 1995). Before the high-resolution structure of GVP

Fig. 6. Stereoscopic diagrams (drawn by QUANTA) of the detailed local structures at the mutation sites. **A:** L32. **B:** R32. **C:** K69. **D:** H69. **E:** R82. **F:** C82. (Figure continues on following pages.)

became available, it had been difficult to obtain a convincing correlation between the thermodynamic data and mutations. It appears that the detailed structural analyses of selected and important mutant proteins will provide a greater understanding of this interesting class of ssDNA-binding proteins that may be useful for future protein engineering.

Materials and methods

The three GVP mutants, L32R, K69H, and R82C GVP, have been produced according to the published procedure (Sandberg & Terwilliger, 1993; Sandberg et al., 1995). The structure determination of the wild-type protein by the multiple wavelength anomalous diffraction (MAD) method has been described previously (Skinner

et al., 1994). The crystals were obtained as follows. L32R and K69H GVPs were crystallized from solutions containing 10 mg/mL protein, 4 mM Tris buffer, pH 7.5, and 16% PEG 4000 (w/v), equilibrated against 12% PEG 4000 by vapor diffusion at room temperature. The R82C GVP was crystallized from solutions containing 10 mg/mL protein, 35 mM Tris, pH 7.0, and 4.7% PEG 4000 (w/v), equilibrated against 6% PEG 4000. Because all large crystals of R82C GVP were twinned, we used small, but clean, crystals as "seeds" to get large single crystals. Single crystals selected for data collection were mounted individually in thin-walled glass capillaries and sealed with a droplet of the crystallization mother liquor.

The crystals of all three mutants are in the space group C2, with the unit cell dimensions listed in Table 1; their diffraction data

Fig. 6. Continued.

have been collected on a Rigaku R-Axis IIc image plate area detector system in our laboratory. One-hundred frames covering 180° of spindle rotation with 1.5° per oscillation step have been collected. The b -axis of the crystals was oriented so that it was tilted by $>15^\circ$ with respect to the spindle rotation axis to ensure that all reflections were collected. The diffraction data have been collected to 2.0, 1.7, and 2.0 Å resolution, respectively, for the L32R, K69H, and R82C mutant proteins. The data have been processed using the software of the R-Axis IIc system provided by Molecular Structure Corporation.

All three structures have been refined by the simulated annealing (SA) procedure incorporated in X-PLOR (Brünger, 1992). The 1.8-Å resolution structure of wt-GVP determined earlier (Guan et al., 1994) was used as the initial model for further refinement of its structure. A new set of diffraction data for the wt-GVP crystal

was collected to extend the resolution to 1.6 Å. The model was refined against the new high-resolution data with $|F_o| > 2\sigma$ in the 8.0–1.6-Å range. SA and individual temperature factor refinements were performed by X-PLOR. Well-ordered water molecules were located using the difference Fourier maps and they were included in the model gradually.

For the mutant structures, even though their unit cell dimensions are generally similar to those from the wt-GVP, significant differences were noted. In particular, the a -axis of the K69H and R82C mutant crystal is about 1 Å longer and the c -axis is 0.6 Å shorter for K69H than the corresponding values of the wt-GVP. The β angle in these two mutant crystals is smaller than that of the wt-GVP. These differences may suggest that the molecular structure of the mutant GVPs is likely to be affected beyond the local area near the mutation sites. We have therefore elected to refine the

Fig. 6. Continued.

crystal structure of the three mutant GVPs using the same approach as in the refinement for the wt-GVP.

The new 1.6-Å wild-type model (without the waters) was used as the initial model with the side chains of the mutated amino acids removed. After rigid-body refinement by X-PLOR, ($2F_o - F_c$) and ($F_o - F_c$) maps were calculated. We used O (Jones et al., 1991) and QUANTA (version 4.0, Molecular Simulation, Burlington, Massachusetts) to examine the electron density maps and the side chains of the mutated amino acids were built into the model. All three mutants have been refined by SA and individual *B*-factor refinements in X-PLOR. During the refinement, some rebuilding of the model was necessary to improve the fitting of the model to the electron density. The Lys 87 in all mutants' models has not been included because it is disordered. Water molecules were added by a method similar to that of the wild-type structure. Crystal data and

refinement summaries are listed in Table 1. Atomic coordinates of the four mutant GVPs have been deposited in the Brookhaven Protein Data Bank (entry numbers: 1gvp, 1ae2, 1gkh, 1ae3).

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Table 1. Crystal and refinement data of the wild-type and three surface mutants of the M13 GVP

	GVP			
	Wild-type	L32R	K69H	R82C
Space group	C2	C2	C2	C2
<i>a</i> (Å)	75.81	76.01	76.66	77.14
<i>b</i> (Å)	27.92	28.03	27.75	27.94
<i>c</i> (Å)	42.40	42.40	41.79	42.19
β (°)	103.1	103.1	102.2	102.5
Resolution (Å)	1.6	2.0	1.7	2.0
Unique reflection no. ($ F_o > 2\sigma$)	9,016	5,539	7,804	4,762
R_{merge}^a	4.3%	7.8%	4.2%	8.8%
Completeness of data	77.9%	92.9%	81.4%	79.2%
<i>R</i> -factor	0.213	0.224	0.221	0.187
R_{free}	0.288	0.326	0.312	0.284
No. of water molecules	53	44	50	47
RMS bond length deviation (Å)	0.017	0.019	0.017	0.018
RMS bond angle deviation (°)	3.4	3.4	3.3	3.4

$$^a R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum \langle I \rangle.$$

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