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Potential use of additivity of mutational effects in simplifying protein engineering

(crystallography/gene V protein/site-specific mutants/mutational additivity)

MATTHEW M. SKINNER AND THOMAS C. TERWILLIGER*

Structural Biology Group, Mail Stop M888, Los Alamos National Laboratory, Los Alamos, NM 87545

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ABSTRACT **The problem of rationally engineering protein molecules can be simplified where effects of mutations on protein function are additive. Crystal structures of single and double mutants in the hydrophobic core of gene V protein indicate that structural and functional effects of core mutations are additive when the regions structurally influenced by the mutations do not substantially overlap. These regions of influence can provide a simple basis for identifying sets of mutations that will show additive effects.**

Substitution of one or several amino acid residues in a protein will often lead to substantial changes in properties such as thermodynamic stability, catalytic activity, or binding affinity (1–3). If these properties could be altered in a rational way then proteins might be readily generated with functions tailored to specific uses. Although considerable progress has been made toward this goal, rational protein engineering still suffers from the lack of a complete understanding of the ways two or more changes in a protein interact (1–8). When several amino acid substitutions are made at well-separated locations in a single protein, their effects are generally additive (1). If the substituted amino acids are near to or in direct contact with each other, the effects are often highly non-additive, because each mutation affects the environment of the other (1, 4, 7).

Additivity of effects of amino acid substitutions can be very useful because it allows the properties of proteins with any combination of substitutions to be inferred directly from those of the proteins with single replacements (1, 8). Fig. 1 illustrates a hypothetical example in which the goal is to increase both the binding affinity of a protein for its substrate and the stability of the folded protein molecule. In Fig. 1, two versions (mutations A and B) of a protein with single amino acid changes from the wild-type (wt) sequence are characterized. Neither has the desired combination of properties, because mutation A increases stability but decreases binding affinity, whereas mutation B does the reverse. If effects of mutations on protein function were known to be additive, it could be expected that the effects of making both mutations A and B in the protein would be the vector sum (A + B in Fig. 1) of the effects of the individual mutations. In this way the changes in protein properties caused by mutation A could be used to offset the unfavorable effects and to enhance the favorable effects of mutation B. This idea could be applied to any protein where a group of variants containing single mutations has been characterized and where additivity of mutational effects applies. An engineered protein with a particular combination of properties could be designed simply by choosing a set of single mutations for which the vector sum of individual effects in a diagram such as Fig. 1 is close to the desired changes from the wt protein (8). This design could be carried out using n-dimensional vectors for n characterized properties of the protein. Although no application to a designed protein is directly demonstrated in this paper, the concept of using additive properties to engineer a protein with specific properties is feasible. Articles by Shih and Kirsch (9) and Dumoulin *et al.* (10), using the fundamentals of combining cumulative properties of individual mutants, have successfully shown engineered proteins with desired properties.

As additivity of mutational effects can simplify the protein engineering problem, it would be of considerable interest to have a means of knowing whether a particular pair of substitutions in a protein are likely to lead to additive effects. Most proteins are remarkably tolerant of amino acid replacements, and even those within the core of a protein usually cause only relatively local changes in protein structure (11, 12). It seems likely that the functional effects of multiple substitutions will generally be additive if the underlying structural effects of the substitutions are additive (2). This might occur, for example, if the regions affected by the various substitutions do not overlap. Here we investigate the additivity of structural changes caused by amino acid substitutions in a protein at sites in the protein core where these substitutions are known to lead to additive functional effects. We find that core substitutions in this protein lead to localized and highly non-isotropic structural effects and conclude that the functional effects of mutations in the cases examined are additive largely because the regions influenced by these mutations do not overlap. These results suggest that an examination of the regions structurally affected by mutations may allow a simple identification of pairs of mutations in a protein that will show additive effects.

MATERIALS AND METHODS

Purification and Crystallization. Wild-type gene V protein (GVP) and all mutants were purified to homogeneity as described (13). Mutant proteins were crystallized as described for the wt protein (14) with varying concentrations of PEG3400. Single crystals with maximum dimensions of 0.6 × 0.3 × 0.2 mm^3 grew in a 1-week period and were used for data collection. Crystals were of the monoclinic space group *C2* and contained one monomer of GVP in the asymmetric unit. The dimer is formed by the crystallographic 2-fold axis. The crystals of all analyzed mutants were isomorphous to those of wt GVP with unit cell dimensions of a = 76.2 Å, b = 28.1 Å, c = 42.6 Å, and β = 103.6°.

Data Collection and Processing. Intensity data were collected at 4°C with a Mar Research Image Plate Scanner on a Rigaku RU-200 rotating anode x-ray generator. Crystals were mounted with their unique b-axis parallel to the axis of phi

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Abbreviations: wt, wild type; GVP, gene V protein.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (references 1VQA, 1VQB, 1VQC, 1VQD, 1VQE, 1VQF, 1VQG, 1VQH, 1VQI and 1VQJ).

*To whom reprint requests should be addressed. e-mail: Terwilliger@LANL.gov.

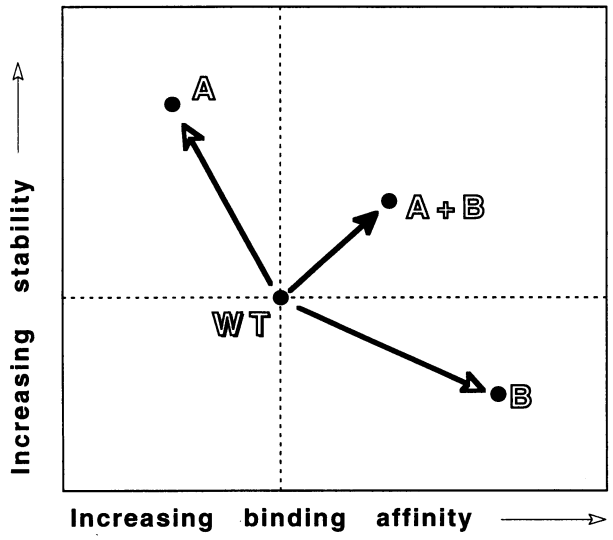

FIG. 1. Use of additive effects of mutations to simultaneously engineer multiple properties of a protein. The abscissa and ordinate represent two properties to be engineered, binding affinity of a protein for its substrate and the stability (free energy of unfolding) of the protein. WT represents the wt protein; the letters A and B represent the properties of proteins with single mutations and A + B represents a protein containing both mutations.

rotation and tilted 25–30° to obtain reflections along the cusp of the unique axis. Wild-type and all mutant data were collected in a similar fashion to minimize errors between the data sets. X-ray diffraction data were collected as close to completion as possible and to a Bragg spacing of 1.8 Å. All data sets were reduced and processed with an in-house Mar600 program package. Overall statistics are given in Table 1.

Structure Determination and Refinement. The wt structure determination and initial refinement have been described (14). Details of the wt and mutant structures and their refinements will be presented elsewhere. The x-ray crystallographic structures of V35I, I47V, and V35I/I47V have been reported elsewhere by Zhang *et al.* (15). Mutant models were built by difference Fourier methods (17) using the program FRODO (18). Difference refinement (16, 19) was carried out using the HEAVY (16) and X-PLOR (20) packages. Refinement statistics are given in Table 1. All mutated side chains adopted rotamer conformations that were most preferred except for I47F of V35I/I47F, which adopted the third most common conformation based on the rotamer library of Ponders and Richards (21). The rms deviation in backbone between mutants and wt ranged from 0.08 Å to 0.19 Å. The estimated error in coordinates based on the method of Luzzati (22) ranged from 0.18 Å to 0.33 Å. As many coordinate errors are correlated among the structures, the errors in coordinate shifts between structures estimated from difference refinement (16, 19) are likely to be considerably lower than this value.

RESULTS AND DISCUSSION

Additivity of Mutational Effects in GVP. The GVP of bacteriophage *f1*, a single-stranded DNA-binding protein, was used as a model system for this investigation (23–25). The GVP contains two identical 87-amino acid subunits. The effects on stability and DNA-binding affinity due to amino acid replacements in the hydrophobic core of GVP have been extensively characterized by mutational studies (13, 26–28). Effects of mutations at residues Val-35 and Ile-47 were found to be very closely additive for both of these properties (Table 2) (26). For example, individual substitutions of Val → Ile at position 35 and Ile → Val at position 47 decrease the stability of the dimeric protein by 0.7 kcal/mol and 2.6 kcal/mol, respectively. Substitution of both Val → Ile at position 35 and Ile → Val at position 47 decreases the stability of the dimeric protein by 3.1 kcal/mol, almost exactly the sum of the effects of the single mutations.

The GVP structure has been determined by x-ray crystallographic (14) and NMR methods (29). Residues Val-35 and Ile-47 are situated near the C-terminal ends of β-strands 3 and 4, respectively (Fig. 2). They are buried in the hydrophobic core of the protein, with the centroids of the side chains separated by about 9 Å. The x-ray crystal structures of the wt and 9 mutants of GVP with substitutions at positions Val-35 and Ile-47 have been determined at a resolution of 1.8 Å (Table 1). The mutant crystal structures are isomorphous to that of wt GVP and were refined by difference refinement (16, 19) to obtain accurate estimates of the small structural differences between the structures.

Structural Basis for Additivity of Mutational Effects. The crystal structures listed in Table 1 were used to examine the structural basis for additivity of mutational effects. The first eight of these structures can be grouped in three partially overlapping sets, each consisting of the wt, the Val 35 → Ile (V35I) mutant, one of three mutants at position 47, and a double mutant with the substitutions at positions 35 and 47. We first examined whether structural effects of mutations are additive in the same way as functional effects, defining structural additivity based on the coordinate shifts of individual atoms in a protein structure. The changes in structure from the wt GVP to the single and double mutants were remarkably

Table 1. Data collection and refinement statistics

Structure	Resolution, Å	R_{sym},* % on I	R_{mut},† %	Completeness of data ($F > 2\ \sigma$), %	R-factor‡ ($F > 2\ \sigma$), %	R_{diff}§ ($F > 2\ \sigma$), %
Wild type	1.80	6.1	—	97.3	19.8	—
V35I	1.80	4.7	10.8	94.7	20.6	6.9
I47L	1.82	6.4	10.0	93.6	20.7	6.4
I47M	1.80	4.3	11.0	86.3	21.2	7.1
I47V	1.80	4.1	10.8	94.0	20.5	6.4
V35I/I47L	1.82	4.1	13.4	91.1	21.0	7.9
V35I/I47M	1.80	4.3	19.5	93.5	21.8	11.5
V35I/I47V	1.80	4.2	16.4	85.5	21.0	9.3
V35I/I47F	1.80	6.1	19.3	89.2	21.8	11.7
V35A/I47L	1.80	3.8	15.6	96.2	21.0	9.3

*$R_{sym} = \Sigma_h \Sigma_i [|I_{h,i} - \langle I_h \rangle|]/\Sigma_h \Sigma_i I_{h,i}$, where $I_{h,i}$ is the intensity value of the i-th measurement of reflection h and $\langle I_{h,i} \rangle$ is the corresponding mean value of reflection h for all i measurements; the summation runs over all measurements.
†$R_{mut} = \Sigma |F^0_{WT} - F^0_{mut}|/\Sigma F^0_{WT}$, where F^0_{WT} and F^0_{mut} are the native and mutant structure factor amplitudes, respectively.
‡R-factor $= \Sigma |F^0 - F^c|/\Sigma F^0$, where F^0 and F^c are the observed and calculated structure factor amplitudes, respectively.
§$R_{diff} = \Sigma |F_{diff} - F^c_{mut}|/\Sigma F_{diff}$ where $F_{diff} = F^0_{mut} - (F^0_{WT} - F^c_{WT})$. This is the R-factor from difference refinement (16).

Table 2. Stabilities and single-stranded DNA binding affinities of core mutants of GVP

Mutation	$\Delta\Delta G°$u, 2 M, kcal/mol	$\Delta\Delta G°$d, 0.15 M, kcal/mol
V35I	−0.7	−0.2
I47V	−2.6	−0.3
V35I/I47V	−3.1	−0.5
I47L	−0.7	0.0
V35I/I47L	−1.2	−0.3
I47M	−2.2	0.6
V35I/I47M	−2.9	0.5
I47F	−2.0	0.9
V35I/I47F	−2.1	0.3
V35A	−2.3	−0.5
V35A/I47L	−3.0	−0.5

Data taken from Sandberg and Terwilliger (8). Changes in stability are reported as the change in free energy upon unfolding in 2 M GuHCl ($\Delta G°$ u, 2 M), and are given in (kcal/mol) of dimeric protein, relative to wt. Mutants with increased stability have positive values of ($\Delta\Delta G°$ u, 2 M). Error estimates of ($\Delta\Delta G°$ u, 2 M) are ±0.4 kcal/mol. Changes in apparent free energies of dissociation from poly(dA) at 0.15 M NaCl ($\Delta\Delta G°$d, 0.15 M), relative to wt GVP are given in kcal/mol. Positive values of ($\Delta\Delta G°$d, 0.15 M) indicate increased binding affinity of single-stranded DNA relative to wt GVP. Error estimates (2 SD) of ±0.1 kcal/mol for ($\Delta\Delta G°$d, 0.15 M).

additive. Fig. 3 illustrates this for the set of structures with Val → Ile at position 35 and Ile → Leu at position 47. Each point in the figure corresponds to one atom in the structure; the abscissa values are the sum of the x-, y-, or z-coordinate shifts for wt → V35I and wt → I47L structures, and the ordinate values are the x-, y-, or z-coordinate shifts from the wt to the double-mutant (wt → V35I/I47L) structure. The coordinate shifts for atoms in the double mutant structure are nearly the sum of the shifts in the two single-mutant structures, with a correlation coefficient of 0.92. Likewise, the correlation coefficients for similar plots for the wt → V35I/I47M and wt → V35I/I47V double mutants are 0.86 and 0.90, respectively. Considering the errors necessarily introduced by the refinement process, the actual structural shifts are likely to be even more closely additive than suggested by Fig. 3. This means that for these side chain replacements, the structures of the double mutants could have been closely predicted from the structures of the wt and the two single-mutant proteins with no prior structural knowledge of the double mutant.

Difference electron density maps in the vicinity of residues 35 and 47 for the wt → V35I and wt → I47L mutants are

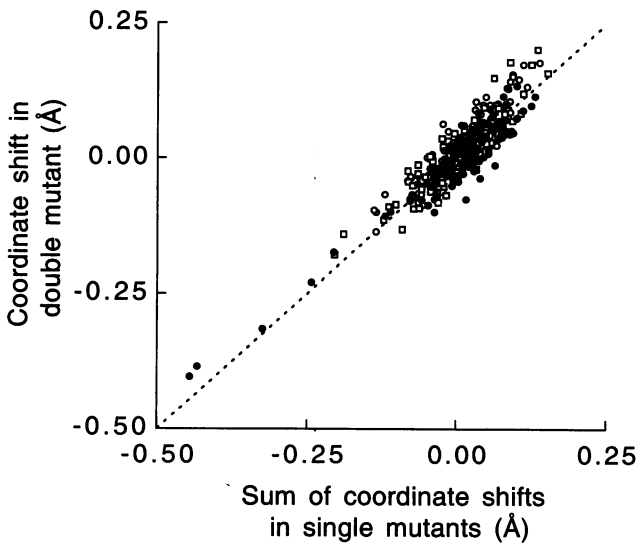

FIG. 3. Additivity of coordinate shifts in single and double mutants. Coordinate shifts for protein atoms from those in the wt structure were calculated for the single mutants V35I and I47L, and the double mutant V35I/I47L. The $C^{\delta 1}$ atom at position 35, the C^{γ} and C^{δ} atoms at position 47, and atoms further than 8 Å from the side chains of residues 35 and 47 were excluded. Each data point corresponds to the x (○), y (□), or z (●) coordinate of one atom in the structures. Abscissas are sums of coordinate shifts for the single mutants, ordinates are coordinate shifts for the double mutant.

illustrated in Fig. 4 (*Top*) and highlight the structural changes that are responsible for this additivity. As shown in Fig. 4 (*Top*), the positive and negative peaks of difference electron density (which indicate the shifts in atomic positions that occur in the single mutants) are clustered near the corresponding sites of mutation, with few structural changes extending as far as the opposite site. The structural effects due to each single mutation are therefore localized to a specific region of the protein, with relatively little influence on the environment of the other side chain. Consistent with this observation, the difference electron density map for the double mutant (not shown) is very similar to the sum of the difference electron density maps for the individual mutants illustrated in Fig. 4 (*Top*). Importantly, the regions affected by each mutation are not spheres about the mutated sites. Instead the effects are highly non-isotropic, evidently depending on which regions of the protein molecule have to adjust to accommodate the newly introduced mutated side chain.

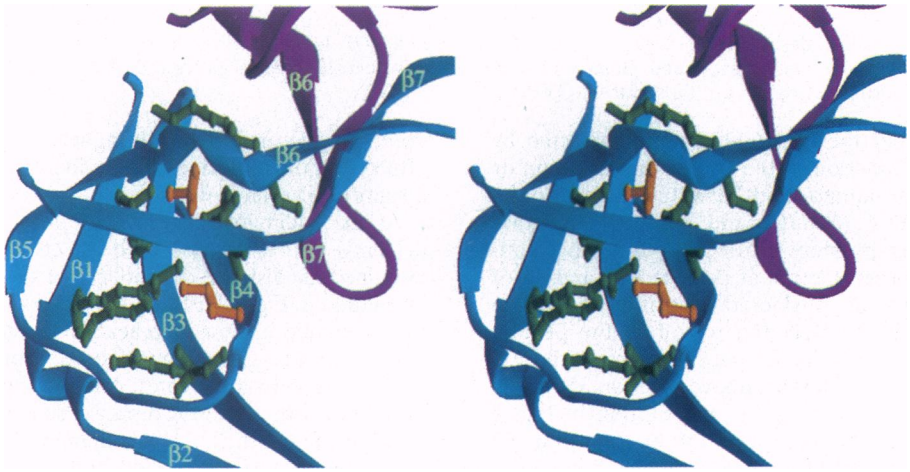

FIG. 2. A stereoview RIBBON representation (30) of a portion of the dimeric GVP showing the environment around residues Val-35 and Ile-47. The dimeric subunits are colored cyan and magenta with corresponding residues of Val-35 and Ile-47 in orange. Selected residues that surround these side chains are shown in green.

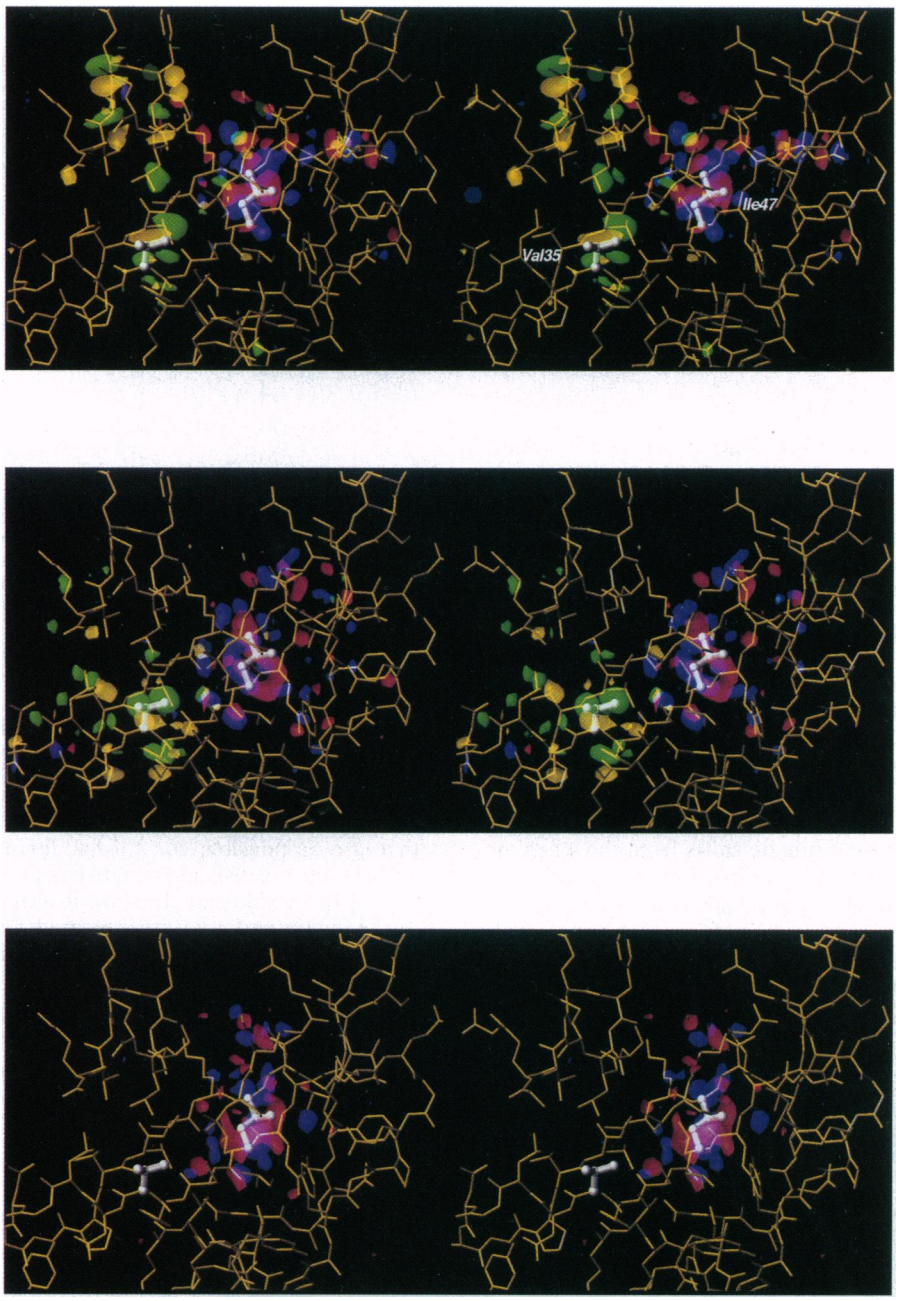

FIG. 4. Difference Fourier syntheses for single amino acid changes in GVP at 1.8-Å resolution. (*Top*) V35I-wt, positive density in green, negative in yellow; and I47L-wt, positive density blue, negative red. (*Middle*) V35A/I47L-I47L, positive density in green, negative in yellow; and V35I/I47F-V35I, positive density blue, negative red. (*Bottom*) I47M-wt, positive density blue, negative red. All contours are at $\pm 3.5\ \sigma$, where σ is the rms of the map. Coordinates shown are those for wt GVP.

We examined whether the region structurally affected by mutations is largely a function of the site of the mutation or whether the particular amino acid substitution is also an important factor. Fig. 4 (*Middle*) and (*Bottom*) illustrate difference electron density maps corresponding to three additional single amino acid changes at positions 35 and 47 of GVP. The two substitutions considered at position 35 (Val → Ile and Val → Ala) both affect the region of the protein immediately surrounding position 35, but the effects of the Val → Ile mutation extend considerably above position 35 in Fig. 4 (*Top*) and those of the Val → Ala substitution in Fig. 4 (*Middle*) extend below it. The three substitutions at position 47 all affect the region around and above residue 47 in Fig. 4 (*Top–Bottom*), but the exact region affected by each substitution is again slightly different than that affected by the others. These structures indicate that the site of mutation is the dominant factor in determining what region of the protein is affected by the mutation, but the identity of the mutation plays a significant role as well.

As the additivity of structural effects of GVP mutations is evidently due to the localized effects of these mutations, we examined the distance-dependence of structural changes caused by mutations. Fig. 5 illustrates the rms coordinate shifts caused by the six unique single amino acid substitutions in GVP obtained from Table 1 as a function of distance from the site of mutation. Coordinate shifts are greatest near the sites of mutation, and fall off rapidly with increasing distance, so that most structural shifts occur within the shell of atoms immediately surrounding the site of the mutation (the region from 4 Å to 8 Å). Smaller changes occur in the 8- to 10-Å zone and very few occur beyond 10 Å. Despite the variation in sizes of the substituted amino acids and the two locations in GVP where substitutions are made, the

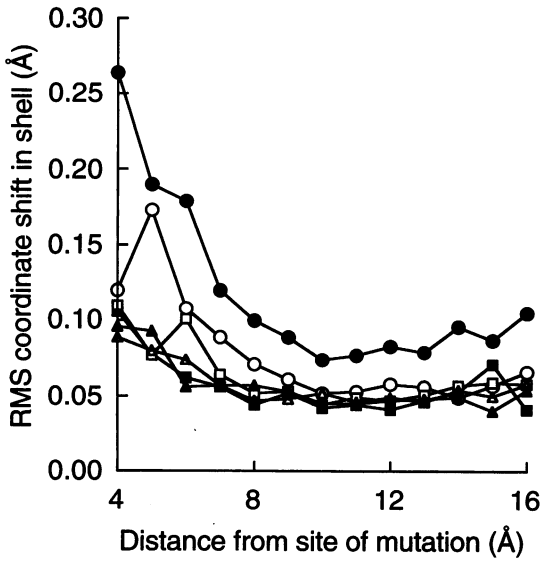

FIG. 5. Propagation of structural changes from sites of mutation. rms coordinate shifts as a function of distance from the site of mutation for wt → V35I (▲), wt → I47V (△), wt → I47M (□), wt → I47L (■), V35I → V35I/I47F (●), and I47L → V35A/I47L (○) structures. This set includes all possible one-amino acid differences among the structures in Table 1. The coordinate shift of each atom was determined after superimposing the 50 atoms surrounding it in the two structures to reduce any systematic errors due to slight changes in unit cell parameters.

overall features of the distance-dependence of these small structural changes are quite similar.

Identifying Pairs of Mutations Likely to Show Additive Effects. Combining the information in Figs. 4 and 5 it is clear that structural changes caused by mutations in the core of GVP fall off rapidly with distance, but in a very non-isotropic way. A specific region of the protein is influenced by mutations at each site, and different mutations at a site affect slightly different regions of the protein. These observations are important because they can now be used as criteria for identifying pairs of amino acid side chains for which mutational effects will be additive. Once the structures of a wt protein and single mutants at each of two sites are known, then the regions of influence of each of the single mutations can be characterized. If these regions of influence do not overlap, then the structural and functional changes in the double mutant can be expected to be the sum of those in the two single mutants. Furthermore, in some cases the regions of influence of several sets of mutations at a characterized pair of sites might be inferred from those of a single pair for which structures are available. In a favorable case where structures of a number of single mutants have been obtained, pairs of mutations likely to show additive effects could be identified in advance of actually making the double mutants, greatly simplifying the engineering of multiple mutants with desired structures and combinations of properties.

The non-isotropic nature of the structural changes illustrated in Fig. 4 (*Top*) could explain how mutations at distant sites can have non-additive effects and how structural changes can in some instances be propagated relatively long distances (31) even though most of the structural effects of mutations are local (see Fig. 5). It is possible that structural changes are propagated preferentially in certain directions or along particular secondary structure elements in most protein molecules, as they appear to be in GVP. This observation may be important in understanding and engineering allosteric effects on protein structure. If a new effector site was to be engineered in a DNA-binding protein, for example, it would be very useful to have characterized the directions of propagation of structural changes so that an effector site likely to influence the environment of the DNA-binding residues in the protein could be chosen.

The mutations examined in this work all consist of replacements of apolar side chains with other apolar residues at two distinct sites in the hydrophobic core of gene V protein. Such mutations can affect the arrangement of side chains on the surface of a protein and therefore can potentially be used to "fine-tune" the catalytic or binding properties of a protein. Some other classes of mutations, such as surface apolar to apolar substitutions, are likely to show properties similar to those examined here. Others, particularly those involving changes in the charge of side chains, often may not show the same extent of additivity of mutational effects because their effects are longer range (3). For substitutions that involve neutral amino acid side chains, however, an examination of the structures and properties of mutants with single amino acid substitutions and the rational combination of mutations that do not affect each other structurally is a very promising approach to rational protein engineering.

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