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Engineering multiple properties of a protein by combinatorial mutagenesis

(protein engineering/additivity/stability/DNA binding protein)

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ABSTRACT **A method for simultaneously engineering multiple properties of a protein, based on the observed additivity of effects of individual mutations, is presented. We show that, for the gene *V* protein of bacteriophage f1, effects of double mutations on both protein stability and DNA binding affinity are approximately equal to the sums of the effects of the constituent single mutations. This additivity of effects implies that it is possible to deliberately construct mutant proteins optimized for multiple properties by combination of appropriate single mutations chosen from a characterized library.**

One of the long-term goals of the study of mutational effects on protein stability and activity is to devise a method by which mutations can be rationally employed to alter or "engineer" the properties of proteins with predictable results. Recombinant DNA technology has allowed the construction of proteins of altered stability *in vitro* (1–16), catalytic efficiency (17–19), substrate specificity (20–23), and resistance to *in vivo* thermal inactivation (24) through the use of single or multiple amino acid substitutions. This effort has been greatly helped by the fact that the effects of amino acid substitutions on such properties of proteins tend to be additive as mutations accumulate, provided that the substituting residues do not interact functionally or by direct contact (25). For example, additive increases in the stability of subtilisin BPN′ have been achieved by combining mutations at six sites in the protein tertiary structure (16). The six mutations individually stabilize the protein by 0.3–1.3 kcal/mol, and the individual effects sum to a stability increase of 3.8 kcal/mol predicted for the hexa-mutant. The observed stabilization of the mutant containing all six substitutions is 4.3 kcal/mol (16). Additive effects of amino acid substitutions have been used to engineer incremental increases in the stability of other proteins including the N-terminal domain of λ repressor (5, 13), T4 lysozyme (9, 12), kanamycin nucleotidyltransferase (6), and neutral protease (26) as well. This strategy of additive mutation has also been employed to alter binding affinities or specificities of proteins, such as λ repressor (20), subtilisin (21–23), and glutathione reductase (27), for their substrates or cofactors, and to alter the pH profile of subtilisin (17).

A factor complicating the effort to engineer proteins by mutation is that most single amino acid substitutions alter multiple properties of the proteins in which they are made. To be functional, a protein must be at once stable, yet flexible, with high catalytic activity balanced against substrate specificity. Because mutants affecting only one of these properties are relatively rare, it appears difficult to optimize one characteristic of a protein through mutations while maintaining adequacy in the others. However, the observation that mutational effects on the *in vitro* properties of proteins are frequently additive suggests that it may be possible to counteract deleterious side effects of desirable (or primary) mutations by including additional mitigating (or secondary) mutations. If the effects of combining multiple mutations display simple additivity, then the net effect of the primary and secondary mutations on a given *in vitro* property of the subject protein should be the algebraic sum of the effects observed in the starting mutants. The objective of the present study is to test this assumption and to explore the possibility of creating proteins that have been optimized with respect to multiple *in vitro* properties.

The gene *V* protein of bacteriophage f1 is a small single-stranded DNA (ssDNA) binding protein that lends itself to this goal because its DNA binding affinity and stability can be readily measured *in vitro* and mutants of the gene *V* protein are readily available. A plasmid-based mutagenesis and expression system allows the rapid production of proteins containing single and multiple substitutions (28, 29). Conditions for monitoring gene *V* protein stability *in vitro* have been established (30), allowing quantitative assessment of the effects of single and multiple substitutions on stability (3, 4, 31). The stability of wild-type (WT) and mutant gene *V* proteins can be estimated as a function of their resistance to guanidine hydrochloride (Gdn·HCl)-induced denaturation, monitored by the disappearance of tyrosine circular dichroism (CD) at 229 nm as the protein unfolds (30). Cooperative binding of the protein to its substrate, ssDNA, can be followed *in vitro* by monitoring the intrinsic tyrosine fluorescence of the protein (32). This fluorescence is quenched as the protein binds to ssDNA and is restored when the protein–ssDNA complex is dissociated by the addition of NaCl. Binding affinities of WT gene *V* protein to a variety of substrates and of WT and several mutant gene *V* proteins to the substrate polydeoxyadenylic acid have been reported (31, 33–36). Thus the gene *V* protein provides a system in which proteins containing amino acid substitutions may be readily obtained and characterized *in vitro*. We used this system, starting with well-characterized single-substitution mutants, to construct doubly substituted proteins displaying predictable and additive changes in both DNA binding affinity and stability.

MATERIALS AND METHODS

Mutagenesis, Strains, and Vectors. Mutagenesis of gene *V* was carried out in the plasmid pTT18 as described (28, 29). Single mutants were constructed by oligonucleotide-directed

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Abbreviations: Gdn·HCl, guanidine hydrochloride; ssDNA, single-stranded DNA; WT, wild type. Substitutions are described in the one-letter code; e.g., Y41F denotes the replacement of tyrosine at position 41 by phenylalanine.

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Table 1. Single- and double-substitution mutants of the gene V protein

Mutation(s)	Minimum separation, Å	$\Delta\Delta G^{\circ}_{u,2M}$, kcal/mol	$\Delta\Delta G^{\circ}_{d,0.15M}$, kcal/mol
I6V		−0.68	0.21
F13T		−0.67	−0.65
L28V		1.09	−0.06
E30F		1.97	2.29
L32Y		1.04	0.37
C33M		−3.49	−0.45
C33V		−0.18	−0.09
V35A		−2.25	−0.45
V35C		−1.45	−0.62
V35F		−3.21	−1.00
V35I		−0.68	−0.24
V35L		−2.72	−0.47
V35M		−1.10	−0.99
Y41F		−0.62	−3.12
V45C		−0.05	0.15
I47C		−5.30	−0.29
I47F		−2.02	0.85
I47L		−0.67	0.03
I47M		−2.21	0.61
I47V		−2.62	−0.25
H64C		0.50	−0.12
L65P		−1.47	−0.73
F68L		−4.30	−0.26
F73W		0.76	1.21
M77I		1.63	0.10
M77V		1.23	0.06
R82C		−1.50	−2.74
A86T		−0.66	−0.26
A86V		0.47	−0.25
I6V/E30F	10.7	0.71	2.27
I6V/M77I	17.3	0.34	0.17
I6V/M77V	17.3	−0.04	0.27
F13T/E30F	5.8	1.59	1.03
L28V/F68L	19.0	−3.46	−0.84
E30F/A86T	21.6	1.17	1.93
E30F/A86V	21.6	2.05	1.94
L32Y/R82C	15.6	0.15	−1.78
C33V/V35C	4.0	−1.57	−0.57
C33M/I47C	3.9	−5.73	0.29
V35A/I47F	6.4	−3.67	0.14
V35A/I47L	6.4	−2.97	−0.50
V35A/I47M	6.4	−4.51	0.10
V35A/I47V	6.4	−4.52	−0.89
V35C/I47C	6.4	−7.20	−0.79
V35F/I47L	6.4	−4.22	−1.28
V35I/I47F	6.4	−2.08	0.30
V35I/I47L	6.4	−1.18	−0.29
V35I/I47M	6.4	−2.85	0.46
V35I/I47V	6.4	−3.12	−0.51
V35L/I47F	6.4	−4.00	0.24
V35L/I47L	6.4	−3.58	−0.51
V35L/I47M	6.4	−5.41	−0.08
V35L/I47V	6.4	−5.10	−1.03
V35M/I47F	6.4	−2.35	0.07
V35M/I47L	6.4	−1.70	−1.08
V35M/I47M	6.4	−3.62	−0.48
Y41F/F73W	21.0	−0.66	−0.73
V45C/R82C	11.7	−1.05	−2.44
H64C/F68L	7.0	−4.07	−0.80
L65P/F68L	4.5	−4.25	−0.83

For double mutants, the closest pair of atoms (minimum separation) in the two side chains in the crystal structure of the WT gene V protein dimer (M. M. Skinner, H. Zhang, D. H. Leschnitzer, Y. Guan, H. Bellamy, R. M. Sweet, C. W. Gray, R. N. H. Konings, A. H.-J. Wang, and T.C.T., unpublished data) is listed. In two cases, Ile-6/Met-77 and Tyr-41/Phe-73, the two side chains are within separate monomers, and in all other cases, they are within the same monomer of the protein. Changes in stability, measured as the change in free energy upon unfolding ($\Delta G^{\circ}_{u,2M}$), are given in kcal/mol of dimeric protein, relative to WT. Mutants with increased stabilities have positive values of $\Delta\Delta G^{\circ}_{u,2M}$ and the magnitude corresponds to making the same substitution twice. Changes in apparent free energies of dissociation from polydeoxyadenylic acid ($\Delta\Delta G^{\circ}_{d,0.15M}$), relative to WT gene V protein, are given in kcal/mol; positive values of $\Delta\Delta G^{\circ}_{d,0.15M}$ indicate enhanced binding of the mutant to ssDNA relative to WT.

mutagenesis and isolated as derivatives of pTT18. Double mutants were obtained by oligonucleotide-directed mutagenesis, recombination of single mutants by the use of intervening restriction sites, or selection (as intragenic suppressors of a conditional lethal mutation) from a pool of random single-amino acid substitution mutants (31). Mutant genes were expressed in *Escherichia coli* K561 (37). Transformation of *E. coli* K561 with pTT18 derivatives was effected using an electroporation device.

Protein Purification. Growth of K561 cultures transformed with pTT18 derivatives encoding gene V protein variants and purification of proteins were carried out as described (30, 31). To confirm that the mutant proteins contained the expected amino acid substitutions, ssDNA was isolated from *E. coli* harvested late in the growth and the gene V region was sequenced.

Measurement of ssDNA Binding Affinity. NaCl-induced dissociation of gene V protein–ssDNA complexes, monitored by fluorescence, was used to estimate the binding affinities of WT and mutant gene V proteins for the substrate polydeoxyadenylic acid as described (refs. 31–36 and unpublished observations). Data are reported as the apparent free energy change upon dissociation in 0.15 M NaCl ($\Delta G^{\circ}_{d,0.15M}$), related to the effective binding constant in 0.15 M NaCl ($K\omega_{0.15M}$) by $\Delta G^{\circ}_{d,0.15M} = +RT\ln(K\omega_{0.15M})$ (where R = 1.987 cal/mol·K; T = 298 K). Differences in binding between mutants are expressed as differences in free energy change upon dissociation ($\Delta\Delta G^{\circ}_{d,0.15M}$), defined as [$\Delta G^{\circ}_{d,0.15M}$ (mutant) − $\Delta G^{\circ}_{d,0.15M}$ (WT)]. Mutants binding more tightly to ssDNA than the WT will have positive values of $\Delta\Delta G^{\circ}_{d,0.15M}$. Error estimates (2 SD) were obtained from seven measurements of the DNA binding affinity of the WT gene V protein leading to an error of ±0.1 kcal/mol for $\Delta\Delta G^{\circ}_{d,0.15M}$.

Measurements of Protein Stability. Stability measurements on mutant gene V proteins were carried out as described (30). The gene V protein is reversibly denatured by Gdn·HCl, and the denaturation can be monitored by the disappearance of a tyrosine CD signal at 229 nm. Unfolding data were fitted to a two-state model (30) with modifications (4) in the case of proteins for which the unfolding is >50% complete when [Gdn·HCl] < 1.5 M. Stabilities are expressed as free energy changes upon unfolding in kcal/mol of dimeric protein. The stability ($\Delta G^{\circ}_{u,2M}$) of the WT gene V protein, given as the average ± 2 SD of 10 measurements, is 9.04 ± 0.3 kcal/mol of dimeric protein. Stabilities of mutant ($\Delta G^{\circ}_{u,2M}$) are compared to that of the WT in the presence of 2.0 M Gdn·HCl to yield the difference ($\Delta\Delta G^{\circ}_{u,2M}$). The estimated error in values of stability changes of mutants, relative to that of the WT protein ($\Delta\Delta G^{\circ}_{u,2M}$) is ±0.4 kcal/mol. Stabilities of mutants at positions 35, 47, 28, 64, 65, and 68 have been reported (4, 31, 38) and are taken from those works.

RESULTS AND DISCUSSION

Mutants of the Gene V Protein. The data compiled in Table 1 include stability and DNA binding affinity measurements for a variety of single mutants of the gene V protein and for a series of double mutants constructed by combination of

these single mutations. These data were used to explore the utility of concomitantly engineering *in vitro* stability and DNA binding affinity of the gene *V* protein through the use of additive mutational effects. Logically, this analysis consists of two steps: (*i*) assessment of the additivity of mutational effects on DNA binding and stability for the mutants listed in Table 1 and (*ii*) comparisons of the combined properties of a group of double mutants with those found in the starting group of single mutants.

Additivity of Mutational Effects on the Gene V Protein. It has been observed that the effects of accumulating mutations on *in vitro* properties of proteins are very nearly additive, provided that the substituted residues do not interact, either functionally (as in the case of catalytic residues) or by direct contact (25). When the substituted residues in multiply substituted proteins do interact or disrupt an interaction present in the WT protein, the effects are not additive. This is a potential stumbling block to protein engineering by accumulating mutations because the *in vitro* properties of such multiple mutants could not be predicted from the effects of the single mutants taken alone. Fortunately, interaction between residues in proteins appears to be relatively rare, with the exception of catalytic residues (25). We assessed the additivity of stability changes among single and double mutants of the gene *V* protein by considering the stabilities of double mutants, along with the stabilities of their constituent single mutants, and comparing these to the stability of the WT. Fig. 1 compares the stability changes, ($\Delta\Delta G^{\circ}_{u,2M}$) relative to WT, of the double mutants listed in Table 1 with the sums of the stability changes of their constituent single mutants. The data generally fall along a straight line with a slope near unity, demonstrating that interactions between substituting residues appear to be minimal in the combinations tested. This simple additivity was unexpected for pairwise substitutions of residues at positions 35 and 47 of the gene *V* protein due to the close proximity of these sites in a published crystallographic model of the protein (39). However, NMR results (40) and a revised crystal structure determined in our laboratory (Skinner *et al.*, unpublished data) show that, instead, Ile-47 is near Cys-33. This in turn may explain the significantly nonadditive stability effect of combining the mutation C33M with I47C (Table 1 and Fig. 1). This double mutant lies far from the line described by the rest of the combinations shown in Fig. 1.

Analogous to the results for stability changes, the DNA binding affinity changes ($\Delta\Delta G^{\circ}_{d,0.15M}$), relative to WT, of the double mutants tested are generally the sums of the binding affinity changes of their constituent single mutants, as shown in Fig. 2.

Presumably, some combinations of single mutations in addition to the C33M/I47C double mutant will lead to interactions between substituting residues. However, the frequent observation of simple additivity for the pairs of sites studied in gene *V* protein (Figs. 1 and 2), and the stepwise accumulation of stability changes observed in other proteins, including variants containing up to six substitutions (16, 25), suggests that interactions between substituents may be relatively rare as long as the sites chosen as targets for substitution are not obviously related by proximity or function (as in the case of catalytic residues). Thus, the additivity of effects of substitutions shown in Figs. 1 and 2 suggests that it should be possible to alter both gene *V* protein DNA binding affinity and stability in an additive and predictable fashion by combining previously characterized single mutants.

Engineering the Gene *V* Protein. To simultaneously adjust gene *V* protein DNA binding affinity and stability by multiple mutagenesis, it is important to know the relationship between the binding affinity changes and stability changes of the starting single mutants. If the two properties are strongly correlated, then the stability change caused by a mutation will always be in the same direction, relative to the WT, as the DNA binding affinity change, restricting the range available in one parameter (binding affinity or stability) relative to the other. On the other hand, if DNA binding affinity changes are loosely correlated or uncorrelated with stability changes in the starting group of single mutants, then it should be possible to generate mutants whose stability changes range widely with respect to DNA binding affinity changes. In this case, the DNA binding affinity and stability of the gene *V* protein can be altered simultaneously yet independently of each other, simply by combining single mutants to give the desired changes in each parameter.

To assess the correlation between DNA binding affinity changes and stability changes in the starting group of single mutants, $\Delta\Delta G^{\circ}_{d,0.15M}$ (the DNA binding affinity change) is plotted against $\Delta\Delta G^{\circ}_{u,2M}$ (the stability change) in Fig. 3*A*. Positive values of $\Delta\Delta G^{\circ}_{u,2M}$ or $\Delta\Delta G^{\circ}_{d,0.15M}$ indicate higher stability or increased DNA binding affinity, respectively,

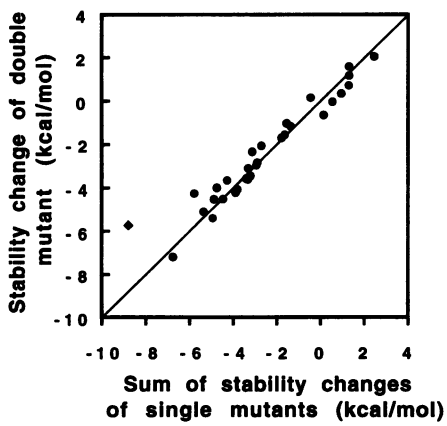

FIG. 1. Additivity of mutational effects on gene *V* protein stability. The stability change ($\Delta\Delta G^{\circ}_{u,2M}$), relative to the WT protein, of gene *V* protein double mutants is shown on the *y* axis. The *x* axis shows the sum of the stability changes, also relative to the WT protein, of the constituent single mutants. Positive values of $\Delta\Delta G^{\circ}_{u,2M}$ indicate proteins with increased stability. The combination of the mutants C33M and I47C is indicated by the diamond (◆). A line with unit slope is shown for reference.

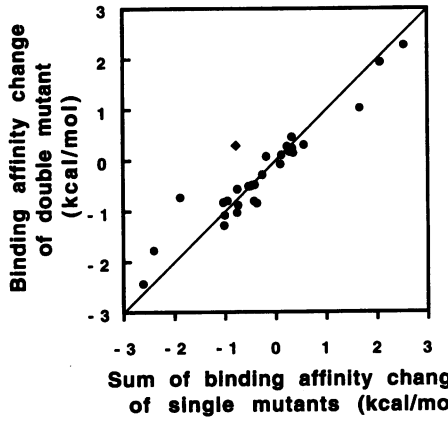

FIG. 2. Additivity of mutational effects on gene *V* protein DNA binding affinity. Binding affinity change ($\Delta\Delta G^{\circ}_{d,0.15M}$), relative to the WT protein, of gene *V* protein double mutants is shown on the *y* axis. The *x* axis shows the sum of the binding affinity changes, also relative to the WT protein, of the constituent single mutants. A positive value of $\Delta\Delta G^{\circ}_{d,0.15M}$ indicates enhanced binding to ssDNA relative to WT. The combination of the mutants C33M and I47C is indicated by the diamond (◆). A line with unit slope is shown for reference.

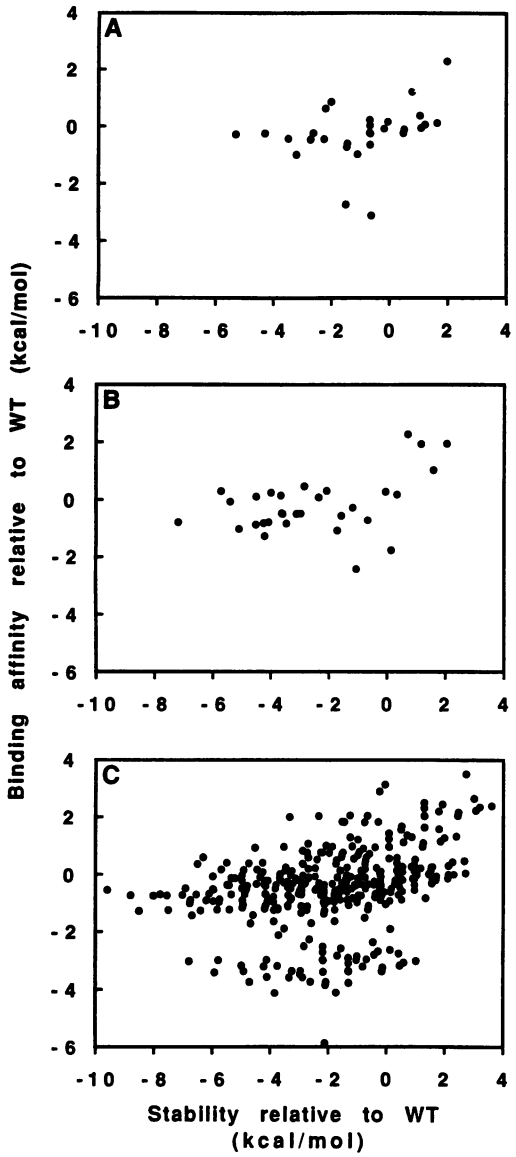

FIG. 3. (*A*) Comparison of ssDNA binding affinity changes ($\Delta\Delta G^{\circ}_{d,0.15M}$) with stability changes ($\Delta\Delta G^{\circ}_{u,2M}$) for single-substitution mutants shown in Table 1. The stability change, relative to the WT, is plotted on the *x* axis while DNA binding affinity change is plotted on the *y* axis. The upper right portion of the graph, therefore, contains mutants with higher stability and DNA binding affinity than the WT. (*B*) Comparison of ssDNA binding affinity changes with stability changes for double-substitution mutants shown in Table 1. (*C*) Calculated stability changes and ssDNA binding affinity changes resulting from all possible pairwise combinations of single mutants shown in Table 1, with assumptions as described in text.

than for the WT protein. Stability changes, relative to the WT protein, are only weakly correlated with binding affinity changes (also relative to WT), indicating that large changes of each parameter with respect to the other can be achieved by combining these mutants. Fig. 3*A* also shows graphically that very few of the single mutants alter stability without a concomitant DNA binding affinity change, or vice versa. Most of the single mutations decrease both stability and DNA binding affinity, but some mutations cause increases in one or both parameters relative to WT, indicating that adjustments of each parameter in either direction are potentially possible.

The results of combining single substitutions to alter two properties of the gene *V* protein are shown in Fig. 3*B*. The double mutants in Fig. 3*B* differ, as a group, in their properties from the starting single mutants in Fig. 3*A*, demonstrating that noncorrelated but additive changes in gene *V* protein DNA binding affinity and stability can be used to create a group of double mutants with distinctive *in vitro* properties. The double mutants shown in Fig. 3*B* are only a small fraction of the possible pairwise combinations of the single mutants shown in Table 1. Fig. 3*C* shows the predicted result, assuming that DNA binding affinity changes and stability changes are always directly additive (meaning no interactions between sites) of all possible pairwise combinations of the single mutants shown in Table 1. Simple pairwise combination of a starting group of 29 single-substitution mutants at 17 sites leads potentially to pairs of proteins differing by as much as 12 kcal/mol in stability or 8 kcal/mol in DNA binding affinity without substantial changes in the other parameter. Stability increases or DNA binding affinity increases relative to the WT of 3 kcal/mol or both are apparently within reach through pairwise combination of single mutants as well. Potential examples are the double mutants L32Y/L28V (increasing stability), I6V/F73W (increasing DNA binding affinity), and L32Y/F73W or F73W/M77I (increasing both parameters) (Table 1). Large destabilizations and reductions in DNA binding could also be achieved in theory. In practice, proteins with $\Delta\Delta G^{\circ}_{u,2M}$ more negative than ≈7.5 kcal/mol are substantially unfolded at 25°C and are difficult to produce *in vivo* (unpublished observations). Similarly, rapid purification of gene *V* protein variants employs ssDNA affinity chromatography, which may impose a lower limit on the obtainable reduction in DNA binding affinity. Within the practical limits of expression and purification, potentially hundreds of proteins with precisely engineered DNA binding affinities and stabilities could be produced by pairwise combination of a relative handful of single-substitution mutants.

Potential Utility of Protein Engineering by Multiple Mutation. Starting from a modest group of characterized single-substitution mutants, we have created double mutants with distinctive pairings of *in vitro* stability and DNA binding affinity. The stabilities and DNA binding affinities of these double mutants are generally additively related to the stability and DNA binding affinity changes of the starting single mutants. These results suggest that large numbers of proteins with precisely tailored properties can be deliberately constructed by the appropriate combination of single-substitution mutants. The properties of the double-substitution proteins can be predicted, based on those of the starting mutants, if care is taken to ensure (as much as possible) that the sites chosen for substitution will lead to simple additive effects (25). Simple additivity may not occur if the substituting residues contact each other, due to a change in the energy of interaction between the two sites (25). However, the potential interactions between amino acid residues, with the exception of charge–charge interactions, are strongly distance-dependent (41). Also, the effects of amino acid substitutions on protein structure are often localized to the immediate vicinity of the substitution (10, 42–45). It has been observed that the effects of substitutions on the properties of proteins are generally additive when the sites of substitution are not in van der Waals contact with each other (25). Consistent with these suggestions, nonadditivity of stability effects in the gene *V* protein is observed for mutations at sites that are close to each other (sites 33 and 47) but not for more distant sites (sites 35 and 47).

The ability to alter multiple properties of the gene *V* protein by combining substitutions is potentially useful in the further characterization of the gene *V* protein as well. For example, some pairwise combinations of substitutions could lead to proteins that differ in sequence and in some properties, yet that possess both WT stability and DNA binding affinity. Combinations of L32Y with V35I, I47L, or L65P might lead to proteins of this type (Table 1). These proteins could be

used to study the effects of substitutions on other properties of the gene *V* protein such as resistance to irreversible thermal denaturation (31) or folding and unfolding rates (30) in the context of WT stability and DNA binding affinity.

Protein engineering through the combination of single-substitution mutants may be most successful at adjusting those properties of concern *in vitro*, rather than *in vivo* activity. This is because activity *in vivo* may involve sequential interactions or parameters such as lifetime and folding/unfolding rates not considered in the *in vitro* analysis of the effects of substitutions. Mutations leading to DNA binding affinity and stability changes probably alter these other properties as well, complicating the task of engineering a particular property as the number of parameters to be maintained near WT values increases. Nevertheless, engineered proteins may find many applications *in vitro* where defects in some properties may be acceptable, and the ability to rapidly adjust the *in vitro* properties of proteins by combining well-characterized single substitutions should facilitate future protein engineering efforts.

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