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Accommodation of Single Amino Acid Insertions by the Native State of Staphylococcal Nuclease

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ABSTRACT **Single alanine and glycine insertions were introduced at 20 randomly selected positions in staphylococcal nuclease. The resulting changes in catalytic activity and in stability to guanidine hydrochloride denaturation indicate that the native state structure is frequently able to accommodate the extra residue without great difficulty, even insertions within secondary structural elements such as alpha helices and beta sheets. On average, an inserted residue reduces the free energy of denaturation (ΔG_{H_2O}) by an amount roughly comparable to an alanine or glycine substitution for one of the residues flanking the site of insertion. Several positions outside of the enzyme active site were found where insertions, but not substitutions, lead to structural changes that modify catalytic activity and the circular dichroism spectrum. Amino acid insertions represent a virtually unexplored class of genetic mutation that may prove complementary to amino acid substitutions for engineering proteins with altered functional and structural properties.**

Key words: protein stability, guanidine hydrochloride denaturation, conformational changes, polypeptide backbone, alanine insertion, glycine insertion, circular dichroism spectra

INTRODUCTION

Two of the most general structural features of globular proteins are the dense packing of backbone and side chain atoms,[1,2] with densities typical of crystals of amino acids, and the complex network of intramolecular hydrogen bonds that includes virtually all buried hydrogen bond donor and acceptor groups.[1,3] Both of these features play critical roles in stabilizing the native state relative to the unfolded state, in much the same way as optimal packing and bonding between small molecules stabilize the crystalline state relative to the liquid state. An implicit analogy between the crystalline state of small molecules and the native state of proteins is often drawn in models for explaining the complex physical chemical properties of proteins, such as the unfolding transition.[4]

This image of an invariant, crystal-like arrangement of atoms can now be tested by applying site-directed mutagenesis and X-ray crystallography to probe the response of protein structures to changes in amino acid sequence. For example, studies on T4 lysozyme mutants by Matthews and colleagues have demonstrated instances in which single amino acid substitutions that dramatically lower the stability of the native state do not undergo significant rearrangements in structure,[5] suggesting that the altered interactions responsible for an increase in free energy of the native state are confined primarily to the site of the substitution. However, in other instances, substitutions which produce relatively little effect on the stability cause significant, and occasionally distant, perturbations in the three dimensional structure.[6]

To obtain a more complete picture of the response of the packing and bonding network of proteins to changes in amino acid sequence, we have constructed and characterized a number of single amino acid insertions at randomly selected positions in staphylococcal nuclease, a small protein whose structure has been solved to 1.65 Å[7,8] and which has been extensively studied by a variety of biochemical, biophysical, and genetic methods.[9–13] Glycine and alanine were chosen for the inserted residue types to minimize the contribution of the side chain to changes in structure and stability. Contrary to expectation, single inserted amino acids had, on average, effects on stability similar to amino acid substitutions at essentially the same position. In addition, while substitutions of residues outside the active site never altered the enzymatic activity of nuclease, several insertions in an alpha helix or a beta strand remote from, but directly connected to, an active site loop had pronounced effects on activity that correlated with structural changes detected by circular dichroism spectroscopy.

MATERIALS AND METHODS

Protein was purified from *Escherichia coli* strain AR120 carrying a mutant allele on the pL9 nuclease expression plasmid by a published procedure.[13] Pu-

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rity was checked by SDS gel electrophoresis and in all cases the nuclease band was judged to represent 95% or more of stained protein. Enzymatic activity was quantitated by the hyperchromicity assay of Cuatrecasas et al.[14] at room temperature in 25 mM Tris.HCl, pH 8.8, 10 mM $CaCl_2$, 50 μg/ml boiled salmon sperm DNA. Wild-type specific activity ranged from 1,700 to 3,300 A_{260} units per minute per milligram. Insertion mutants at 36/37, 60/61, 61/62, and 90/91 showed no increase in activity at 250 micrograms/ml of DNA.

ΔG_{H_2O} in kcal/mole (at 20°C, pH 7.0, 100 mM NaCl, 25 mM Na_2HPO_4) was estimated for each mutant by linear extrapolation of ΔG_{app} determined by intrinsic fluorescence to zero GuHCl concentration using the mutant value of m_{GuHCl}.[11] Values of ΔG_{H_2O} obtained in this way are reproducible to ± 0.1 to 0.2 kcal/mole and have been shown to agree with values from thermal denaturation.[12] For mutants with ΔG_{H_2O} less than 2.0 kcal/mole, a second estimate was obtained by using the ratio between the gain in fluorescence on addition of $(NH_4)_2SO_4$ and the loss of fluorescence on addition of GuHCl.[15,16] In effect, the fluorescence intensity of the native state is determined by refolding all of the unfolded molecules, and this value is then used for calculating the apparent equilibrium constant in the absence of denaturant. In all such cases, ΔG_{H_2O} agreed to within ±0.4 kcal/mole with the value of ΔG_{H_2O} estimated by linear extrapolation to zero denaturant concentration after adjusting the fluorescence intensity of the native state to a value that gives the straightest line fit of the data. For mutants at 126/127 and 131/132 that are near to the single tryptophan residue at position 140, an additional denaturation experiment was done using circular dichroism at 222 nm to estimate ΔG_{H_2O}; the results from fluorescence and CD agreed to within 0.5 kcal/mole or less. $\Delta\Delta G$ is calculated by subtracting the wild-type value of ΔG_{H_2O} (+5.6 kcal/mole) from the mutant value. m_{GuHCl} is the first derivative of ΔG_{app} with respect to GuHCl concentration.

RESULTS AND DISCUSSION

Insertion Mutants

To assure a representative sampling of the effects of single alanine and glycine insertions, 20 peptide bonds were randomly selected for sites of modification. Their positions relative to the secondary structure of staphylococcal nuclease are shown in Figure 1. Mutations at the corresponding sites in the nuclease gene were constructed in one of two ways: 1) A modification of the method of Heffron et al.[17] employing DNase I plus Mn^{2+} was used to make random double-stranded breaks, followed by covalent addition of an oligonucleotide by blunt end ligation. This method, which will be published elsewhere, yielded 9 mutants at 8 different sites. 2) Oligonucleotide-directed mutagenesis by the method of

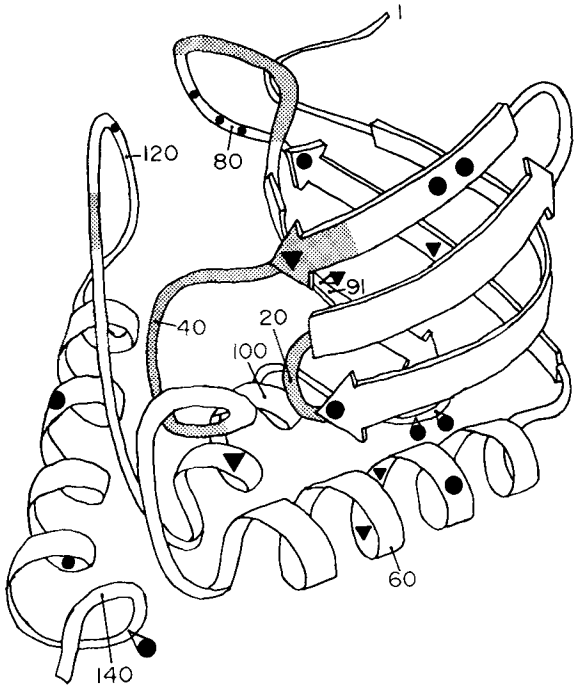

Fig. 1. Ribbon diagram of the secondary structural features of staphylococcal nuclease (copyrighted by Dr. Jane Richardson and used with her permission): ●, sites of alanine and glycine insertions with essentially wild-type enzyme activity; ▼, sites of insertions with significantly reduced activity. Numbers indicate positions of the C-alpha carbon of the corresponding residues. Chain segments that include active site residues are shaded.

Kunkel[18] was employed with a degenerate oligonucleotide encoding both alanine and glycine codons. Four peptide bonds were chosen randomly (literally from a hat) from all bonds that, by the criteria of Kabsch and Sander,[19] link two residues both of which are in an alpha helix. Likewise, four sites in beta sheets and four sites in neither helices nor sheets were also picked at random.

To simplify the analysis, the 20 different sites modified in the resulting collection of insertion mutants are divided into 3 groups based on secondary structure. Six sites are unequivocally within regions of alpha helix, and four fall within beta strands by all standard criteria. The remaining ten sites are a much more heterogeneous group that includes peptide bonds within or abutting turns, loops, and extended segments of chain. Mutant proteins are designated by a label consisting of the extra amino acid type (in the one letter code) inserted between the position numbers of the two residues that flank the site of insertion. For example, a mutant nuclease with an alanine inserted between residues 18 and 19 is designated 18A19.

Table I lists the results of enzyme assays on purified protein using single-stranded DNA as substrate. Insertions at 14 of the 20 sites display catalytic activity comparable to wild-type nuclease.

TABLE I. Insertion Mutants of Staphylococcal Nuclease*

Mutant	Secondary structure	Specific activity	ΔΔG	m_{GuHCl}
wt		1.0	0	1.0
18G19	C	0.2	−2.8	1.10
31A32	B	2.0	−4.5	1.14
31G32	B	0.9	−4.8	1.17
32A33	B	0.3	−6.2	
32G33	B	0.6	−6.8	
36A37	C	<.002	−2.6	.89
36G37	C	<.002	−4.3	.89
60A61	A	<.002	−2.0	.89
60G61	A	.005	−2.6	.97
61A62	A	.05	−4.1	.94
61G62	A	<.002	−5.9	
63A64	A	0.3	−7.0	
63G64	A	1.0	−7.4	
73A74	B	.05	−7.6	
73G74	B	.01	−7.9	
76A77	C	0.9	−3.1	.79
76G77	C	0.6	−2.7	.88
79G80	C	0.4	−1.9	.95
80A81	C	0.7	−2.2	.93
80G81	C	0.7	−2.4	.91
82G83	C	0.5	−4.0	.83
90A91	B	.002	−2.7	.86
94A95	C	0.2	−6.0	1.05
95A96	C	0.4	−6.0	1.09
95G96	C	0.5	−5.3	1.06
103A104	A	<.002	<−8.0	
118A119	C	0.3	−1.9	.90
118G119	C	0.5	−1.5	.95
126A127	A	0.5	−6.1	.77
126G127	A	0.4	−6.1	.78
131A132	A	0.3	−7.3	
138A139	C	0.4	<−8.0	
138G139	C	0.5	−6.0	

*Enzymatic activity and stability parameters for reversible denaturation of insertion mutant forms of staphylococcal nuclease. The secondary structural class of each peptide bond is characterized by the criteria of Kabsch and Sander[19] as alpha-helical (A), beta-strand (B), or neither helical nor beta-strand (C). Enzyme activity is expressed relative to the wild-type value. Changes in stability ($\Delta G_{mutant} - \Delta G_{wt} = \Delta\Delta G$) are given in kilocalories per mole. m_{GuHCl} is expressed relative to the wild-type value of 6.8 kcals/mole *M*. As observed earlier with substitution mutants of staphylococcal nuclease,[11] the m_{GuHCl} varies from 0.65 to 1.25 of the wild-type value, a phenomenon of uncertain origin.[15,28]

From this observation alone, the conclusion can be drawn that these mutant proteins are either stably folded or can be stabilized in a folded state on binding of substrate. (Since the conditions of assay include concentrations of substrate and calcium ion cofactor that are 10 to 20 times the K_m values measured for wild-type enzyme, the potential for "substrate stabilization" is quite large.) In either case, the enzyme-substrate complexes for mutant proteins with normal activity have undergone no significant structural changes effecting the important catalytic residues.

Among the six sites where insertions significantly reduced activity, the extra residue introduced into peptide bond 36/37 may lower activity directly by modifying the structure of the active site. (The leucine at 37 forms the hydrophobic floor of the nucleotide binding cleft.) The alanine insertion at 103/104 appears to be highly unstable even in the presence of very high concentrations of substrate (data not shown). The remaining four sites (60/61, 61/62, 73/74, and 90/91) are outside the active site region. Nuclease mutants at these positions are saturated at low concentrations of substrate and have specific activities which are much lower than can be accounted for by their reduced stabilities.

The stability of the native state to reversible denaturation was measured for each mutant protein by guanidine hydrochloride denaturation. The changes in ΔG_{H_2O} from the wild-type value of +5.6 kcal/mole[11,12] are listed in Table I. Although the sample size is small, the loss of stability ($\Delta\Delta G_{H_2O}$) on average was −5.7 kcal/mole for insertions which fell in alpha helices and approximately −5.8 kcal/mole for those in beta sheets. In other words, the average mutant in these two classes has a stabilization free energy of zero, meaning that in the absence of added denaturant, roughly one half of the protein chains are folded and the other half are unfolded. For insertions into regions of staphylococcal nuclease which have no regular secondary structure, such as loops, turns, and irregularly structured regions, the stability consequences are smaller—approximately −3.8 kcal/mole. No correlation was found between the magnitude of $\Delta\Delta G_{H_2O}$ and the relative surface exposure of the insertion site, defined either by the average solvent accessibility of the side chains of the two flanking residues or by the solvent accessibility of the peptide backbone.[8]

Sites were identified in both an alpha helix (60/61 and 61/62) and a beta sheet (90/91) where the energetic consequences of an insertion were surprisingly small in view of the peptide bond's central location in the secondary structure and its buried position. Since all insertions at these three positions, which are not adjacent to residues within the active site, showed large reductions in catalytic activity, it seems likely that significant structural rearrangements involving one or more active site residues may have occurred to accommodate the extra amino acid. As can be seen in Figure 2, mutants 60A61 and 60G61 have ultraviolet circular dichroism spectra consistent with small reductions in alpha helical content, whereas mutant 90A91 exhibited some nonspecific spectral changes.

In addition to the sites in a helix and sheet where an extra residue could be stably accommodated, sites in a solvent exposed turn—94/95 and 95/96—were found where an extra residue is surprisingly destabilizing (−5.3 to −6.0 kcal/mole). Although plausible arguments on the origins of the instability caused by any change in amino acid sequence can be proposed in terms of changes in the native state

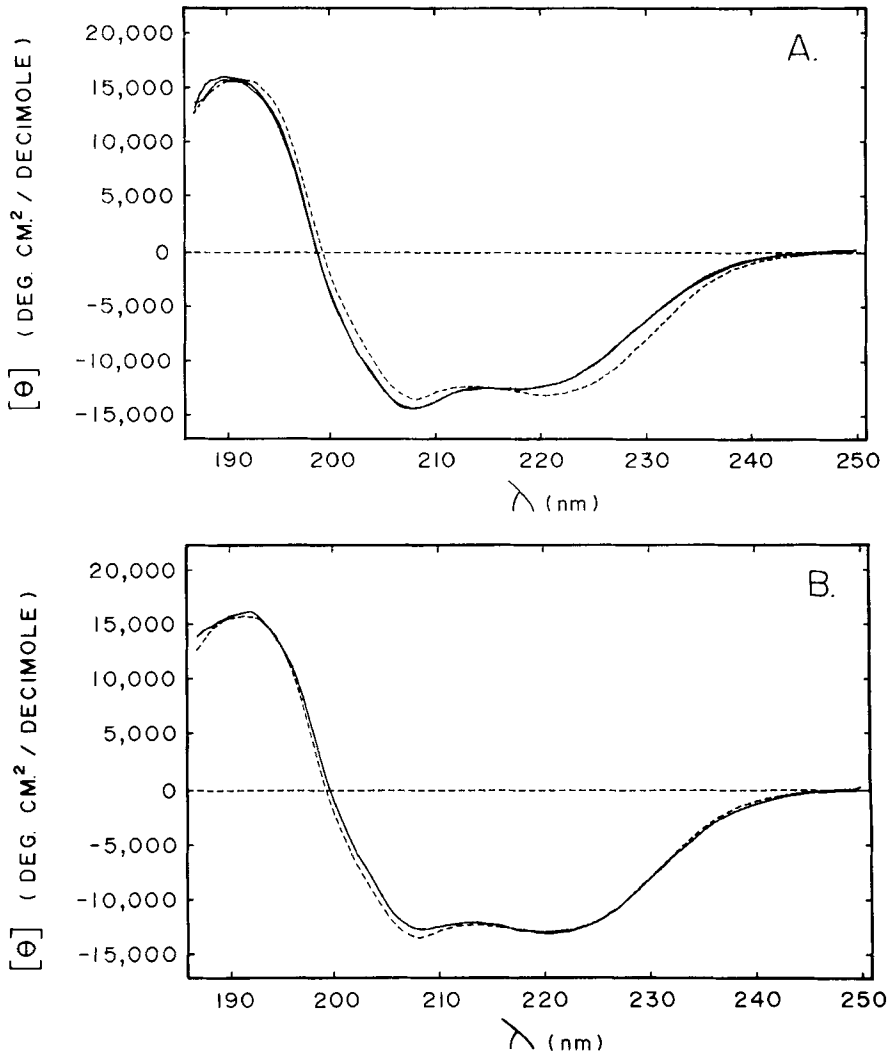

Fig. 2. Circular dichroism spectra of wild-type (– – –) and mutant forms (—) of staphylococcal nuclease. **A:** Insertion mutants 60A61 and 60G61. **B:** Insertion mutant 90A91. The abscissa is wavelength in nanometers; the ordinate is mean molar residue ellipticity expressed in degrees*cm^2 per decimole. Spectra are the average of seven scans on an AVIV 60DS spectrometer using a 0.1mm path cuvette thermostatted at 20°C. Protein concentration was 1.0 mg/ml in 50 mM NaCl/1 mM Na_2PO_4, pH 7.0.

structure, the possibility must be considered that the energetic effects of such mutations may be exerted in part on the free energy of the denatured state.[12] Simply lengthening the chain increases the entropy of the denatured state, and the increased flexibility produced by an extra amino acid might permit new interactions in the denatured state, such as contacts between hydrophobic residues on opposite sides of the insertion, that alter its structure or free energy.

Substitution mutants at residues which flank an insertion

In order to compare the magnitude of the stability effects of an inserted residue with that of a simpler sequence change at the same site, alanine or glycine substitutions were introduced into wild-type length nuclease for each of the two residues that flank the site of five different insertions. Values of $\Delta\Delta G_{H_2O}$ and specific activity for these mutant proteins are listed in Table II. In three cases—36A37, 60A61, and 76A77—the insertion mutant is more stable than one of the flanking alanine substitutions and less than or equal in stability to the other. In the other two cases—61A62 and 61G62—the insertion mutant is less stable than either of the two flanking substitution mutants. Single substitutions involving sites flanking other insertions are also listed for comparison, and in several cases, they lower stability by roughly the same amount as an adjacent insertion—18G19 ≅ I18G, 79G80 ≅ G79S, 82G83 ≅ D83A, 118A119 ≅ N118D.

With regard to possible mechanisms used by the native state to accommodate the inserted residue,

TABLE II. Substitution Mutants of Staphylococcal Nuclease*

Mutant	Secondary structure	Specific activity	ΔΔG	m_{GuHCl}
I18G	C	0.8	−2.6	1.06
T33S	B	0.6	−1.4	1.07
L36A	B	0.9	−3.6	1.15
L36G	B	0.5	−5.4	1.24
L37A	C	0.3	−1.8	.90
L37G	C	0.3	−3.9	.95
A60G	A	0.6	−1.5	1.00
A60V	A	1.3	−2.9	.89
F61A	A	1.0	−2.4	1.00
F61G	A	1.0	−4.7	1.04
T62A	A	0.7	−2.4	.92
T62G	A	1.4	−3.4	1.09
E73A	B	0.9	−1.6	1.00
F76A	B	0.5	−4.1	1.07
F76G	B	0.6	−4.7	1.14
D77A	C	0.7	−3.2	.78
D77G	C	0.9	−2.2	.83
G79S	C	0.9	−2.4	.92
D83A	C	0.1	−3.9	.90
D83G	C	0.2	−2.8	.93
A90S	B	0.9	−2.0	1.10
Y91S	B	0.4	−5.3	1.08
D95A	C	1.1	−3.6	1.07
D95G	C	0.8	−3.1	1.03
L103A	A	0.6	−4.6	1.0
N118D	C	n.d.†	−2.5	.89

*Enzymatic activity and stability parameters of substitution mutants of staphylococcal nuclease. See Table I for the legend.
†n.d., not determined.

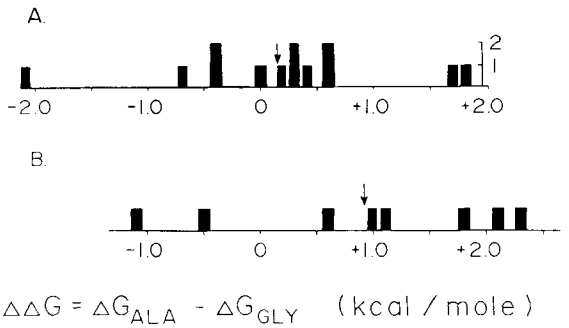

Fig. 3. Histogram of the stability difference between pairs of alanine and glycine mutations at the same position ($\Delta G_{ala} - \Delta G_{gly}$) expressed in kcal/mole. The y-axis designates the number of residue positions; the mean value is marked with an arrow. All data are taken from Tables I and II. **A:** Insertions. **B:** Substitutions.

several influences can be drawn from these data. For example, a segment of polypeptide chain could shift its register by one residue in a direction away from the inserted residue until the left-over residue is extruded at a solvent-exposed end, such as a turn or loop. Such a register shift would effectively equal a series of substitutions plus an end insertion. Given the magnitude of the effect of the first one of the requisite series of substitutions (i.e., the alanine or glycine substitution for one of the two immediately adjacent residues), this seems an unlikely mechanism except when the inserted residue is very near one end. In the cases of 18G19, 36A37, 76A77, and 76G77 for which the insertions occur at the end of beta strands, the values of $\Delta\Delta G_{H_2O}$ are very similar to those of the corresponding substitution of one of the flanking residues, raising the possibility of a register shift of a single amino acid.

Alternatively, inserted residues might be accommodated by localized bulges, such as the "beta bulge" found occasionally at ends or at edges of antiparallel beta sheets.[20] If bulges are a common local structural response to an extra residue, one might expect on average that an inserted glycine would be more readily accommodated than an alanine because of its greater range of allowable phi,psi angles. As can be seen in Figure 3A, for a few alanine-glycine pairs at the same site of insertion (most notably 138/139), the Gly insertion is more stable. However, since the values of $\Delta G_{ala} - \Delta G_{gly}$ are fairly symmetrically distributed around a mean of only +0.2 kcal/mole, this mechanism may not provide a general solution for accommodating the extra residue.

In Figure 3B, the difference in ΔG_{H_2O} between eight pairs of Ala and Gly substitutions ($\Delta G_{ala} - \Delta G_{gly}$) also shows a wide distribution, with a mean of approximately +0.9 kcal/mole greater stability for Ala substitutions. The greater hydrophobic character of alanine and its more restricted phi-psi angles presumably make it a more stable substitution than glycine for most amino acids types and positions. However, in the context of the altered structure surrounding the inserted residue, one or both of these two differences may no longer make a net contribution to stability. The different mean values for these two distributions of $\Delta G_{ala} - \Delta G_{gly}$ may reflect the less significant role of the side chain for insertions, where the extra main chain atoms and the change in spacing are presumably the major determinants of the reductions in ΔG_{H_2O}.

CONCLUSIONS

These data suggest that the insertion of an additional small amino acid, with the accompanying 3–4 Å increase in separation of the two flanking residues, seldom interferes with the ability of staphylococcal nuclease to fold into a more or less stable native state. Although occasionally fairly large structural changes may be required to accommodate the extra residue, the solution to this problem in the majority of instances appears to be a relatively confined perturbation of structure that does not grossly alter the active site. This surprising ability to adjust to such large modifications in covalent structure implies that the folded chain must be able to access a number of alternative bonding and packing arrangements of only slightly higher free energy than

the wild-type native state. Put more simply, the packing and bonding network of staphylococcal nuclease behaves more like a glass that deforms locally than a rigid crystal. Attempts to identify the detailed structural changes in these insertion mutant proteins by X-ray crystallography are currently underway.

Attention is called to the insertions remote from the active site which show evidence of small structural changes by circular dichroism (60A61, 60G61, and 90A91). Unlike substitution mutants at these same positions (or at any position outside the active site), the insertion mutants display markedly reduced levels of catalytic activity. Since the sites of insertion fall within segments of secondary structure directly connected to surface loops that play critical roles in forming the active site (residues 40–49 and 83–87, respectively; see Fig. 1), it seems likely that changes in positioning of catalytic residues has occurred. Perhaps in the evolution of a new enzymatic activity, similar small distortions of loops caused by distant insertions or deletions might play a role in fine-tuning the active site of a protein to achieve higher catalytic efficiencies in ways that complement the larger re-positionings of a loop produced by amino acid insertions or deletions within the loop itself.

Over the past ten years a number of reports have appeared in the literature describing single, double, and multiple codon insertions that cause temperature-sensitive behavior and/or altered functional activity for a variety of proteins.[21–27] In a systematic study of the effects of insertion mutations, Barany found that mutants that inserted two amino acids into the TEM beta-lactamase of *E. coli* retained some residual activity at six out of eight random sites,[25] whereas for the Taq I restriction endonuclease, eight out of eleven insertion mutants were active.[26] A similar fraction of active proteins was found by Tanese and Goff among two amino acid insertion mutants in the genes encoding DNA polymerase and RNase H of Moloney murine leukemia virus.[27] Unfortunately, the three-dimensional structures of these proteins have not yet been determined, so the positions of the tolerated insertions are not known. Nevertheless, the very high frequency of active insertions in these four proteins suggests that the majority of proteins may, like staphylococcal nuclease, exhibit a high degree of adaptability to insertions. If so, amino acid insertions may prove to be a new and useful class of genetic probe of protein structure and function that occasionally produces perturbations which are not achievable by amino acid substitutions alone.

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