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Increasing and Decreasing Protein Stability: Effects of Revertant Substitutions on the Thermal Denaturation of Phage λ Repressor

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The thermal denaturations of five revertant λ repressors containing single amino acid substitutions in their N-terminal domains have been studied by differential scanning calorimetry. Two substitutions slightly decrease stability, and the remaining three render the protein more stable than wild type. The $Gly_{48} \rightarrow Asn$ and $Gly_{48} \rightarrow Ser$ proteins are 4°C more stable than wild type. These two substitutions replace an α helical residue, and in each case a poor helix forming residue, glycine, is replaced by a residue with a higher helical propensity. We also present data showing that one revertant, $Tyr_{22} \rightarrow Phe$, has reduced operator DNA binding affinity despite its enhanced stability.

Key words: mutant repressors, differential scanning calorimetry, protein stability, thermal denaturation

Questions concerning the structure and function of proteins can often be addressed by studying mutant proteins containing single amino acid substitutions. For example, the effects of mutations on protein stability have been investigated for a number of proteins, including hemoglobin, T4 phage lysozyme, the α subunit of tryptophan synthetase, phage P22 tail protein, and staphylococcal nuclease [1–9]. We have previously described mutations in the N-terminal domain of λ repressor that reduce protein stability and/or interfere with the binding of the folded mutant repressor to operator DNA [10–13]. In general, when side chains that are buried in the wild type structure are substituted, the thermal stability of the N-terminal domain is substantially decreased. Conversely, most solvent-exposed substitutions have little or no effect on thermal stability [12]. One repressor mutation, $Gln_{33} \rightarrow Tyr$, was found

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to increase the thermal stability of N-terminal domain by 6°C, apparently by introducing favorable tertiary interactions not present in the wild-type protein [12].

In recent work, we have isolated same-site and second-site revertants of many of the mutant repressors [14,15]. In same-site revertants, repressor activity is restored by replacing the mutant amino acid with an amino acid other than wild type. These revertants provide information about the chemical and steric properties that are required at a given side chain position in order to maintain protein stability and activity. In second-site revertants, the original mutation is maintained, and activity is rescued by an amino acid substitution elsewhere in the protein. The second-site substitutions that we have studied increase the operator affinity of otherwise wild-type repressor [15]. These results suggest intragenic reversion as a general method for obtaining proteins with greater than wild-type activity.

In this paper, we report thermal denaturation studies for five revertant λ repressor proteins containing single amino acid substitutions in their N-terminal domains. Some of the revertants are more stable than wild type, and some are less stable. We interpret the effects of the amino acid substitutions in terms of the crystal structure of the N-terminal domain [16].

MATERIALS AND METHODS

Revertant Proteins

The isolation of revertants in the N-terminal domain of λ repressor and the separation of second-site revertants from original mutations have been described elsewhere [14,15]. Substituted repressor proteins were purified from overproducing strains using procedures similar to those devised for the wild type protein [17].

Thermal Stabilities

The thermal denaturations of wild type and five substituted repressors were observed with a DASM-4 scanning microcalorimeter in a buffer containing 10 mM potassium phosphate, pH 8.0, 200 mM KCl, and 1 mM NaN_3. We have previously shown that the temperature, t_m, of maximal excess heat capacity is slightly dependent on protein concentration and scan rate [12]. Therefore, in this work the protein concentration was 2.0 mg/ml and the scan rate was 1.0°C/min in all cases.

Filter Binding Assays

The repressor-operator DNA filter binding assay has been described elsewhere [13,15,17]. In this study, assays were performed at 22°C using a ^{32}P radiolabeled λ O_R1 DNA fragment, which was dissolved at less than 10^{-12} M in a buffer containing 10 mM PIPES, pH 7.0, 50 mM KCl, 0.1 mM EDTA, 100 μg/ml bovine serum albumin, and 5% DMSO.

RESULTS

The $Tyr_{22} \rightarrow$ Phe and $Gly_{48} \rightarrow$ Asn repressor proteins were obtained by same-site reversion from the primary mutants $Tyr_{22} \rightarrow$ Ser and $Gly_{48} \rightarrow$ Asp, respectively [14]. The $Glu_{34} \rightarrow$ Lys, $Gly_{48} \rightarrow$ Ser, and $Glu_{83} \rightarrow$ Lys repressors were obtained by second-site reversion and were separated from the primary mutations by recloning [14,15].

The wild type sequence of the N-terminal domain [18,19] and the revertant substitutions are shown in Figure 1.

In differential scanning calorimetry (DSC) experiments, the N-terminal and C-terminal domains of λ repressor show separate denaturational endotherms at approximately 50°C and 70°C, respectively [20]. Tracings of DSC curves for wild type and the five revertant repressors are shown in Figure 2. For the $Glu_{34} \rightarrow Lys$ and $Glu_{83} \rightarrow Lys$ revertants, the N-terminal domain denaturational endotherm occurs at a lower temperature than for the wild type protein. Conversely, the $Tyr_{22} \rightarrow Phe$, $Gly_{48} \rightarrow Asn$, and $Gly_{48} \rightarrow Ser$ N-terminal domains are more thermally stable than wild type. The C-terminal domain denaturational endotherms of all five revertants are the same as for wild type within experimental uncertainty. The t_ms and calorimetric enthalpies for the N-terminal and C-terminal domain transitions are summarized in Table I. Enthalpies for both the N-terminal and C-terminal domain transitions are within experimental uncertainty of the wild type values.

We have previously reported that λ repressor proteins containing the $Glu_{34} \rightarrow Lys$, $Gly_{48} \rightarrow Asn$, $Gly_{48} \rightarrow Ser$, or $Glu_{83} \rightarrow Lys$ substitutions have greater than wild type affinities for λ operator DNA [15]. Figure 3 shows equilibrium operator binding experiments for wild type and the $Tyr_{22} \rightarrow Phe$ repressor. Fourfold higher concentrations of the $Tyr_{22} \rightarrow Phe$ repressor are required to achieve the same degree of operator binding as wild type. In this experiment, the free repressor is predominantly monomeric and must dimerize before binding to the operator. We have assumed that the wild type and revertant repressors have the same dimerization constant. This seems a fair assumption in that the $Tyr_{22} \rightarrow Phe$ substitution does not affect the C-terminal domain, which mediates strong dimerization, nor the helix 5 region of the N-terminal domain, which mediates the weak dimerization of the N-terminal domain [see 13,15

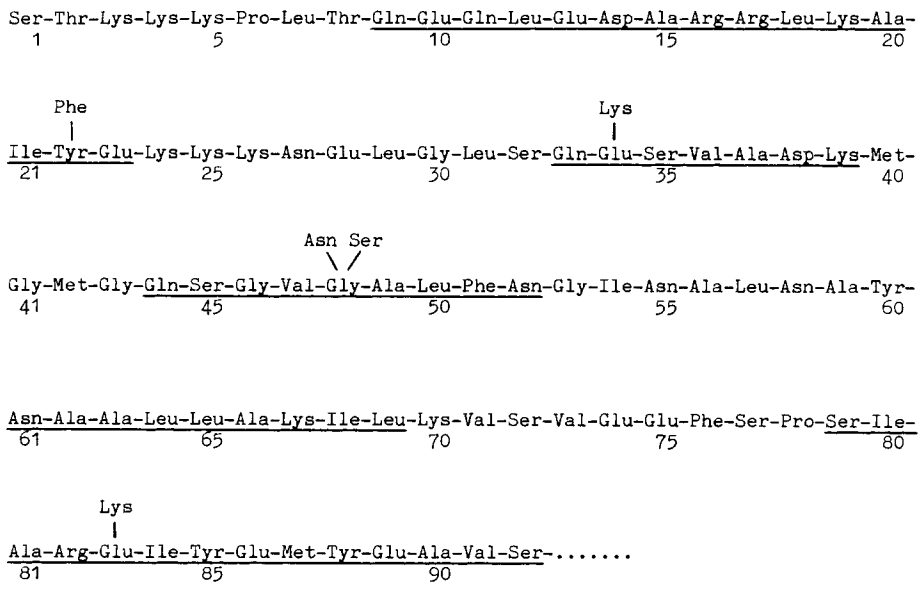

Fig. 1. Sequence of the wild type N-terminal domain of λ repressor with the five α helices underscored. Revertant substitutions are shown above the wild type amino acid that they replace.

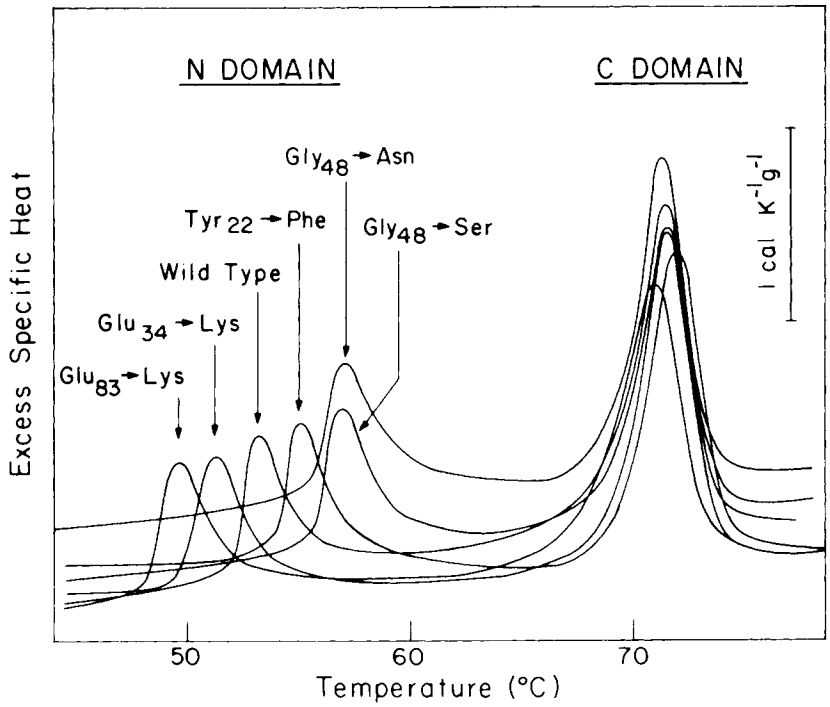

Fig. 2. Differential scanning calorimetry curves of excess specific heat vs temperature for wild type and five revertant repressors.

TABLE I. Properties of Wild Type and Mutant Proteins

	N-terminal domain			C-terminal domain	
Substitution	t_m (°C)	Δt_m[a] (°C)	ΔH (kcal/mol)	t_m (°C)	ΔH (kcal/mol)
Wild type	53.2	—	52	71.4	136
Tyr_{22}→Phe	55.05	+ 1.85	56	71.9	124
Glu_{34}→Lys	51.3	− 1.9	51	71.7	135
Gly_{48}→Asn	56.95	+ 3.75	62	71.3	118
Gly_{48}→Ser	56.85	+ 3.65	55	71.45	122
Glu_{83}→Lys	49.65	− 3.55	59	71.05	137

[a] $\Delta t_m = t_m$(mutant) − t_m(wild type).

for further discussion]. Thus the binding constant of the Tyr_{22}→Phe repressor dimer for operator is about 16-fold weaker than wild type. This weaker binding is consistent with our previous finding that the Tyr_{22}→Phe-substituted protein is about 25-fold less active *in vivo* than wild type [14].

DISCUSSION

In λ repressor, the N-terminal and C-terminal domains are structurally independent. Proteolytic fragments corresponding to each domain remain stably folded and active; the N-terminal fragment binds operator DNA, and the C-terminal fragment

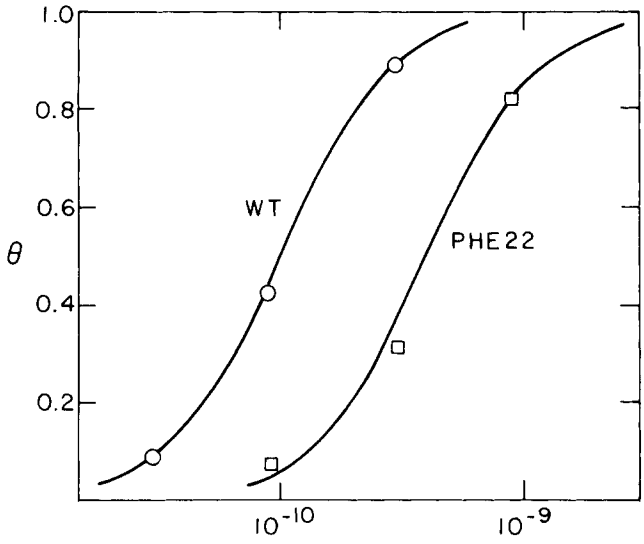

Fig. 3. Equilibrium operator DNA binding experiments for wild type (WT) and the $Tyr_{22} \rightarrow Phe$ revertant repressor. θ, the fractional saturation of operator, is calculated from the fraction of radioactive operator DNA that is retained on filters by bound repressor. The abscissa gauges total repressor (M).

forms oligomers [20,21]. The crystal structure of the N-terminal domain is predominantly α helical [16]. The first four helices enclose a hydrophobic core and form a compact globular structure. The fifth α helix interacts with the fifth helix of a neighboring molecule to stabilize a dimer. The substitutions discussed here replace residues in four of the five α helices of the N-terminal domain. In addition, all of the substitutions except $Tyr_{22} \rightarrow Phe$ replace residues that are solvent exposed in the wild type crystal structure. In the following section, we will discuss the revertant substitutions in terms of the wild type crystal structure and mechanisms that might account for the changes in thermal stability.

$Gly_{48} \rightarrow Asn$ and $Gly_{48} \rightarrow Ser$

Residue 48 is found on the solvent-exposed face of α helix 3. The wild-type residue, Gly_{48}, is one of two glycines in this nine-residue α helix. The t_ms for the N-terminal domain in the Asn_{48} (56.95°C) and Ser_{48} (56.85°C) revertants are almost 4°C higher than that of the wild type protein (Fig. 2, Table I).

Glycine has the lowest helical propensity (s = 0.6 [22], P_α = 0.57 [23]) of all the amino acids. (s is an equilibrium constant derived from synthetic copolymer studies for the addition of one random coil residue to a nucleated α helix [22] P_α is the normalized frequency at which a given amino acid is found in α helices in a set or proteins with known structures [23].) The increased thermal stability of the Asn_{48} and Ser_{48} proteins could be caused by enhanced stability of α helix 3. Neither asparagine (s = 0.79, P_α = 0.67) nor serine (s = 0.77, P_α = 0.77) are good helix formers, but both are significantly better than glycine. Although the increased stability of $Gly_{48} \rightarrow Asn$ and $Gly_{48} \rightarrow Ser$ proteins is in accordance with helix-coil studies, the magnitude of the stability increase is larger than would be predicted from s values alone. For example, at 53.2°C the free energy (ΔG) of stabilization for the $Gly_{48} \rightarrow Asn$

N-terminal domain is 0.7 kcal/mol greater than wild type. Differences in s values between glycine and asparagine could account for less than 0.2 kcal/mol of the increased energy of stabilization. Other stabilizing mechanisms might involve helix nucleation and/or favorable interactions between the revertant side chain and other repressor side chains.

If the enhanced thermal stabilities of the $Gly_{48} \rightarrow Asn$ and $Gly_{48} \rightarrow Ser$ proteins result predominantly from helix stabilization, then the site-directed replacement of Gly_{48} by alanine, which has a high helical propensity ($s = 1.06$, $P_\alpha = 1.42$), would be expected to increase stability to a greater extent than the $Gly_{48} \rightarrow Asn$ or $Gly_{48} \rightarrow Ser$ substitutions. This experiment is currently in progress.

$Glu_{34} \rightarrow Lys$

Glu_{34} is on the solvent-exposed face of α helix 2. The t_m (51.3°C) for the N-terminal domain of the $Glu_{34} \rightarrow Lys$ revertant protein is about 2°C lower than wild type. The $Glu_{34} \rightarrow Lys$ substitution changes the net charge of the protein by two, and the altered distribution of surface charges could be responsible for the small destabilizing effect of this substitution. However, Hollecker and Creighton [24] have shown that 10 or more lysines can be converted to acidic groups by succinylation with little or no effect on the stability of several different proteins. Alternatively, the small decrease in stability caused by the $Glu_{34} \rightarrow Lys$ substitution might result from the replacement of a residue with a high helical propensity ($P_\alpha = 1.51$) by one with a moderate helical propensity ($P_\alpha = 1.16$) [23]. Glu_{34} is the second residue of α helix 2, and its negatively charged side chain might interact favorably with the partial positive charge at the N-terminal end of the α helix. In this case, the $Glu_{34} \rightarrow Lys$ substitution would remove a favorable interaction and introduce an unfavorable interaction. It is not clear to what extent this electrostatic factor is already included in the P_α values of Chou and Fasman [23] and the s values of Scheraga [22].

$Glu_{83} \rightarrow Lys$

Glu_{83} is found in α helix 5. Because this helix is involved in N-terminal domain dimerization [16], the effects of the $Glu_{83} \rightarrow Lys$ substitution could be complex. As shown in Figure 2, this substitution decreases the thermal stability of the N-terminal domain by 3.5°C. If dimerization is coupled to stability as shown in the reaction:

$$\text{Native Dimer} \leftrightarrow \text{Native Monomer} \leftrightarrow \text{Denatured Monomer},$$

then the decrease in stability caused by the $Glu_{83} \rightarrow Lys$ substitution could be accounted for by a decrease in dimerization energy. As discussed above for the $Glu_{34} \rightarrow Lys$ substitution, it is also possible that helix-coil, electrostatic or side chain interactions are responsible for the decreased stability of the $Glu_{83} \rightarrow Lys$ revertant protein.

$Tyr_{22} \rightarrow Phe$

The Tyr_{22} side chain is buried in the hydrophobic core of the wild type N-terminal domain. (In calculations of solvent-exposed surface area, only the $C\delta 1$ and $C\epsilon 1$ atoms show any solvent accessibility.) The aromatic ring of Tyr_{22} packs against Phe_{51}, and, as is shown in Figure 4, the hydroxyl group points towards the polypeptide backbone near the N-terminus of α helix 2. It appears that the hydroxyl group is involved in a hydrogen bond with the backbone, but at the current resolution of the crystal structure [16] this is not certain.

Fig. 4. Stereo pair showing the α carbon backbone and side chain Tyr_{22} in the wild type N-terminal domain. The positioning of repressor with respect to the backbone of B-form DNA is according to the repressor-operator model of Lewis et al [25].

Most substitutions at position 22 appear to destabilize the N-terminal domain. For example, a $Tyr_{22} \rightarrow$His mutant repressor is about 20°C less thermally stable than wild type, and genetic evidence suggests that the Cys_{22}, Asp_{22}, and Ser_{22} mutant repressors are even less stable [12,14]. The data presented here show that the N-terminal domain of the $Tyr_{22} \rightarrow$Phe-substituted repressor is approximately 2°C more stable than wild type (Fig. 2, Table I). This suggests that packing interactions involving the aromatic ring of the wild type Tyr_{22}, and not a hydrogen bond involving the hydroxyl group, are of primary importance in maintaining the stability of the folded structure. Perhaps the hydroxyl group forms a strained hydrogen bond with the polypeptide backbone of α helix 2, and this bond is detrimental to protein stability in that it occurs at the expense of a good hydrogen bond that might be formed with water in the unfolded state.

Tyr_{22} is found in α helix 1, and the revertant Phe has a higher helical propensity ($P_\alpha = 1.13$) than Tyr ($P_\alpha = 0.69$). Furthermore, according to the host-guest copolymer experiments of Scheraga [22], this difference in helical propensity is magnified at elevated temperatures. Thus it is possible that the stability of α helix 1 in the $Tyr_{22} \rightarrow$Phe revertant is enhanced and that this accounts for the increased thermal stability of this substituted protein.

Despite its increased thermal stability, the Phe_{22} revertant has a lower affinity for operator DNA than the wild type Tyr_{22} repressor (Fig. 3). In that several of the side chains on the solvent-exposed face of α helix 2 interact with operator DNA [25]

(see Fig. 4), it is possible that interactions mediated by the Tyr_{22} hydroxyl group position helix 2 to allow side chains to interact optimally with operator DNA. In the Phe_{22} revertant, helix 2 might assume a slightly different position that increases protein stability at the expense of favorable interactions with the operator.

CONCLUSION

The five revertant substitutions that we have characterized result in small (2–4°C) but significant changes in the thermal stability of the N-terminal domain of λ repressor. There are a variety of mechanisms that could account for the increases and decreases in thermal stability, and considerable further work will be required to support or rule out any particular mechanism. However, one correlation is striking. The residue substitutions that reduce α helical propensity reduce thermal stability, and those that increase α helical propensity increase stability. The significance of this correlation remains to be determined.

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