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Effect of single amino acid replacements on the thermal stability of the NH_2-terminal domain of phage λ repressor

(scanning calorimetry/circular dichroism/proteolysis/temperature-sensitive mutants)

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ABSTRACT **The thermal stabilities of mutant phage λ repressors that have single amino acid replacements in the NH_2-terminal domain have been studied by means of circular dichroism and differential scanning calorimetry. The variations in stability determined by these physical methods correlate with the resistance to proteolysis at various temperatures and can be compared with the temperature-sensitive activity of the mutants *in vivo*. In general, mutant proteins bearing solvent-exposed substitutions have thermal stabilities identical to wild type, whereas buried substitutions reduce stability. In one case, a single amino acid replacement increases the thermal stability of the repressor.**

The three-dimensional structure of a protein is determined by its amino acid sequence. However, the forces and interactions that cause a protein to adopt and maintain one structure out of an enormous number of possible structures are understood only in a general sense. One approach to understanding the contributions of specific interactions to the stability of protein structures is to study mutant proteins with single amino acid substitutions. In this paper, we examine the effect of mutations on the thermal stability of the NH_2-terminal domain of phage λ repressor.

λ repressor is a 236-amino-acid protein consisting of an NH_2-terminal DNA binding domain and a COOH-terminal oligomerization domain (1, 2). The two domains are structurally and dynamically independent; they show widely separated thermal denaturation transitions (1), they reorient independently (3), and proteolytic fragments corresponding to each domain remain stably folded and functionally active (1, 2). The *de novo* folding of the NH_2-terminal domain must also be independent of the COOH-terminal domain since cloned NH_2-terminal fragments are active *in vivo* (2). The crystal structure of the NH_2-terminal domain is predominantly α-helical (4). The first four helices form a compact globular structure, whereas the fifth α helix is directed away from this structure and is packed against the fifth helix of a neighboring molecule in a dimer.

We have previously reported the sequences of mutations coding for 52 different single amino acid substitutions in λ repressor's NH_2-terminal domain (5, 6). In this paper we determine the thermal stabilities of 15 purified mutant repressors and report the temperature-sensitive activities *in vivo* of these and eight additional mutant repressors.

MATERIALS AND METHODS

Protein Purification. Wild-type and mutant λ repressor genes were subcloned onto the overproducing plasmid vector pEA300 (7). In this background, cells can be induced with isopropyl β-D-thiogalactoside to produce λ repressor as 10% of their total protein. Mutant proteins were purified by procedures similar to those described for wild-type repressor (8).

Thermal Stabilities. CD measurements were made with a Cary model 60 spectrapolarimeter equipped with a Cary model 6001 CD accessory. A water-jacketed cylindrical cell with a 1-cm pathlength was used for protein concentrations <0.1 mg/ml, and a similar cell with a 1-mm pathlength was used for more concentrated samples. Mean residue ellipticity, $[\theta]$, was calculated as:

$$[\theta] = \frac{100 \times \theta \times m}{c \times L}, \qquad [1]$$

where θ is the observed ellipticity, m is the mean residue weight, c is the concentration in mg/ml, and L is the pathlength in cm. Melting curves were routinely determined at repressor concentrations of 0.02–0.05 mg/ml in 10 mM phosphate, pH 7.0/100 mM KCl. CD spectra of repressor and proteolytic fragments of repressor were taken from 280 nm to 200 nm and analyzed by the method of Greenfield and Fasman (9). Melting curves were obtained by measuring $[\theta]_{222}$ as a function of temperature. The temperature was adjusted with a thermostated circulating water bath and checked with a thermocouple inserted directly into the cell. The temperature was raised in 3–6°C increments, allowing time for equilibration before measurements were made.

The thermal denaturations of wild-type and seven mutant repressors were observed with a DASM-1M scanning microcalorimeter (10) in 10 mM K phosphate, pH 8.0/200 mM KCl/1 mM NaN_3. The protein concentration in all cases was 4.44 mg/ml, except for the wild-type repressor for which concentrations of 9.27 and 13.9 mg/ml were also studied. In previous studies of protein denaturation with the DASM-1M calorimeter, a scan rate of 1°C/min usually has been employed. However, at this scan rate we encountered difficulties with exothermic peaks, which presumably result from protein aggregation. These exotherms were greatly reduced or eliminated at a scan rate of 0.25°C/min; therefore, we used this rate throughout the present work. At this reduced scan rate, the temperature, t_m, of maximal excess heat capacity for the denaturational endotherm of the NH_2-terminal domain is approximately 0.5°C lower than at a scan rate of 1°C/min, and that of the COOH-terminal domain is about 1.5°C lower.

Mutant Phenotypes. The activity of mutant repressors *in vivo* was monitored by the resistance to infection by phage λ of cells harboring wild-type or mutant copies of the repressor-producing plasmid pKB280 (11). Wild-type and missense mutant repressors were assayed in *Escherichia coli* strain 294 (11), and suppressed amber mutants at positions 33 and 44 were assayed in the amber-suppressing strains DB6431 (inserts serine) and DB6433 (inserts tyrosine). Cells were

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Abbreviation: DSC, differential scanning calorimetry.

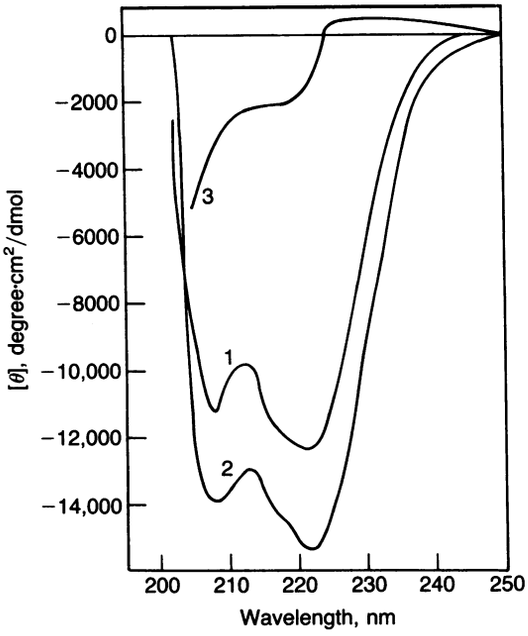

FIG. 1. CD spectra of intact repressor (curve 1) and proteolytic fragments corresponding to the NH_2-terminal domain residues 1–92 (curve 2) and COOH-terminal domain residues 93–236 (curve 3). Spectra were measured at 25°C.

grown to midlogarithmic phase in L broth containing tetracycline (5 μg/ml). Wild-type phage λ at about 200 plaque-forming units per plate was added to 0.25 ml of cells and plated at 26°C, 30°C, 37°C, and 42°C. Cells resistant to phage infection showed no plaques, whereas sensitive cells gave plaques after overnight growth.

RESULTS

Circular Dichroism. Fig. 1 shows the CD spectra of λ repressor and of proteolytic fragments corresponding to its NH_2-terminal and COOH-terminal domains. The peaks of large negative ellipticity at 208 and 222 nm in the spectra of the intact repressor and the NH_2-terminal domain are indicative of substantial α-helical structure (9). The spectrum of the COOH-terminal domain indicates little, if any, α helix. Since the 208- and 222-nm peaks in the spectrum of the intact repressor are dominated by the NH_2-terminal domain, these peaks provide a sensitive monitor of the denaturation of this domain in the intact repressor.

Melting curves measuring $[\theta]_{222}$ as a function of temperature were obtained for wild-type and 10 mutant repressors. Each curve was corrected by subtracting the small contribution of the COOH-terminal domain and its denaturation at all temperatures. Values for the melting temperatures (t_m) and the van't Hoff enthalpy (ΔH_{vH}) were obtained by fitting the experimental points to the van't Hoff relation

$$RT \ln \left[\frac{\theta_N - \theta_T}{\theta_T - \theta_D}\right] = \Delta H_{vH} \left(\frac{T}{T_m} - 1\right), \qquad [2]$$

where θ_N is the mean residue ellipticity at 222 nm for the native protein; θ_D, for the denatured protein; and θ_T, for the protein at temperature T. Values of $t_m = T_m - 273.2$ and ΔH_{vH} are summarized in Table 1. The reversibility of the denaturation was demonstrated for the wild-type protein by the recovery of the native ellipticity upon cooling of the heat-denatured protein.

The NH_2-terminal domain in the wild-type protein melted with a t_m of 55°C (Fig. 2). The ΔH_{vH} for this transition was 80 kcal/mol (Table 1). Values similar to those obtained for wild type were found for mutants Lys-4 → Gln, Gln-33 → Ser, Gln-44 → Leu, Gln-44 → Tyr, and Ser-45 → Leu (Table 1). Thus, these mutants, which contain solvent-exposed amino acid substitutions, have wild-type thermal stabilities.

Fig. 2 also shows that mutants Tyr-22 → His and Ala-66 → Thr, which contain buried substitutions, had t_ms significantly lower than that of wild type. The melting transition of the Tyr-22 → His mutant was somewhat steeper than that of wild type, whereas the Ala-66 → Thr transition was much shallower than wild type. Two of the other mutant repressors also were found to be less stable than wild type: the Ala-

Table 1. Properties of wild-type and mutant proteins

Mutant	CD		Calorimetry				Proteolysis temperature, °C	Activities *in vivo*		
	t_m, °C	ΔH_{vH}, kcal/mol	t_m, °C	ΔH_{cal}, kcal/mol	$\Delta G°$ at 51.5°C, kcal/mol	ΔH_{vH}, kcal/mol		30°C*	37°C	42°C
Wild type	55	80	51.5	65	0	290	47–51	R	R	R
Surface substitutions										
Lys-4 → Gln	56	50	53.5	70	−0.43	272	47–51	S	S	S
Gln-33 → Ser	55	80	—	—	—	—	45–47	S	S	S
Gln-33 → Tyr	61	80	57.4	74	−1.32	368	51–56	R	R	R
Gly-43 → Glu	—	—	—	—	—	—	47–51	S	S	S
Gln-44 → Leu	58	70	—	—	—	—	47–51	S	S	S
Gln-44 → Ser	—	—	—	—	—	—	47–51	R	S†	S
Gln-44 → Tyr	56	60	51.4	60	+0.02	406	47–51	S	S	S
Ser-45 → Leu	55	60	—	—	—	—	47–51	S	S	S
Ala-49 → Val	49	45	38.5	29	+1.22	491	37–42	R	S	S
Ala-49 → Asp	—	—	—	—	—	—	47–51	S	S	S
Asn-52 → Asp	—	—	—	—	—	—	47–51	S	S	S
Asn-55 → Lys	—	—	—	—	—	—	47–51	S	S	S
Buried substitutions										
Tyr-22 → His	34	105	28.8	30	+2.25	232	30–34	R	R	S
Ala-66 → Thr	42	20	29.0	40	+2.99	56	26–30	R	R	R
Ile-84 → Ser	46	55	37.2	49	+2.25	218	37–42	S	S	S

Thermal properties of wild-type and mutant repressors were measured as described in the text. Proteolysis results indicate the temperatures at which the NH_2-terminal domain changed from being resistant to thermolysin digestion to being sensitive. Activities *in vivo* indicate resistance (R) or sensitivity (S) to infection by phage λ.

*Phenotypes at 26°C were identical to those at 30°C.

†Temperature sensitivity of this suppressed nonsense mutation may result from temperature-sensitive suppressor activity.

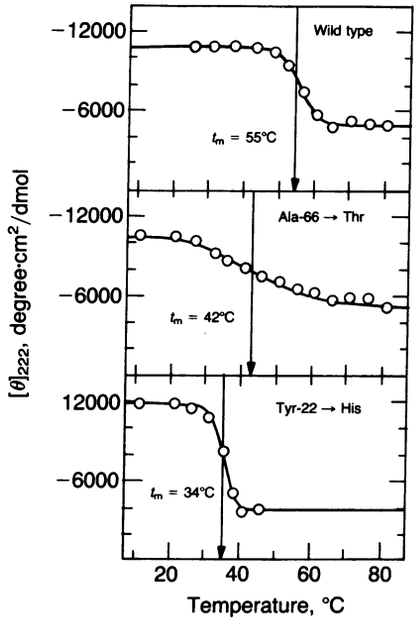

FIG. 2. Thermal denaturation curves for wild-type and two mutant repressors. Mean residue ellipticities at 222 nm are shown as a function of temperature, and the melting temperatures (t_ms) are indicated.

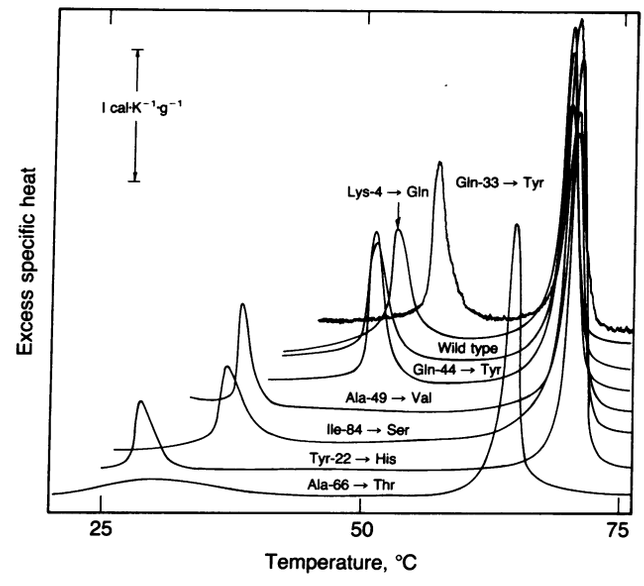

FIG. 3. DSC curves of excess specific heat vs. temperature for seven mutant forms and wild-type repressor. The uppermost curve, for Gln-33 → Tyr, is an actual tracing and illustrates the noise level of all the curves. A scan rate of 0.25°C/min was used throughout, and the protein concentration was 4.44 mg/ml.

49 → Val mutant contained a surface change and melted six degrees (t_m = 49°C) lower than wild type, and the Ile-84 → Ser mutant melted with a t_m (46°C) that was reduced by nine degrees compared to wild type. This last mutation replaced a residue that was buried as part of the dimer interface in the crystal structure (4).

One mutant protein, Gln-33 → Tyr, was found to have a value for t_m six degrees higher than that of the wild-type protein. This substitution introduces a new aromatic side chain in close proximity to two existing aromatic side chains.

Differential Scanning Calorimetry (DSC). In DSC experiments the NH_2-terminal and COOH-terminal domains of λ repressor show separate denaturational endotherms at approximately 50°C and 70°C, respectively (1). Tracings of DSC curves for the wild-type repressor and for seven mutant repressors are shown in Fig. 3. The uppermost curve indicates the low-noise level found in these experiments. The low-temperature endotherms for mutants Tyr-22 → His, Ala-49 → Val, Ala-66 → Thr, and Ile-84 → Ser occurred at 29–39°C. Thus, the DSC experiments, like the CD experiments, show that the NH_2-terminal domains of these mutants are less thermally stable than those of wild type. Compared with the NH_2-terminal domain of wild type, the NH_2-terminal domain of the Gln-44 → Tyr mutant denatured at the same temperature, that of the Lys-4 → Gln mutant denatured at a t_m two degrees higher, and that of the Gln-33 → Tyr mutant denatured at a t_m six degrees higher. These results also correlate well with those obtained from the CD experiments.

For six of the seven mutants studied by DSC, the endotherms for the COOH-terminal denaturation were the same as for wild type within experimental uncertainty. However, the COOH-terminal endotherm for the Ala-66 → Thr mutant occurred at a lower temperature than for wild type or the other mutants. Investigation of the DNA sequence of the Ala-66 → Thr mutant gene revealed a second mutation, Tyr-210 → His. This substitution presumably accounts for the reduced thermal stability of this mutant COOH-terminal domain.

The denaturations of wild-type and mutant repressors were irreversible under the conditions of the calorimetric experiments; at the completion of the scans, the proteins had precipitated, and no denaturational endotherms were observed on rescanning. Denaturation observed by CD was reversible, but these experiments were performed at approximately 1/100th of the concentrations used in the calorimetric experiments.

Despite the irreversibility of the calorimetric transitions, we have nevertheless applied equilibrium thermodynamics, in the form of the van't Hoff equation, to the data. Some empirical justification for this unorthodox procedure can be given as follows: The denaturational behavior of the tetrameric core protein of *lac* repressor, although irreversible, follows the dictates of equilibrium thermodynamics with respect to the changes resulting from variations in the concentrations of the protein and its ligand isopropyl β-D-thiogalactoside (unpublished results). The same is true for both the catalytic and regulatory subunits of aspartyl transcarbamoylase (unpublished results). Moreover, in the model $N_m \rightleftarrows mU$; $U \rightarrow X$, where the native oligomer, N_m, is denatured to m molecules of the unfolded form, U, and then U is irreversibly converted to X, calculations show that when parameters are chosen to give 95% of the protein in the irreversible form X, the assumption of complete reversibility does not lead to serious error in the evaluation of the van't Hoff enthalpy, ΔH_{vH}, by means of the equation

$$\Delta H_{vH} = ART_m^2\, C_{max}/\Delta H_{cal}, \qquad [3]$$

where C_{max} is the maximal value of the excess apparent heat capacity, C_{ex}, and ΔH_{cal} is the calorimetric enthalpy obtained by integration of the observed curve of C_{ex} *vs.* temperature. The value of the constant A depends on the value of m in the model, being 4.0 if $m = 1$ and 9.0 if $m = 4$.

The calorimetric parameters for the denaturation of the NH_2-terminal domain are listed in Table 1. Since the DSC experiments gave no consistent indication of heat capacity changes that accompanied denaturation, the denaturational enthalpies were taken to be independent of temperature. By using the calorimetric enthalpies, we calculated the standard free energies for the denaturation of wild-type and mutant repressors at 51.5°C (the wild-type t_m). These free energies reflect the stabilization or destabilization caused by the vari-

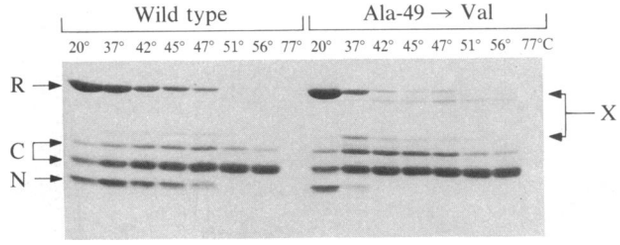

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of products of thermolysin digestion of wild-type repressor and the Ala-49 → Val mutant. Each panel shows digestion at eight temperatures as described (5, 6). Positions: R, intact repressor; C, COOH-terminal fragments; N, NH_2-terminal fragment; X, fragments of unknown sequence position.

ous amino acid replacements under these conditions. The mean values of the calorimetric parameters for the COOH-terminal domain transitions (excluding the Ala-66 → Thr + Tyr-210 → His mutant, which contains a substitution in the COOH-terminal domain) were t_m = 70.4 ± 0.08°C, ΔH_{cal} = 131.8 ± 3.4 kcal/mol, and ΔH_{vH} = 437 ± 39 kcal/mol (A = 4.0 in Eq. 3).

An outstanding feature of the calorimetric results is the large ratios of ΔH_{vH} to ΔH_{cal}. Values of this ratio >1 indicate intermolecular cooperation and presumably reflect varying degrees of oligomerization of the mutant repressors and/or aggregation of their denatured forms. The markedly reduced cooperativity seen in the NH_2-terminal transition of Ala-66 → Thr may indicate reduced oligomerization caused by the Tyr-210 → His replacement in its COOH-terminal domain. The increased cooperativity observed for the NH_2-terminal transition of the Ala-49 → Val mutant ($\Delta H_{vH}/\Delta H_{cal}$ = 17) is difficult to understand.

Proteolytic Sensitivity of Mutants. The t_m of the NH_2-terminal domain of λ repressor also can be approximated from the temperature at which it becomes sensitive to digestion by the protease thermolysin (5, 6). Such an experiment is shown in Fig. 4. For wild-type repressor, the fragment corresponding to the NH_2-terminal domain became thermolysin sensitive between 47°C and 51°C. For the Ala-49 → Val mutant, this domain became sensitive between 37°C and 42°C. The temperatures at which 14 additional mutants became thermolysin sensitive are listed in Table 1. These data extend and reinforce the findings from the CD and DSC experiments. The mutants Tyr-22 → His, Ala-49 → Val, Ala-66 → Thr, and Ile-84 → Ser were less stable than wild-type, Gln-33 → Tyr was more stable, and the remaining 10 mutants were essentially wild type with respect to their sensitivity to thermolysin at different temperatures.

Concentration Dependence of Denaturation. Increasing concentration resulted in decreased thermal stability for the NH_2-terminal domain and an increased ΔH_{vH} for its denaturation (Table 2). These results suggest that denaturation of the NH_2-terminal domain is accompanied by aggregation of the unfolded state. Since the NH_2-terminal domain is initially dimeric, the overall unfolding reaction can be modeled as:

$$N_2 \rightleftarrows N \rightleftarrows U \rightleftarrows U_2 \rightleftarrows U_3 \ldots \rightleftarrows U_n.$$

By this model, increasing the concentration should stabilize the aggregated, unfolded state relative to the native form and, thus, reduce the t_m. This model is supported by the finding that in several cases the calorimetric peak due to the NH_2-terminal melting is asymmetric, with the low-temperature side being steeper (12).

The calorimetric denaturation experiments used ≈100-fold higher concentrations than were used to obtain the CD melting curves, and the t_ms obtained by DSC were consistently lower than those obtained by CD for both mutant and wild-type repressors. The experiments reported here were performed near physiological pH and ionic strength; thus, the concentration dependence is probably relevant to the stability *in vivo* of the mutant proteins. To minimize complications, the denaturations of wild-type and mutant repressors were compared at similar, physiologically relevant, concentrations. For example, repressor concentrations used in the CD experiments approximate those of phage λ lysogens, whereas the calorimetric experiments used concentrations approximating those found in overproducing cells harboring plasmid pKB280.

Temperature Dependence of Mutant Phenotypes. Cells harboring the wild-type repressor producing plasmid pKB280 synthesize λ repressor at levels about 100-fold higher than those necessary to render the host cell resistant to infection by phage λ (11). Thus, a mutant repressor need have only 1% of wild-type activity to render its host cell immune. Many of the mutant proteins did mediate immunity to phage infection, and this immunity was often temperature sensitive (Tables 1 and 3). For a mutant to show immunity, we assume that some of the mutant repressor molecules must assume a folded, active structure at the permissive temperature.

For proteins whose only defect is instability, the temperature at which the phenotype changes from resistant to sensitive should be higher than the t_m measured *in vitro* because thermal denaturation would need to be nearly complete to account for the loss of immunity. For example, the Tyr-22 → His mutant lost immunity between 37°C and 42°C, whereas its t_m, measured by calorimetry, was about 29°C. The Ala-66 → Thr mutant also had a calorimetrically determined t_m near 29°C, but its melting transition was very shallow, and cells containing this mutant were immune at 42°C. The Ala-49 → Val and Ile-84 → Ser mutants were not immune at temperatures near their calorimetrically determined t_ms. However, in these cases the amino acid substitutions affect the DNA

Table 2. Concentration dependence of the denaturation of the NH_2-terminal domain of wild-type repressor

Method	Concentration, mg/ml	t_m, °C	ΔH_{vH}, kcal/mol	ΔH_{cal}, kcal/mol
CD	0.05	57.0	60	—
	0.35	56.5	90	—
	1.49	53.5	180	—
DSC	4.44	51.5	290	65
	9.27	50.9	370	48
	13.90	50.7	330	58

Table 3. Additional mutants with temperature-sensitive phenotypes *in vivo*

Mutants	Activities *in vivo* 30°C	37°C	42°C
Buried substitutions			
Ala-15 → Glu	R	S	S
Leu-18 → Phe	R	S	S
Tyr-22 → Cys	R	S	S
Leu-31 → Ser	R	R	S
Gly-53 → Cys	R	R	S
Ser-77 → Asn	R	S	S
Prolines in helices			
Leu-12 → Pro	R	S	S
Ser-35 → Pro	R	S	S

Activities *in vivo* indicate resistance (R) or sensitivity (S) to infection by phage TL.
*Phenotypes at 26°C were identical to those at 30°C.

binding activity of the folded protein as well as its stability (5, 6).

DISCUSSION

A number of mechanisms have been proposed to account for differences in the thermal stabilities of related proteins (13–15). These include changes in internal hydrophobicity and packing interactions, changes in the helix-forming potential of residues in α helices, and the introduction or removal of hydrogen bonds and salt bridges.

Several generalizations can be made concerning the thermal stabilities of mutant λ repressors. Almost all of the mutants that are temperature sensitive *in vivo* contain substitutions that would be expected to reduce stability; these include replacement of residues buried in the hydrophobic core and replacement of residues in α helices by proline residues. Conversely, all of those mutants that we have shown to have wild-type thermal stabilities contain substitutions of solvent-exposed residues. These substitutions can increase hydrophobicity (Ser-45 → Leu) or exchange charged and uncharged residues (Ala-49 → Asp; Lys-4 → Gln) and still not affect stability. We have previously argued that these mutations that change solvent-exposed residues define parts of the DNA binding surface of the protein (5, 6). Our results concerning the stabilities of mutant λ repressors are in agreement with the finding that abnormal hemoglobins with surface substitutions also have normal thermal stabilities (14).

Four of the 15 mutant repressors that we have characterized *in vitro* show reduced thermal stability. These are Tyr-22 → His, Ala-49 → Val, Ala-66 → Thr, and Ile-84 → Ser. Tyr-22 is buried in the hydrophobic core. It interacts with Phe-51, and its hydroxyl group appears to hydrogen bond to the polypeptide backbone near the NH_2-terminal end of helix 2 (Fig. 5). The reduced stability of the Tyr-22 → His protein could result from the loss of this hydrogen bond, from reduced packing interactions, and/or from the burying of the more polar histidine residue. (The phenotype *in vivo* of the Tyr-22 → Cys mutant suggests that this substituted protein is even less stable than Tyr-22 → His).

The Ala-49 → Val mutant protein contains the only solvent-exposed substitution, other than with proline, that renders the NH_2-terminal domain thermolabile. The β-branched side chain of Val-49 might interfere with local surface packing, but at the current resolution of the wild-type crystal structure, there are no obvious destabilizing interactions (C. O. Pabo, personal communication). The reduced helical propensity of Val-49 relative to Ala-49 (16, 17) also might cause instability. However, aspartic acid and valine residues have similar helical propensities (16, 17), and yet the Asp-49-substituted repressor has wild-type thermal stability (Table 1). A final possibility, which is suggested by the highly cooperative calorimetric transition of this mutant, is that the Val-49 substitution increases aggregation of the unfolded protein and thus destabilizes the native structure. If this is true, then it is surprising that in other cases the introduction of surface hydrophobic residues (e.g., Gln-44 → Leu; Ser-45 → Leu) does not decrease stability.

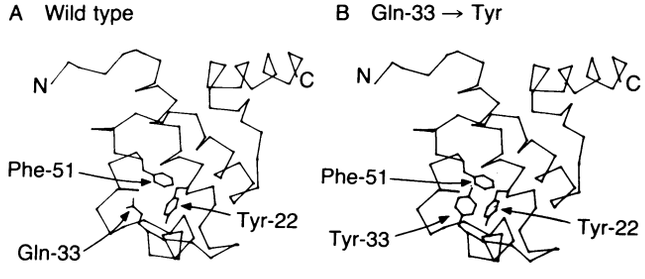

FIG. 5. Computer-generated models of the α-carbon backbone and side chains 22, 33, and 51 for the NH_2-terminal domain of the wild-type and Gln-33 → Tyr repressors. Atomic coordinates for the wild-type structure were provided by C. O. Pabo (4).

The Ala-66 → Thr substitution replaces a buried residue in an α helix. The reduced stability of this mutant protein could result from the introduction of a larger and more polar residue into the hydrophobic core or from the replacement of alanine residue, a very good helix former, by threonine residue, a residue with a low helical propensity (16, 17).

The Ile-84 → Ser mutation replaces a residue that appears to stabilize the helix 5–helix 5 packing, which mediates formation of the NH_2-terminal domain dimer. In the dimer, Ile-84 packs against the Met-87 side chain from the symmetrically related monomer (4). The reduced thermal stability of the Ile-84 mutant may result from destabilization of the dimer and/or from a decrease in the stability of the monomer itself.

The Gln-33 → Tyr mutant, obtained by suppression of an amber nonsense mutation, was found to be more thermally stable than the wild-type repressor. Fig. 5*A* shows the positions of Tyr-22, Gln-33, and Phe-51 in the wild-type crystal structure. Fig. 5*B* shows that the aromatic ring of the Tyr-33 mutant side chain can be positioned perpendicular to Tyr-22 at a distance of about 2.5 Å. This configuration of aromatic rings is similar to that found in crystalline benzene (18), is frequently observed in proteins, and has been implicated as an important interaction in the stabilization of protein structures (S. K. Burley and G. A. Petsko, personal communication). We presume that the Gln-33 → Tyr-substituted repressor owes its increased stability to this favorable interaction.

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