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Structural and Energetic Consequences of Disruptive Mutations in a Protein Core†

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ABSTRACT: We have characterized the properties of a set of variants of the N-terminal domain of λ repressor bearing disruptive mutations in the hydrophobic core. These mutations include some that dramatically alter the total core residue volume (by up to six methylene groups) and some that place a single polar residue into the otherwise hydrophobic core. The structural properties of the purified proteins have been studied by CD spectroscopy, biological activity, recognition by conformation-specific monoclonal antibodies, and ^{1}H NMR spectroscopy. The stabilities of the proteins have been measured by thermal and guanidine hydrochloride denaturation. Proteins with disruptive core mutations are found to display a continuum of increasingly nonnative properties. Large internal volume changes cause both significant conformational rearrangements and destabilization by up to 5 kcal/mol. Variants with polar substitutions at core positions no longer behave like well-folded proteins but rather display characteristics of molten globules. However, even proteins bearing some of the most disruptive mutations retain many of the crude secondary and tertiary structural features of the wild-type protein. These results indicate that primitive elements of native structure can form in the absence of normal core packing.

The interiors of proteins are packed very tightly. Such packing has long been thought to play an essential role in stabilizing a protein's native conformation (Richards, 1974, 1977, Chothia, 1975b). Tight packing maximizes van der Waals interactions and, by efficiently excluding solvent, may also contribute to hydrophobic stabilization. In recent studies, site-directed mutagenesis has been used to replace wild-type core residues with other apolar residues that have different steric properties or slightly smaller volumes (Garvey & Matthews, 1989; Karpusas et al., 1989; Kellis et al., 1988, 1989; Lim & Sauer, 1991; Matsumura et al., 1988, 1989; Sandberg & Terwilliger, 1989, 1991a; Shortle et al., 1990). These studies yield two general conclusions. First, packing interactions make significant contributions to the free energy of folding. Mutants with sterically altered residues are often destabilized by more than would be expected solely due to changes in hydrophobic interactions as modeled by transfer free energies. Second, although destabilizing, these types of core mutations alter the overall protein structure relatively little. Mutant proteins that can stably fold usually adopt a structure that is sufficiently native-like to maintain similar spectroscopic properties and at least partial biological activity. Structural analyses show that subtle main-chain rearrangements can allow new side-chain combinations to maintain reasonable packing densities (Eigenbrot et al., 1990; Katz & Kossiakoff, 1990; Daopin et al., 1991). Thus, while modest changes in packing can destabilize a protein, the overall structure seems quite tolerant to such perturbations.

Given the apparent plasticity of protein cores, we were interested in probing the limits to which a protein can tolerate disruptions in its core. How does a protein respond to mutations that severely alter core volume? For example, what happens if much larger side chains are inserted into the tightly packed interior of a protein? Does the structure rearrange and expand to accommodate the greater volume or is the protein simply unable to fold? How does a protein respond to the insertion of polar residues into the core? To investigate these questions, we have constructed and purified a set of potentially disruptive core mutants of the N-terminal domain of λ repressor (Figure 1). These mutant proteins can be

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FIGURE 1: Structure of the N-terminal domain of λ repressor (Jordan & Pabo, 1988). (a, left) Stereodiagram showing seven residues that constitute part of the hydrophobic core. These are Leu 18, Val 36, Met 40, Val 47, Phe 51, Leu 65, and Phe 76. Note that residues are numbered starting from 1000. (b, right) Ribbon diagram of the N-terminal domain shown in the same orientation as in part a. The surfaces recognized by the monoclonal antibodies 51F and 62F are indicated (Breyer, 1989; Breyer & Sauer, 1989).

Table I: Hydrophobic Core Mutants of λ Repressor[a]

class	mutant	activity (26 °C)[b]	Δvol (Å³)[c]	$\Delta\Delta G_{tr}$ (kcal/mol)[d]	$[\theta]_{222} \times 10^{-3}$ (deg·cm²·dmol)[e]	T_m (°C)	ΔH_{vH} (kcal/mol)[f]	25 °C ΔG_{H_2O} (kcal/mol)	25 °C m (kcal/mol·M)	25 °C c_m (M)	5 °C ΔG_{H_2O} (kcal/mol)	5 °C m (kcal/mol)	5 °C c_m (M)
	wild-type	5	0	0	−18.9	55.7	62	4.8	2.4	2.03	6.0	2.5	2.52
steric	1 MV^{40} VL^{47}	2	−3	0.6	−17.7	51.3	41	3.7	2.3	1.57			
	2 VL^{36} MV^{40} VI^{47}	3	+24	1.4	−16.9	53.6	44	3.7	2.2	1.73			
	3 VI^{36} MV^{40} VL^{47}	2	+24	1.4	−17.5	53.4	44	3.9	2.2	1.75			
	4 VI^{36} MV^{40} VI^{47}	5	+25	1.6	−19.6	53.7	47	4.0	2.1	1.92			
	5 VI^{36}	5	+27	0.8	−17.1	59.1	52	5.8	2.5	2.38	6.6	2.5	2.69
	6 VL^{36} ML^{40} VI^{47}	2	+50	2.1	−17.3	59.6	51	5.3	2.3	2.27	5.9	2.4	2.55
	7 VF^{36} ML^{40}	0	+58	1.4	−18.7	51.6	43	3.4	2.1	1.62			
extreme volume	8 MA^{40}	4	−79	−1.3	−17.6	47.1	46	2.8	2.1	1.37	3.9	2.3	1.89
	9 VF^{36} MF^{40} VI^{47}	1	+120	2.2	−16.8	47.2	38	2.6	1.8	1.48			
	10 VF^{36} MF^{40} VF^{47}	0	+154	2.3	−14.8	45.4	34	1.8	1.6	1.21	2.9	1.9	1.87
	11 LA^{18} MA^{40}	0	−155	−3.8	−16.0	23.2	27	−0.2[g]	–	–			
	12 VF^{36} MF^{40} VI^{47} LF^{65}	0	+155	2.5	−15.6	49.1	38	3.0	2.0	1.50	4.4	2.5	2.25
polar	13 LN^{18}	0	−33	−2.7	−9.7	–	–	–	–	–			
	14 VN^{36}	0	−7	−2.5	−15.5	–	–	–	–	–			
	15 VD^{36}	0	−17	−3.1	−15.0	–	–						

[a] Thermodynamic measurements were performed in 50 mM potassium phosphate (pH 7.0) and 100 mM KCl. Errors are estimated to be less than ±1 °C for T_m, ±10 kcal/mol for ΔH_{vH}, ±0.3 kcal/mol for ΔG_{H_2O}, ±0.1 kcal/(mol·M) for m, and ±0.02 M for c_m. A dash indicates that parameters could not be determined because of difficulties in obtaining native or denatured state baselines. A blank indicates that values were not measured. [b] Activity level in vivo is discussed under Materials and Methods. [c] Volumes calculated from values of Chothia (1975a). [d] Calculated from values of ΔG_{tr} from *n*-octanol to water (Fauchere & Pliska, 1983). [e] Measured at 0 °C. [f] van't Hoff enthalpy at the T_m of each protein. [g] From thermal denaturation data.

divided into three classes: (1) *steric mutants*, which have different or rearranged core residues but approximately equal core volumes; (2) *extreme volume mutants*, which have core volumes differing from the wild-type by ±3–6 methylene group equivalents; and (3) *polar mutants*, which have a single polar residue inserted into the otherwise hydrophobic core. The basic structural properties of these mutants have been analyzed using circular dichroism (CD) spectroscopy, biological activity, reactivity to a set of conformation-specific monoclonal antibodies, and, in some cases, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. The thermodynamic stabilities of the mutants have been measured by both thermal and guanidine hydrochloride denaturation.

We find that both the steric mutants and the extreme volume mutants fold into α-helical structures similar to the wild-type. The steric mutants maintain near wild-type stabilities ($\Delta\Delta G$ = −1.5 to +1.0 kcal/mol), but most have slightly reduced biological activity and antibody reactivity, indicating moderate structural perturbations. The extreme volume mutants are destabilized significantly relative to the wild-type ($\Delta\Delta G$ = −1.8 to −5.0 kcal/mol). Most of these mutants show no detectable biological activity, and all are poorly recognized by antibodies, indicating that their detailed structural features, i.e., those involved in ligand binding, are more significantly perturbed. The polar mutants are found to have α-helical structures at low temperature (50–80% of the wild-type ellipticity) and in some cases residual native-like tertiary structure. However, these proteins undergo highly noncooperative thermal transitions and have properties more characteristic of molten globules than of properly folded proteins.

Materials and Methods

Construction and Purification of Mutants. Mutants were constructed in pWL105, a pBR322-derived plasmid bearing the gene for residues 1–102 of λ repressor under the control of an inducible tac promoter (Lim & Sauer, 1991). The 15 mutants studied here are listed in Table I. Amino acid changes are indicated using the one-letter code, with the

wild-type residue listed first, the mutant residue second, and the site of the change third. The mutants LN^{18}, VN^{36}, VD^{36}, MA^{40}, $MV^{40}VL^{47}$, $VL^{36}MV^{40}VI^{47}$, $VI^{36}MV^{40}VL^{47}$, $VI^{36}MV^{40}VI^{47}$, VI^{36}, $VL^{36}ML^{40}VI^{47}$, $VF^{36}ML^{40}$, $VF^{36}MF^{40}VI^{47}$, and $VF^{36}MF^{40}VF^{47}$ (Lim & Sauer, 1989, 1991) were isolated previously. The mutants $LA^{18}LA^{40}$ and $VF^{36}MF^{40}VI^{47}LF^{65}$ were constructed by ligating together the appropriate fragments of plasmids bearing the mutations LA^{18}, LA^{40}, LF^{65}, and $VF^{36}MF^{40}VI^{47}$ (Lim & Sauer, 1989, 1991). Mutant proteins were purified by column chromatography using Affi-Gel blue (Bio-Rad), Bio-Rex 70 (Bio-Rad), and Sephadex G-75 sf (Pharmacia) as previously described (Lim & Sauer, 1991). Proteins were purified to greater than 90% homogeneity, except for LN^{18}, which was only ~80% pure, due to low expression and losses by precipitation during the purification.

Biological Activity. Activity of the mutant proteins in vivo was measured by determining the immunity of *Escherichia coli* bearing the mutant gene to infection by a series of clear or virulent phage λ derivatives (Hecht & Sauer, 1985; Lim & Sauer, 1991). Active repressor molecules confer immunity by binding the λ operators of the infecting phage, thereby shutting off expression of its lytic genes. Assays were performed at 26 °C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as described (Lim & Sauer, 1991). IPTG induces high-level expression of the repressor gene and thus allows for more sensitive detection of intracellular repressor activity. The activity levels 0–5 correspond to the following phenotypes: 0, λ KH54 sensitive; 1, λ KH54 resistant and λ cIc17 sensitive; 2, cIc17 resistant and λ vir sensitive; 3, λ vir resistant and λ 3v sensitive; 4, λ 3v resistant and λ 4v sensitive; 5, λ 4v resistant and λ 5v sensitive. Loss of activity (level 0) has been found to correspond to a decrease in DNA-binding activity of approximately 100-fold or greater (Lim & Sauer, 1991).

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectroscopy was used to monitor the secondary structure of mutant proteins and changes in structure during thermal and guanidine hydrochloride denaturation. An Aviv 60DS spectropolarimeter fitted with a Hewlett Packard temperature controller was used for all CD experiments. Spectra were collected at 0 °C between 200 and 300 nm in 1-nm steps with 1-s averaging time. Spectra shown are the average of seven scans. Samples contained 25 μg/mL protein in 50 mM potassium phosphate (pH 7.0) and 100 mM KCl. From the variation in three independently prepared samples of the wild-type protein, the error in measured intensities is estimated to be approximately ±2%.

Thermal Denaturation. CD ellipticity at 222 nm was monitored as a function of temperature as described previously (Lim & Sauer, 1991). Denaturation was performed at a protein concentration of 25 μg/mL in 50 mM potassium phosphate (pH 7.0) and 100 mM KCl. Ellipticity was measured in 1° steps with a 1-min equilibration time or 2° steps with a 2-min equilibration time. The signal was averaged over 30 s. Thermal denaturation was found to be between 70 and 100% reversible. Irreversible denaturation was due to time-dependent aggregation at high temperatures. Unfolding curves were analyzed assuming a two-state model. T_m is defined as the midpoint temperature of the thermal unfolding transition. The van't Hoff enthalpy at T_m (ΔH_{vH}) was calculated from the dependence of the folding equilibrium constant on temperature (Schellman et al., 1981).

Guanidine Hydrochloride (GuHCl) Denaturation. CD ellipticity at 222 nm was measured as a function of guanidine hydrochloride concentration at 25 or 5 °C using the method of Shortle and Meeker (1986), except that both the starting sample and the stock denaturant contained 50 μg/mL protein. Samples were in 50 mM potassium phosphate (pH 7.0) and 100 mM potassium chloride. Stock denaturant was prepared from an 8 M guanidine hydrochloride solution, purchased from Pierce Biochemicals, dibasic potassium phosphate, and KCl. Each sample was allowed to equilibrate at the correct temperature for at least 5 min, and the CD signal was averaged over 100 s. GuHCl unfolding transitions were fit to a two-state model by a nonlinear least-squares analysis, using the NONLIN module of the program SYSTAT (Systat, Inc.). Data were fit to

$$\theta_{222} = [(a_N[\text{GuHCl}] + b_N) + (a_D[\text{GuHCl}] + b_D)\exp(-\Delta G_{app}/RT)]/[1 + \exp(-\Delta G_{app}/RT)]$$

where b_N is the ellipticity of the native state at 0 M GuHCl, a_N is the slope of the native state baseline, b_D is the (extrapolated) ellipticity of the denatured state at 0 M GuHCl, a_D is the slope of the denatured state baseline, and ΔG_{app} is the free energy change upon unfolding at the given concentration of GuHCl. ΔG_{app} is assumed to be a linear function of denaturant concentration, as described by

$$\Delta G_{app} = \Delta G_{H_2O} - m[\text{GuHCl}]$$

where ΔG_{H_2O} is the free energy of unfolding at 0 M GuHCl calculated by extrapolation (Schellman, 1978; Santoro & Bolens, 1988). The parameter c_m is defined as the GuHCl concentration at the midpoint of the unfolding transition.

Competition Radioimmunoassay. To probe structural perturbations, mutant proteins were tested for binding to two monoclonal antibodies, 51F and 62F, which recognize distinct conformational epitopes in the folded wild-type protein (Breyer & Sauer, 1989a,b). Binding was measured using a competition radioimmunoassay, as described (Breyer & Sauer, 1989a). Briefly, antibody is adsorbed to a polystyrene well, and the remaining nonspecific binding sites are blocked with bovine serum albumin. The antibody-coated wells are incubated with a fixed concentration of ^{125}I-labeled wild-type protein and a variable concentration of cold mutant protein. After equilibration for 1 h, wells are washed, and bound radioactivity is determined by scintillation counting. Results are reported as the concentration of mutant protein required to achieve 50% inhibition, relative to inhibition by unlabeled wild-type protein. Since the assays are performed at protein concentrations several orders of magnitude below the dimerization constant, only changes in monomer structure should be detected.

^{1}H Nuclear Magnetic Resonance Spectroscopy. Proteins were dialyzed against 50 mM ammonium bicarbonate, lyophilized, resuspended in D_2O, and lyophilized again. The sample was prepared by dissolving the appropriate amount of dry protein in 50 mM potassium phosphate (pD 7.0, direct meter reading), 100 mM KCl, and 1 mM sodium azide, in 99.96% D_2O. One-dimensional spectra were obtained for wild-type and the mutants MA^{40}, $VF^{36}MF^{40}VI^{47}LF^{65}$, VD^{36}, and VN^{36} using a Bruker AM 500 spectrometer. Protein concentrations of approximately 100 μM were used. Spectra were taken at 14 °C, except where noted, and are the sum of 2048 scans. A 3-Hz line broadening was applied to all spectra. Chemical shifts are relative to 3-(trimethylsilyl)-1-propanesulfonic acid (TSP) at 0.0 ppm.

RESULTS

Secondary Structure. The secondary structure of purified mutant proteins was studied using circular dichroism spectroscopy at 0 °C, as shown for several mutant proteins in

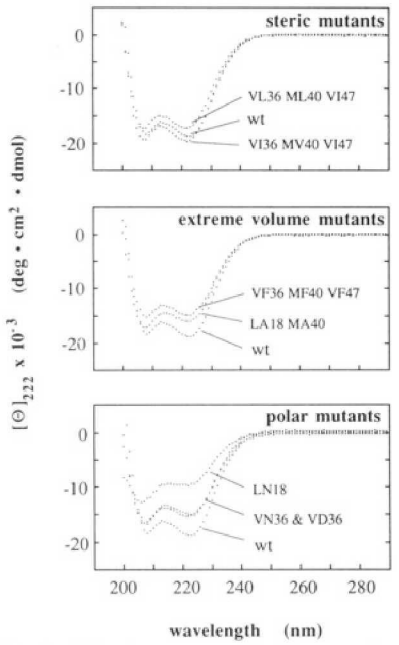

FIGURE 2: Circular dichroism spectra of wild-type and several mutants from each class, measured at 0 °C in 50 mM potassium phosphate (pH 7.0) and 100 mM KCl.

Figure 2. All of the proteins have CD spectra characteristic of significantly α-helical structures, with minima at 208 and 222 nm. Using mean residue ellipticity at 222 nm as a probe for helical content, we find that each of the steric mutants retains approximately 90–100% of the wild-type α-helical content, while each of the extreme volume mutants retains approximately 80–90% of the wild-type α-helical content. The two polar mutants VN^{36} and VD^{36} show about 80% of the wild-type α-helicity, while the polar mutant, LN^{18}, shows only about 50%.

Biological Activity. One probe for maintenance of a structure similar to wild-type is biological activity in vivo. These data for the mutant proteins, at 26 °C, are summarized in Table I and in Figure 3a. Of the seven steric mutants, two are fully active (level 5), four have detectable activity, and one is inactive. Only two of the five extreme volume mutants are active, and none of the polar mutants is active.

Antibody Recognition. As another probe of structure, purified mutant proteins were tested for their ability to compete for the binding of two conformation-specific monoclonal antibodies (Breyer & Sauer, 1989a,b). The inhibition of antibody binding by the mutant proteins, relative to unlabeled wild-type protein, is shown in Figure 3b (antibody 51F) and Figure 3c (antibody 62F). All of the mutations appear to have perturbed the local structure recognized by antibody 51F. For the steric and extreme volume mutants, antibody binding is reduced by roughly 10- to 100-fold. The three polar mutants are bound with affinities reduced by over 100-fold.

The different mutant classes show widely varying affinities for antibody 62F. None of the steric mutations appears to be significantly altered at the epitope recognized by 62F, since binding of these mutants is reduced by no more than 2-fold. Two extreme volume mutants, those with the smallest changes in volume, also show less than 3-fold reductions in binding. Of the remaining extreme volume mutants, however, one shows a 9-fold reduction in binding, and the other two show roughly 200- to 500-fold reductions. 62F binding to the polar mutants is reduced by approximately 1000-fold or greater.

Thermal Denaturation. The thermal denaturation of the mutant proteins was monitored by changes in ellipticity at 222 nm. The thermal transitions of the steric and extreme volume mutants were analyzed assuming a two-state model. Both the native and denatured states of the proteins have ellipticities that show a linear dependence on temperature (see Figure 4a,b). The values of T_m and ΔH_{vH} derived from this analysis are listed in Table I.

The steric mutants have T_m's ranging from 51 to 60 °C, compared to the wild-type T_m of 56 °C. The van't Hoff enthalpies of these mutants are reduced by 10–20 kcal/mol, relative to the wild-type value. The extreme volume mutant T_m's range from 45 to 48 °C, with the exception of the extremely small volume mutant, $LA^{18}MA^{40}$, which has a T_m of only 23 °C. The van't Hoff enthalpies of the extreme volume mutants are reduced by 15–35 kcal/mol, relative to wild-type.

The thermal unfolding of the polar mutants does not appear to be well fit by a two-state model. The thermal melts of these proteins are unusually broad and show very little cooperativity.

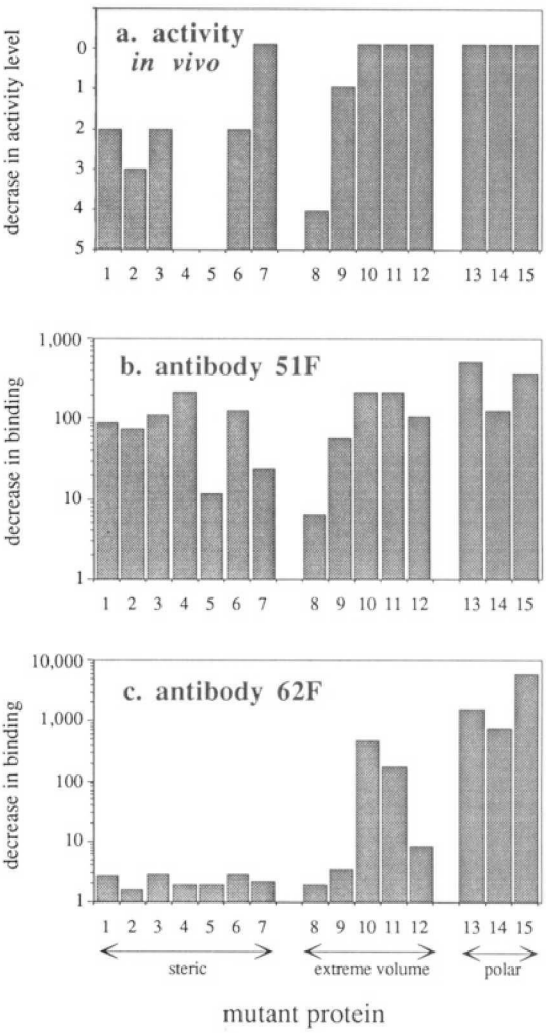

FIGURE 3: Structural perturbations in mutant proteins as monitored by activity in vivo and antibody recognition. Larger bars reflect larger decreases in binding or activity and, presumably, more significant structural changes at the relevant binding surfaces. The regions involved in binding to these probes are described under Discussion and shown in Figure 1. The numbers representing mutant proteins are those listed in Table I. Panel a shows the activity levels in vivo at 26 °C (Materials and Methods), where level 5 represents wild-type activity and level 0 represents no detectable activity. Decreases in binding by the monoclonal antibodies 51F and 62F, relative to wild-type, are shown in panels b and c, respectively. A competition RIA was used to determine the concentration of unlabeled mutant protein required to inhibit binding to ^{125}I-labeled protein by 50%. Plotted values are normalized to the concentration of unlabeled wild-type protein required to give the same level of inhibition.

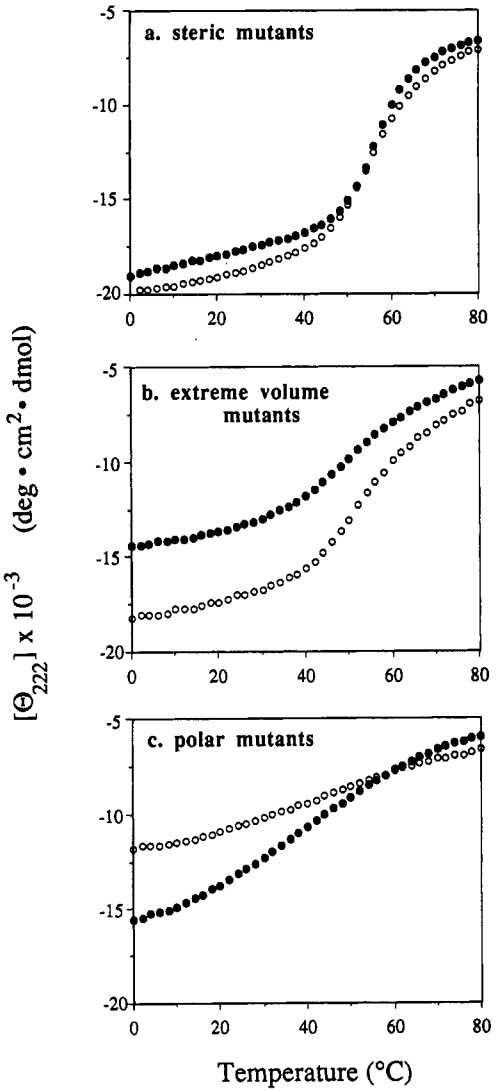

FIGURE 4: Thermal denaturation of several core mutants as measured by CD spectroscopy. (a) Steric mutants: wild-type (●) and $VI^{36}MV^{40}VI^{47}$ (○). (b) Extreme volume mutants: $VF^{36}MF^{40}VF^{47}$ (●) and $VF^{36}MF^{40}VI^{47}LF^{65}$ (○). (c) Polar mutants: VD^{36} (●) and LN^{18} (○).

The data for two of these mutants are shown in Figure 4c. For comparison, the uncorrected thermal melts of several steric (Figure 4a) and extreme volume (Figure 4b) mutants are also shown. Included is the extreme volume mutant $VF^{36}MF^{40}VF^{47}$, which had the broadest transition of all the nonpolar mutants.

Guanidine Hydrochloride Denaturation. The GuHCl denaturation of mutant proteins at 25 °C, and in some cases at 5 °C, was monitored by ellipticity at 222 nm. The values for ΔG_{H_2O}, m and c_m calculated from these experiments are given in Table I. As indicated, several of the proteins, including the polar mutants, were not stable enough to yield a good native baseline and could not be analyzed by this method. GuHCl denaturation data for several mutant proteins are shown in Figure 5.

The ΔG_{H_2O} values of the steric mutants at 25 °C vary from 3.4 to 5.8 kcal/mol, clustered around the wild-type value of 4.8 kcal/mol. Most of the extreme volume mutants have unfolding free energies of 1.8–3.0 kcal/mol. GuHCl denaturation at 25 °C was not performed on the extreme volume mutant $LA^{18}MA^{40}$ because the protein was greater than 50% unfolded at this temperature. However, its stability at 25 °C, calculated from the thermal unfolding transition, is −0.2 kcal/mol ($\Delta\Delta G$ = −5.0 kcal/mol).

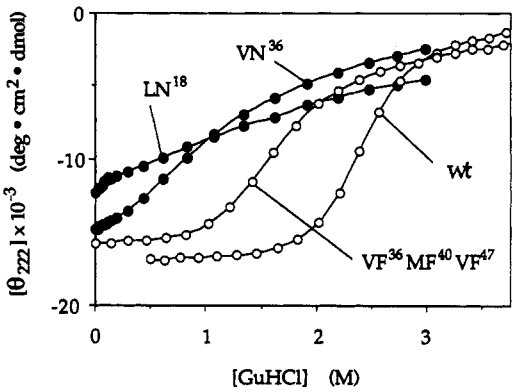

FIGURE 5: Dependence of mean residue ellipticity at 222 nm on guanidine hydrochloride concentration for the polar mutants of LN^{18} and VN^{36} and the extreme volume mutant $VF^{36}MF^{40}VF^{47}$, at 5 °C.

The steric and extreme volume mutations also lead to changes in m, the slope describing the dependence of ΔG_{app} on GuHCl concentration. The value of m is effectively a measure of the steepness of the unfolding transition. For all of the steric mutants and two of the extreme volume mutants, m values are within 15% of the wild-type value. More significant changes in m are observed for the two least stable extreme volume mutants (excluding $LA^{18}MA^{40}$). These mutants, $VF^{36}MF^{40}VI^{47}$ and $VF^{36}MF^{40}VF^{47}$, have m values that are reduced by 25% and 34%, respectively.

^1H NMR Spectroscopy. One-dimensional ^1H NMR spectroscopy was used to probe the tertiary structure of the extreme volume mutants MA^{40} and $VF^{36}MF^{40}VI^{47}LF^{65}$ and the polar mutants VD^{36} and VN^{36}. The aromatic region of the wild-type, MA^{40}, and $VF^{36}MF^{40}VI^{47}LF^{65}$ spectra are shown in Figure 6. Both of the extreme volume mutant spectra show a high degree of dispersion, indicative of well-ordered tertiary structure, and many of the observed resonances are identical to those observed for wild-type. The spectrum of VD^{36} (Figure 7) shows much less overall dispersion (the spectrum of VN^{36}, which is not shown, is nearly identical to that of VD^{36}). There are, however, two markedly shifted resonances, which disappear upon heating (Figure 8). These peaks (marked a* and b*) appear to correspond to resonances in the wild-type spectrum assigned to the para proton of Phe 76 and the methyl protons of Leu 18 (Weiss et al., 1987a).

Discussion

We have studied 15 λ repressor variants bearing amino acid substitutions at positions that are completely buried in the hydrophobic core of the wild-type structure (Jordan & Pabo, 1988). Their positions are illustrated in Figure 1. The mutants and their properties are listed by class in Table I.

The stabilities of these core mutants have been determined by thermal and GuHCl denaturation. Their structures have been studied by four different probes. CD spectroscopy has been used to assay for secondary structure content. Dispersion in the one-dimensional ^1H NMR spectrum has been used to probe for tertiary structure (Bundi & Wüthrich, 1979). Dispersion in the aromatic region is an especially good probe for tertiary structure, since Tyr 22, Phe 51, and Phe 76 are buried in the core (see Figure 1), and their resonances have been assigned (Weiss et al., 1987a).

Activity in vivo has also been used as a third probe for structure, since an active repressor mutant must be capable of specifically binding λ operator DNA. Mutants that have no detectable activity are likely to have significantly altered structures or to be highly unstable, either thermodynamically

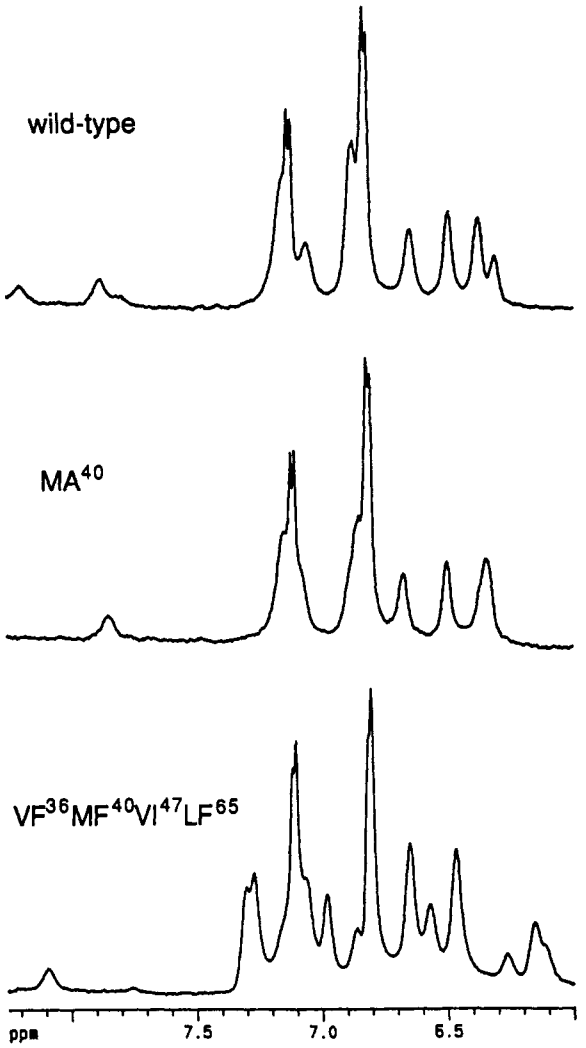

FIGURE 6: Aromatic ^{1}H NMR spectra of wild-type and the extreme volume mutants MA^{40} and VF^{36}MF^{40}VI^{47}LF^{65}. Aromatic random coil shifts are expected to be between 6.9 and 7.4 ppm (Bundi & Wüthrich, 1979).

or with respect to intracellular proteolysis. Mutants that retain only partial activity most likely have undergone structural rearrangements. Activity should be sensitive to many different structural perturbations, since critical protein–DNA and dimer contacts are distributed throughout the protein (Jordan & Pabo, 1988).

Recognition by conformation-specific monoclonal antibodies serves as a fourth probe for structure (Breyer & Sauer, 1989a). Antibody binding assays complement the assay for biological activity as methods for detecting how sequence changes alter the structure of the protein at different surfaces. The epitope recognized by antibody 51F includes the surface of helix 4 in λ repressor (residues 59–70). This surface patch is on the opposite side of the structure from most of the core sites that have been substituted in this study (positions 18, 36, 40, and 47). It is, however, directly external to position 65. The epitope recognized by antibody 62F includes the surface near the beginning of helix 2 (approximately residues 26–35). This region is adjacent in sequence to the substituted sites 36 and 40. The regions recognized by these antibodies are indicated in Figure 1b.

It is important to note that the antibody binding assay, as well as the activity assay, does not yield a geometric measure of structural change. Rather, these assays assess the degree of complementarity of a particular part of the protein surface to a specific ligand, or the energetic cost required for the protein to adopt a complementary surface.

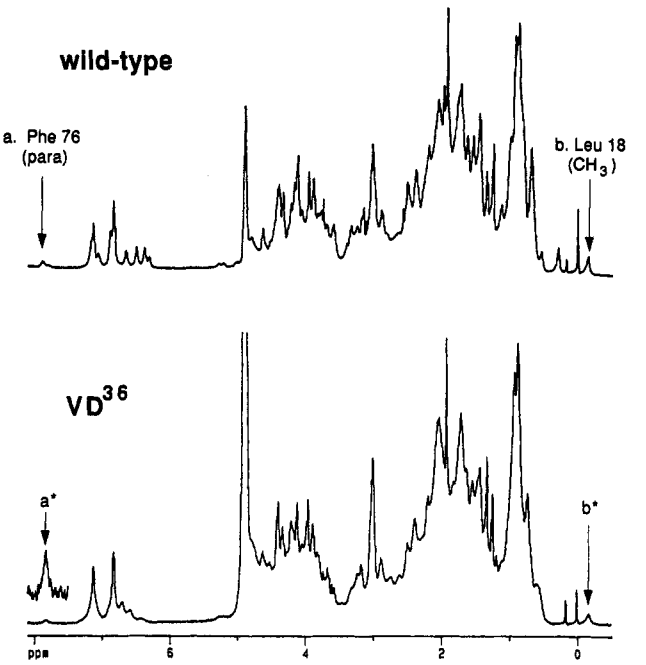

FIGURE 7: ^{1}H NMR spectra of wild-type and the polar mutant VD^{36}. The labeled peaks in the wild-type spectrum have been assigned as (a) Phe 76 para; and (b) Leu 18 methyl (Weiss et al., 1987). The peaks in the mutant spectra labeled a* (also shown on an expanded scale) and b* are thought to correspond to the same protons. The sharp resonance at 0.0 ppm is TSP, and that at 0.2 ppm is thought to be a TSP impurity.

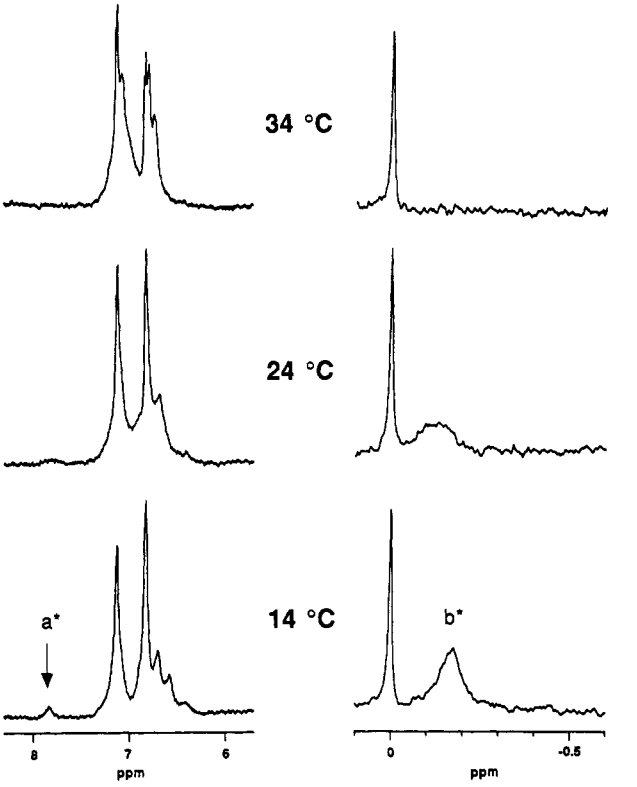

FIGURE 8: Temperature dependence of dispersion in the VD^{36} ^{1}H NMR spectrum. The aromatic and aliphatic regions are shown. Note that the two regions are shown on different ppm and intensity scales. The peaks labeled a* and b* are those indicated in Figure 7. The sharp resonance at 0.0 ppm is TSP.

Below we summarize the structural and energetic consequences of different types of core mutations, as monitored by these probes.

Steric Mutants. The steric mutant proteins bear sequence

changes that alter the precise arrangement of atoms in the core without significantly changing the total core residue volume (±58 Å^3) or hydrophobicity. By several probes, these mutants appear to adopt the overall wild-type fold. Seven of the eight steric mutants maintain detectable DNA-binding activity in vivo, and all have an α-helical content within 10% of the wild-type. These mutant proteins exhibit cooperative unfolding transitions and have stabilities near wild-type, ranging from 1.4 kcal/mol less stable to 1.0 kcal/mol more stable than wild-type (Table I).

Although these steric variants resemble wild-type in their general structural and thermodynamic features, their detailed structures appear to be different. These mutations must cause moderate structural rearrangements in the protein, since the level of biological activity and/or monoclonal antibody binding is reduced for each protein (Figure 3). As is shown in Figure 3, five of the seven steric mutants have reduced biological activity. In addition, all of the mutants show 10- to 200-fold reduced binding to the antibody 51F, which recognizes an epitope that includes the surface of helix 4. Thus these core mutations seem to propagate structural changes to the opposite side of the protein. Interestingly, binding of this set of mutants by the antibody 62F, which recognizes the turn before helix 2, is largely unchanged. These mutations therefore appear to alter the local structure at some binding interfaces but not at others.

Internal mutations in other proteins have also been found to cause similar structural rearrangements. Some subtle core mutations in dihydrofolate reductase disrupt ligand binding (Garvey & Matthews, 1989). These types of rearrangements have been observed crystallographically in T4 lysozyme and subtilisin BPN′. Internal substitutions in these proteins are often found to cause both side-chain and main-chain shifts which help either to fill voids created by mutation or to expand around inserted volume (Eigenbrot et al., 1990; Daopin et al., 1991). In some cases, detectable shifts occur as far away as 15 Å from the site of mutation.

Just as the steric mutants show slight variations in structure, they also show variations in stability. What features of particular core combinations lead to these moderate differences in stability? Two simple hypotheses are that changes in stability are the result of changes in core residue hydrophobicity or changes in the core residue volume. From the data given in Table I, however, we have found a very poor correlation between $\Delta\Delta G_{H_2O}$ for the steric mutants and either $\Delta\Delta G_{transfer}$, the change in hydrophobic energy calculated from amino acid free energies of transfer from water to *n*-octanol (Fauchère & Pliska, 1983), or against Δvol, the change in core residue volume (Chothia, 1975a). The poor correlations suggest that features other than simple hydrophobicity or volume are playing an important energetic role. Presumably, the shape and arrangement of interacting core residues are also critical. If the steric complementarity of core residues is energetically important, one would expect that substitutions at interacting core positions might exhibit nonadditive free energy changes (Carter et al., 1984; Wells, 1990). Such nonadditive behavior is indeed observed for several of the steric mutants. For example, wild-type and the three mutants VI^{36}, MV^{40}VL^{47}, and VI^{36}MV^{40}VL^{47} make up the thermodynamic cycle illustrated in Figure 9. The effect of mutating residue 36 from Val to Ile in the wild-type context is to increase the stability of the protein by 1.0 kcal/mol, perhaps because the additional methyl group can contribute both better packing and more buried hydrophobic surface area without introducing strain. However, if the same change is made in the context of Val 40 and Leu

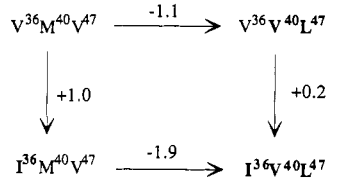

FIGURE 9: Core mutations showing nonadditive effects. Differences in free energies (kcal/mol) are calculated from the values in Table I. Mutant residues are indicated in boldface.

47, the stability of the protein only increases by 0.2 kcal/mol. Thus the contribution of a particular core residue is highly context dependent. Similar nonadditive mutations have been found in the core of T4 lysozyme (Daopin et al., 1991).

Extreme Volume Mutants. The extreme volume mutants have core residue volumes that differ from wild-type by three to six methylene group equivalents. Such large alterations in volume are expected to be highly disruptive. Decreases in core residue volume could lead to losses in hydrophobic stabilization and favorable van der Waals interactions that occur as a result of the tight packing in the core. Large increases in core residue volume might act to pry open the structure so that steric overlaps could be avoided. Such changes would not only disrupt packing of the core but might also act to break a wide array of other specific interactions throughout the protein.

Despite these more severe alterations, the extreme volume mutants are found to retain many of the global structural properties of the wild-type protein. Each has at least 80% of the wild-type α-helicity as determined by CD spectroscopy. NMR studies indicate that these mutants also retain tertiary structure similar to that of wild-type. The pattern of aromatic resonance dispersion observed in the MA^{40} spectrum is nearly identical to that of wild-type, indicating that the structures are very similar. The VF^{36}MF^{40}VI^{47}LF^{65} spectrum is somewhat more difficult to interpret, because these mutations introduce three additional aromatic side chains into the protein. Still, the high degree of chemical shift dispersion and the sharpness of the resonances are characteristic of a folded protein in which aromatic residues participate in specific interactions within the core.

It is remarkable that the protein is able to accommodate many of these large changes in volume within the same overall fold. As might be expected, these variants appear to accomplish this by undergoing significant structural rearrangements. The conformational shifts in these mutants, inferred from activity and antibody affinity data, are more severe than those observed in the steric mutants. Three of the five mutants have no detectable activity in vivo. In addition, two of the mutants are recognized by the antibody 62F with affinities reduced by greater than 100-fold. In contrast, binding of the steric mutants by 62F is reduced by only up to 2-fold. Thus, compared to the steric mutations, the extreme volume mutations generally propagate more severe rearrangements to the 62F surface epitope and to the DNA binding interface. Like the steric mutants, most of the extreme volume mutants are also poorly recognized by the antibody 51F.

The extreme volume proteins are significantly destablized relative to wild-type. The least stable mutant is LA^{18}MA^{40}, which has a core residue volume six methylene groups smaller than the wild-type protein (Table I). This mutant has a T_m of 23 °C, which corresponds to a stability loss of 5.0 kcal/mol at 25 °C. Other extreme volume mutants are between 1.8 and 3.0 kcal/mol less stable than wild-type and have T_m's between 44 and 50 °C. Part of this destabilization presumably results from the change in core residue hydrophobicity, which can be estimated from free energies of transfer of amino acids from

water to *n*-octanol (Fauchère & Pliska, 1983). The remaining effect on stability, which has been referred to as the *interior packing energy* (Sandberg & Terwilliger, 1991), must result from the disruption of internal packing and the cost of any associated structural rearrangements. In the case of decreased volume mutants, most of the observed destabilization may be accounted for by decreases in core residue hydrophobicity. For the larger volume mutants, the hydrophobic contribution to stability is expected to be higher than that of wild-type, yet these mutants are significantly destabilized. Thus the introduction of larger side chains into the core results, not surprisingly, in a much higher interior packing energy cost.

It is interesting to note that the unfolding transitions of two extreme volume mutant proteins, $VF^{36}MF^{40}VI^{47}$ and $VF^{36}MF^{40}VF^{47}$, are significantly broader than that of the wild-type. The GuHCl denaturation *m* values for these mutants are 1.8 and 1.6, respectively, compared to the wild-type value of 2.4. Changes in *m* can be interpreted in several different ways. By one model, *m* values are thought to reflect the difference in the exposed surface area of the native and denatured states (Schellman, 1978; Shortle & Meeker, 1986). Thus one interpretation of these significantly reduced *m* values is that the native states of these mutants are unusually expanded or that their denatured states are more compact. An expanded native state would not be surprising, considering the extremely large volumes inserted into these proteins. A second explanation for these low *m* values is that these mutants no longer exhibit two-state behavior. If an additional state (or states) of intermediate structure and stability were populated, then the unfolding transition would appear broader and lead to a lower apparent *m* value. A third interpretation is that the altered *m* values reflect changes in the preferential interactions of denaturant molecules with the denatured state compared to the native state (Arakawa & Timasheff, 1984). Whatever the mechanism, it is clear that the insertion of large volumes in the core can cause the protein to exhibit denaturation behavior that deviates significantly from that of the native structure.

Polar Mutants. We expected that inserting polar residues into the hydrophobic core might prevent folding of the protein. By two criteria the polar mutants studied here indeed appear to be denatured. First, their thermal unfolding transitions are considerably less cooperative than those of most other core mutants. Such gradual changes in ellipticity with temperature may not reflect a transition between two distinct states. The unfolding curves for VD^{36} and LN^{18} are shown in Figure 4. VN^{36} unfolding is nearly identically to that of VD^{36}. The thermal unfolding transitions of mutants in the other classes are shown for comparison, including the extreme volume mutant $VF^{36}MF^{40}VF^{47}$, which exhibited the least cooperative transition among all of the extreme volume mutants. VN^{36} is found to have a small cooperative GuHCl unfolding transition, while LN^{18} shows almost no cooperativity (Figure 5). A second indicator that these proteins are nonnative is that they are very poorly recognized by both monoclonal antibodies and show no DNA binding activity. All three bind to the antibody 51F with affinities reduced by more than 100-fold and to the antibody 62F with affinities reduced by more than 1000-fold. Thus they are even more poorly recognized than most of the extreme volume mutants.

Despite these nonnative properties, however, these proteins are not completely unstructured. All three mutants still adopt partially α-helical conformations. The mutant LN^{18} retains about 50% of the wild-type ellipticity at 0 °C, while the mutants VN^{36} and VD^{36} maintain approximately 80% of the wild-type ellipticity. This latter helicity corresponds to approximately the same amount of secondary structure as is observed in many of the extreme volume mutants.

One-dimensional ^{1}H NMR studies of the mutants VD^{36} and VN^{36} suggest that, in addition to native-like secondary structure, the position 36 mutants may also retain some native-like tertiary structure (insufficient LN18 was obtained for NMR studies). The VD^{36} and wild-type spectra at 14 °C are shown in Figure 7. The VN^{36} spectrum is nearly identical to that of VD^{36}. Compared to the wild-type, the resonances of the polar mutants are considerably broader and less disperse, indicating some loss of internal structure. Nonetheless, the mutant appears to have some tertiary structure, since several aromatic and aliphatic resonances display significant shifts from their expected random coil positions. As shown in Figure 8, these resonances converge toward random coil shifts at higher temperature, as expected for unfolding of this residual structure.

Is the residual structure observed at low temperature native-like? Tentative assignments suggest that it is. In the mutant spectrum at 14 °C, there are two markedly shifted resonances that are also observed in the wild-type spectrum. The first resonance, labeled a, has been assigned in the wild-type to the para proton of Phe 76 (Weiss et al., 1987a). This residue, shown in Figure 1, lies in the sequence following helix 4 and is fully buried in the core. Its para proton resonance is shifted far downfield from the random coil position, presumably as a result of its environment in the core. The second resonance, labeled b, has been assigned in the wild-type to the methyl protons of Leu 18. This resonance is shifted far upfield from its normal position because this core side chain is packed between the aromatic rings of Phe 51 and Phe 76. The presence of two comparable resonances in the VD^{36} spectrum (marked a* and b*), if they still correspond to Phe 76 and Leu 18, suggests that some native tertiary interactions are maintained.

The behavior observed for the polar mutant proteins, particularly VN^{36} and VD^{36}, is characteristic of molten globule states. The properties of these states have been reviewed by Kuwajima (1989). Molten globules are partially folded states that are found to have native-like secondary structure but to have fluctuating and somewhat expanded tertiary structure. Thermal unfolding of such states shows very little cooperativity, with near zero ΔH as measured by calorimetry. GuHCl unfolding, however, often shows partial cooperativity. Such states have been identified for many different proteins and are obseved under a variety of relatively mild denaturing conditions, such as extreme pH or low GuHCl concentration (Kronman et al., 1965; Kuwajima et al., 1976; Dolgikh et al., 1981; Goto & Fink, 1989). Similar states have also been observed for mutated or truncated proteins (Craig et al., 1985; Shortle & Meeker, 1989). Presumably the above conditions or alterations disrupt a limited number of the important stabilizing interactions in the native structure. Many key interactions, however, are unperturbed, leading to some native-like structural features.

In the cases of α-lactalbumin (Baum et al., 1989), β-lactamase (Goto & Fink, 1989), staphyloccocal nuclease (Shortle & Meeker, 1989), and apomyoglobin (Hughson et al., 1991), nonspecific hydrophobic interactions, without tight packing, appear to play a key role in stabilizing partially structured states. We suspect that this type of hydrophobic clustering also plays a role in stabilizing the structure observed in VN^{36} and VD^{36}. Most likely the substitutions at position 36 result in the expulsion of this site from the core. This notion is

supported by the observation that both the charged Asp and uncharged Asn substitutions have essentially identical properties—if position 36 were still buried in these structures, the charged substitution would be expected to be more disruptive. These substitutions therefore probably cause severe disruption of the local core packing. However, it appears that the clustering of remaining core residues, such as Leu 18 and Phe 76, may be sufficient to stabilize a primitive λ repressor topology. Some core residues, such as Leu 18, may play a more critical role in stabilizing such primitive structure, since the polar LN^{18} substitution at this site results in a much lower degree of residual structure than the VD^{36} and VN^{36} substitutions.

A growing body of evidence suggests that the denatured states of proteins under physiological conditions still retain a moderate amount of structure, similar to a molten globule state (Dill & Shortle, 1991). Some of the mutants studied here may therefore serve as good models for the denatured state under physiological conditions. The thermally denatured state of the wild-type N-terminal domain of λ repressor in fact still shows a significant amount of α-helix, which after the unfolding transition melts noncooperatively. It is interesting to note that, for the polar mutants, the dependence of secondary structure on temperature is very similar to that observed for the wild-type protein in the posttransition range (Figure 4).

Conclusions

Folding of the N-terminal domain of λ repressor is quite tolerant to substitutions in the hydrophobic core. All of the mutant proteins containing core residues with hydrophobic character are capable of adopting the same overall fold. This includes mutants with sterically altered core residues, as well as mutants with much larger or smaller core volumes. This observed plasticity is particularly remarkable for mutants that have much larger core volumes, since these substitutions must force the structure to expand, and no doubt place strain on many interactions throughout the protein. Many of the proteins bearing more drastic substitutions are, however, considerably less stable than the wild-type protein.

The protein appears to accommodate the different core substitutions through conformational rearrangements. These rearrangements are not simply local; effects can be detected at surfaces distant from the sites of mutation. As assessed by changes in ligand binding, rearrangements appear to be more severe for those proteins with significantly increased or decreased core volumes. Moderate stuctural changes, however, are still observed for fairly conservative steric mutations. The exact shapes and sizes of interacting core residues thus appear to play an important role in determining the precise structure of the protein.

Even the insertion of single polar residues into the core does not completely prevent folding. Some polar mutants show properties similar to molten globules—native-like secondary structure and, perhaps, some native-like tertiary contacts. Although it is virtually impossible for wild-type packing to occur around the sites of these mutants, the remaining interactions appear to cause the protein to maintain a partially folded structure. Resonance broadening and loss of dispersion in the NMR spectra indicate that much of this structure is poorly ordered. Nonetheless, this ensemble of conformations still appears to retain some basic wild-type features. These results suggest that some primitive elements of the protein's fold are remarkably overdetermined; they are adopted even in the absence of normal core packing. Understanding the forces that determine primitive conformation is clearly an important part of the protein folding problem and may be a prerequisite for understanding the details of protein stability and activity.

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