Stabilization of λ Repressor Against Thermal Denaturation by Site-Directed Gly→Ala Changes in α-Helix 3

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ABSTRACT **Oligonucleotide-directed mutagenesis has been used to replace α-helical glycines in the N-terminal domain of λ repressor with alanines. Since alanine is a significantly better helix-forming residue than glycine, these changes were predicted to have a stabilizing effect. We show that the Gly46→Ala substitution, the Gly48→Ala substitution, and the double substitution increase the melting temperature of the N-terminal domain by 3-6°.**

Key words: protein stability, helix-coil, mutant, calorimetry

INTRODUCTION

Glycine plays a special role in many protein structures because it can assume many backbone dihedral angles (φ, ψ angles) that are energetically unfavorable for other amino acids.[1,2] However, some glycines in folded proteins have backbone conformations that can be adopted by any amino acid. This is the case, for example, when glycine occurs in an α-helix or β-sheet. Glycines of this type may limit protein stability by increasing the number of conformations that are accessible to the unfolded form of the protein. In such cases, substitution of the glycine by another residue would be expected to stabilize the protein as long as the new side chain did not introduce unfavorable tertiary interactions. The third α-helix of the N-terminal domain of λ repressor contains two solvent-exposed glycine residues, Gly46 and Gly48.[3] Our attention was focused on these positions when we found that revertant repressors in which Gly48 was replaced by Asn or Ser were more stable than wild-type.[4] To investigate further the relationship between α-helical glycines and protein stability, we have used oligonucleotide-directed mutagenesis to engineer mutant repressors with the single Gly46→Ala and Gly48→Ala substitutions and the double Gly46/Gly48→Ala/Ala substitution (Fig. 1). Alanine was chosen to replace glycine because it is one of the best α-helix-forming residues[5,6] and because it is the most structurally conservative replacement for glycine.

MATERIALS AND METHODS

Directed Mutagenesis

A P_{tac}-λ repressor cI gene fusion[7] was cloned between the *Eco*R1 and *Cla*1 sites of the M13 origin plasmid pZ150[8] to generate plasmid pMH236. Single-stranded pMH236 template DNA was prepared by infecting *Escherichia coli* strain US3/F′ *lac*I^q/pMH236 with M13RV1 helper phage.[9] The Gly46→Ala, Gly48→Ala, and Gly46/Gly48→Ala/Ala changes were introduced into the repressor gene by using antisense primers (3′CGTCAGTC*GG*CAACCAC5′; 3′TCCGC-AAC*GA*CGAAATA5′; and 3′CCGTCAGTC*GG*CAA-C*GA*CG5′) and standard methods of oligonucleotide-directed mutagenesis.[10,11] Kinased missense primers and a second primer complementary to bases 3′ to the cI gene were annealed to the template, and second-strand synthesis was carried out by using DNA polymerase large fragment and DNA ligase. This mixture was treated with S1 endonuclease and transformed into *E. coli* strain MH90 (an AB1157 derivative obtained from L. Marsh; relevant genotype *mut*S, *sr*1:Tn10, F′*lac*I^q). Transformants were combined and infected with M13RV1, phages were purified, and pMH236 was introduced into strain UA2F by transfection. Individual colonies were picked, single-stranded pMH236 was prepared by helper phage infection, and candidates were screened by dideoxy DNA sequencing. The desired mutations were recovered at frequencies from 5 to 10%. The complete repressor genes of a few candidates with the desired changes were sequenced to ensure that additional substitutions had not occurred.

Protein Purification

Overproducing plasmids encoding the Gly46→Ala, Gly48→Ala, as well as Gly46/Gly48→Ala/Ala repressors, were transformed into the *lac*I^q *E. coli* strain UA2F[12], and the substituted repressors were purified from IPTG-induced cultures by using the purification protocol described for wild-type repressor.[13] Each of the three mutant repressors was purified to greater than 95% homogeneity and behaved like wild-type during the purification.

Differential Scanning Calorimetric (DSC) Experiments

DSC experiments were performed by using a DASM-4[14] scanning microcalorimeter and a scan rate of 1° K min^{-1}; proteins were dissolved at a concentration of 2.0 mg ml^{-1} in a buffer containing 10 mM potas-

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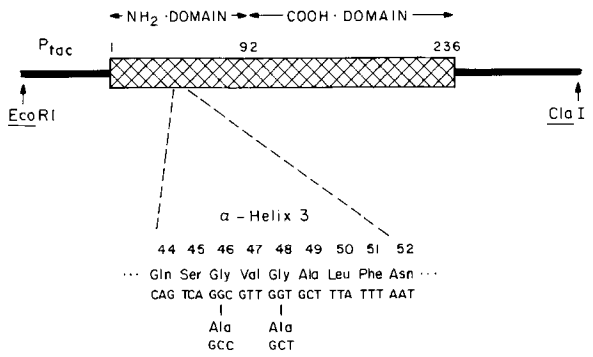

Fig. 1. Schematic of λ repressor gene and site-directed changes in α-helix 3.

sium phosphate (pH 8), 200 mM KCl, and 1 mM sodium azide. The DSC curves showed no visible noise at the sensitivity employed.

RESULTS AND DISCUSSION

Nucleotide substitutions were introduced into the region of the λ repressor gene encoding the N-terminal domain by standard methods of oligonucleotide-directed mutagenesis (see Methods), and repressor proteins with the Gly46→Ala, Gly48→Ala, and Gly46/Gly48→Ala46/Ala48 substitutions were purified.

Tracings of DSC curves for wild-type repressor and each of the alanine-substituted mutants are shown in Figure 2. Inspection of this figure shows that the mutant N-terminal domains are thermally more stable than wild-type. The temperature of half-denaturation, $t_{1/2}$, for the mutant N-terminal domains are from 3.4° to 6.5° higher than for wild-type, whereas the C-terminal domains of all the repressors show very similar denaturation profiles. At 57.5°C, for example, the wild-type N-terminal domain (curve A) is almost completely unfolded while that of the double mutant (curve D) has barely begun to undergo denaturation.

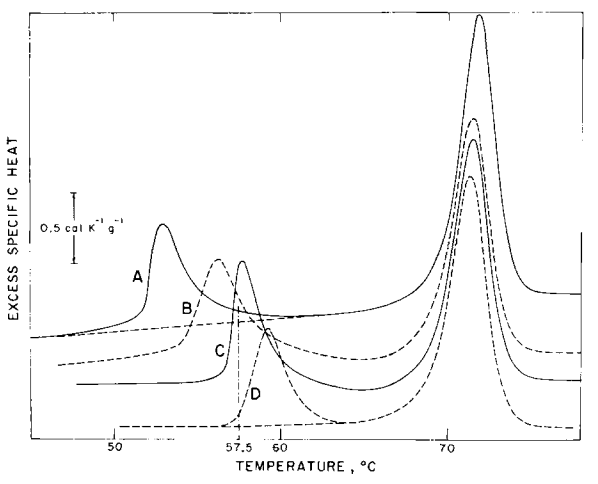

Fig. 2. Tracings of differential scanning calorimetric (DSC) curves of excess specific heat vs. temperature for wild-type repressor (curve A), the Gly46→Ala (curve B), Gly48→Ala (curve C), and Gly46/Gly48→Ala/Ala (curve D) mutant proteins. The transitions that peak below 60°C represent denaturation of the N-terminal domain, and those at about 72°C represent denaturation of the C-terminal domain.[18] Arbitrarily drawn baselines (see text) are shown for the lower transitions in curves A and D.

Table I lists mean values obtained in three experiments with each protein for $t_{1/2}$, for the calorimetric denaturational enthalpies, and for the change in the standard free energy of denaturation calculated at 53.4°C, the $t_{1/2}$ for the wild-type. These last ΔΔG° values may be interpreted as standard free energies of stabilization relative to the wild-type protein. It is evident in Figure 2 that no information is obtainable as to the appropriate final baseline for the N-terminal transition or the appropriate initial baseline for the C-terminal transition. Because the calorimetric enthalpy is calculated from the area under the denaturational curve and is used in the calculation of ΔΔG°, this baseline ambiguity is the major source of uncertainty in the values listed in Table I. In addition, the assignment of values to ΔΔG° in the present case is to some extent model dependent since the denaturation is not a simple reversible transition between monomeric folded and unfolded states.[15]

Although the Gly46→Ala and Gly48→Ala substitutions both increase the thermal stability of the N-terminal domain, they do so to different degrees. This difference may arise as a consequence of tertiary interactions or sequence-specific effects on helix nucleation or propagation. It is also noteworthy that ΔΔG° for the double mutant is less than that expected from the sum of the single mutant ΔΔG° values. This could be explained if one or more of the mutant substitutions altered the conformation of the native protein (see reference 11 for related discussion) *or* altered the ensemble of conformations accessible to the unfolded protein.

Amino acids are generally classed as poor, moderate, or good helix-forming residues based on the Zimm-Bragg "s" value derived from host-guest copolymer experiments[5] or on the normalized frequency at which they occur in helices of proteins with known structures.[6] By either scale, glycine is one of the worst helix-forming residues; serine and asparagine are moderately good; and alanine is one of the best. As shown in Table II, there is a rough correlation between the rank order of the Zimm-Bragg "s" value for these four residues, the temperatures of half-denaturation, and ΔΔG° values for N-terminal domains that bear these residues at position 48 of α-helix 3. Helix propagation and nucleation parameters from copolymer experiments may not be appropriate for calculating the stability of short α-helices of defined sequence.[16,17] It is reassuring, nevertheless, that there is some correlation between the copolymer data and

TABLE I. Thermodynamic Properties of Wild-Type and Mutant N-Terminal Domains*

Protein		$t_{1/2}$ (°C)	ΔH (kcal mol^{-1})	ΔΔG° (kcal mol^{-1})
Wild-type . . .	Gly46/Gly48	53.4 ± 0.1	65.5 ± 9.8	0
	*Ala*46/Gly48	56.5 ± 0.1	70.2 ± 10.5	0.66 ± 0.12
	Gly46/*Ala*48	58.1 ± 0.1	61.6 ± 9.2	0.87 ± 0.13
	*Ala*46/*Ala*48	59.6 ± 0.1	58.9 ± 8.8	1.10 ± 0.16

*$t_{1/2}$, the temperature at which the denaturation of the N-terminal domain is half completed; ΔH, the enthalpy of denaturation, proportional to the area under the denaturational curve; ΔΔG°, the standard free energy of denaturation corrected to $t_{1/2}$ for the wild-type protein using the Gibbs-Helmholtz equation, assuming the denaturational enthalpy to be independent of temperature:

$$\frac{\Delta G^{\circ}(T_2)}{T_2} - \frac{\Delta G^{\circ}(T_1)}{T_1} = \Delta H \left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

where T_1 and T_2 are absolute temperature.

TABLE II. Properties of N-Terminal Domains With Different Residues at Position 48

Protein	"s"*	$t_{1/2}$ (°C)	ΔΔG° (kcal mol^{-1})
Gly48	0.60	53.4 ± 0.1	0
Ser48	0.77	57.4 ± 0.1	0.68 ± 0.12
Asn48	0.79	57.5 ± 0.1	0.79 ± 0.14
Ala48	1.06	58.1 ± 0.1	0.87 ± 0.16

*Zimm-Bragg constant for the equilibrium between the coil and helical states of a residue at the boundary of a nucleated α-helix.[5]

the relative stabilities of proteins that differ at a single position in an α-helix.

One question of current interest is whether protein stability directly influences ligand binding. The DNA binding properties of the alanine-substituted repressors were indistinguishable from those of wild-type: at 37°C, cells containing the mutant repressors had the same superinfection immunity properties as wild-type and, at 23°C, the purified mutant proteins bound to the O_R1 operator site with wild-type affinities as assayed by nitrocellulose filter binding (H.C.M. Nelson, personal communication). Since the Gly→Ala substitutions in α-helix 3 stabilize repressor without affecting its binding to operator DNA, there is no obvious relationship between stability and binding. However, since helix 3 of repressor forms much of the protein's DNA binding surface, it is possible that one or both of the Gly→Ala substitutions introduce contacts with the operator that mask some effect of the stability changes on DNA binding.

Since the alanine-substituted repressors are more stable than wild-type and have comparable operator binding properties, then why haven't these variants replaced wild-type? One possibility is that these molecules have no selective advantage because wild-type repressor is sufficiently stable to maintain a folded, protease-resistant structure at the temperatures where its host bacterium, *E. coli*, is viable.

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