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Combining Thermostable Mutations Increases the Stability of λ Repressor†

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ABSTRACT: We have combined three mutations previously shown to stabilize λ repressor against thermal denaturation. Two of these mutations are in helix 3, where Gly-46 and Gly-48 have been replaced by alanines [Hecht, M. H., et al. (1986) *Proteins: Struct., Funct., Genet. 1*, 43–46]. The other mutation, which replaces Tyr-88 with cysteine, allows the protein to form an intersubunit disulfide bond [Sauer, R. T., et al. (1986) *Biochemistry 25*, 5992–5998]. Calorimetric measurements show that the two alanine substitutions stabilize repressor by about 8 °C, that the disulfide bond stabilizes repressor by about 8 °C, and that the triple mutant is 16 °C more stable than wild-type repressor.

Do we understand proteins well enough to systematically design thermostable variants? Can changes be combined to generate "hyperstable" variants? Many interactions are important for stabilizing proteins—hydrophobic interactions, van der Waals contacts, hydrogen bonds, and salt bridges. Since each interaction might contribute only a few kilocalories per mole to the net stability of a protein, we expect that a number of changes must be combined to generate significant increases in the thermostability. To test this approach, we have combined several existing mutations in the amino-terminal domain of λ repressor (residues 1–92) and measured the thermostability of the new protein. In a previous study, two glycines in helix 3 of repressor were replaced with alanines in order to stabilize that helix (Hecht et al., 1986). Each Gly → Ala substitution increased the thermostability of intact repressor by 3–5 °C, while the double mutant increased the thermost-

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ability by 6 °C. Another thermostable mutant had been constructed by replacing a tyrosine with a cysteine at the dimer interface of repressor. As predicted by computer model building, an intersubunit disulfide bond formed spontaneously, and the thermostability increased by 8 °C (Pabo & Suchanek, 1986; Sauer et al., 1986). In this paper, we show that combining these mutations generates a significantly more stable protein.

Materials and Methods

Constructions and Mutagenesis. A gene encoding the first 92 residues of λ repressor was constructed by using site-directed mutagenesis to place 2 termination codons after residue 92. M13mp8 derivatives containing the genes for the wild-type or Cys-88 mutant repressors (residues 1–102) served as the templates for this experiment. A 29-base antisense primer (5′-CTAAGTGACGGCTATCAACTAACCGCTTC) was annealed to these templates, and second-strand synthesis, ligation, transfection, and plaque hybridization were performed according to standard protocols (Zoller & Smith, 1983; Carter et al., 1984). Several plaques were chosen by hybridization with labeled primers and sequenced by the dideoxy method (Sanger et al., 1977). After the mutant sequence was confirmed, the repressor gene was subcloned into an expression vector which directs repressor synthesis under the control of the *tac* promoter and also contains the *lac* i and β-lactamase genes. The Gly-46/Gly-48 → Ala/Ala mutations were introduced into these plasmids by subcloning a restriction fragment from pMH147 (Hecht et al., 1986; Sauer et al., 1986).

Protein Purification and DNA Binding. Plasmids expressing the amino-terminal domain of repressor (residues 1–92) were grown in *Escherichia coli* strain DB4729. Production of repressor was induced by IPTG. Wild-type and mutant proteins were purified as described for repressor residues 1–102 (Sauer et al., 1986), except that the final Sephadex G-75 step was omitted. All proteins were purified to greater than 95% homogeneity.

The DNA binding activity of each repressor was determined by using a gel retardation assay. Various concentrations of protein were incubated with 500 ng of the 20 base pair DNA operator site which had been used for cocrystallization (Jordan et al., 1985). After 30 min at room temperature, the samples were electrophoresed on a 20% polyacrylamide gel, and the gel was stained with ethidium bromide. The protein–DNA complex migrates significantly slower than the uncomplexed DNA (Jordan et al., 1985), and the binding activity was assessed by comparing the amount of protein required to give 50% complex formation. The buffer used in these reactions contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, and 5% glycerol.

Thermal Denaturation. The thermal stability of all the protein samples was determined by high-sensitivity differential scanning calorimetry using a Microcal MC2 instrument interfaced to a microcomputer for automatic data collection and instrument control via a 12-bit A/D converter (Data Translation DT2801). This instrument has been modified for heating/cooling operation as well as quick cooling after heating scans by using a computer-controlled Haake F3C refrigerated water circulator connected to the calorimeter cell jacket (Myers et al., 1987). All the experiments in these studies were performed in a buffer containing 10 mM potassium phosphate (pH 8.0), 200 mM KCl, and 1 mM sodium azide. All proteins were at a concentration of approximately 1.0 mg/mL, and protein concentrations were determined by measuring the OD_{280}.

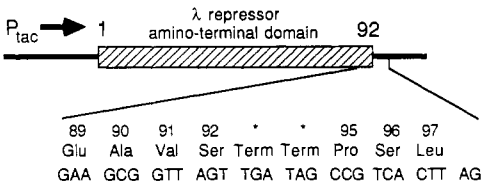

FIGURE 1: Schematic representation of the gene encoding the amino-terminal domain of λ repressor. The sequence of the oligonucleotide used to place two termination codons after residue 92, and the carboxyl-terminal sequence of the protein are shown. Expression of the protein is driven by the *tac* promoter.

Results and Discussion

We are interested in designing thermostable variants of the λ repressor, and we have chosen to work with the N-terminal domain (residues 1–92) since the structure of this fragment has been determined (Pabo & Lewis, 1982). Several thermostable variants of λ repressor have already been designed, but these studies used either the intact protein (Hecht et al., 1986) or an N-terminal fragment containing residues 1–102 (Pabo & Suchanek, 1986; Sauer et al., 1986). To allow direct use of the known crystal structure and to simplify comparison of different mutants, we constructed the appropriate plasmids to specifically produce residues 1–92. Oligonucleotide-directed mutagenesis was used to place two termination codons after residue 92 of the wild-type and Cys-88 repressor genes (Figure 1). The Gly-46/Gly-48 → Ala/Ala (Hecht et al., 1986) mutations were introduced into the gene by subcloning the appropriate restriction fragment (see Materials and Methods). Wild-type, Cys-88, Ala-46/Ala-48, and Cys-88/Ala-46/Ala-48 repressors were then purified.

Since we are interested in designing thermostable variants that still bind tightly to the operator site, the DNA binding of each protein was checked using a gel retardation assay. The Ala-46/Ala-48 mutant bound slightly less well (2–5-fold) than wild-type repressor, whereas the Cys-88 mutant and the triple mutant bound more tightly than the wild-type repressor (data not shown). Tighter binding has also been observed for the Cys-88 mutant in the 1–102 repressor fragment (Sauer et al., 1986), since the intermolecular disulfide bond stabilizes the dimeric DNA binding species.

Thermal denaturation curves for the wild-type and mutant repressors were determined by differential scanning calorimetry (DSC). The raw calorimetric data, prior to filtering and base-line subtraction, for the triple mutant are shown in Figure 2. The two curves in the figure correspond to two different protein preparations and illustrate the degree of reproducibility of these experiments. At the concentrations used in the calorimetric experiments (1 mg/mL), the repressor protein shows a strong tendency to aggregate in the unfolded state. This aggregation appears to be responsible for the negative slope of the heat capacity of the unfolded state and constitutes the major source of uncertainty in the determination of the calorimetric enthalpy. (This effect has been observed in other proteins; aggregation may bury hydrophobic residues that otherwise would be exposed to solvent.) In agreement with previous calorimetric data for the wild-type repressor as well as other mutants (Pabo et al., 1979; Hecht et al., 1984, 1986), we found no clear evidence for the existence of a ΔC_p for the transition, and therefore the excess heat capacities were obtained by projecting the initial and final base lines under the transition region using a cubic splines interpolation procedure.

Under the conditions of these experiments, the unfolding transition of the amino-terminal fragment of the wild repressor molecule is approximately 95% reversible provided that the sample is rapidly cooled immediately after the transition is

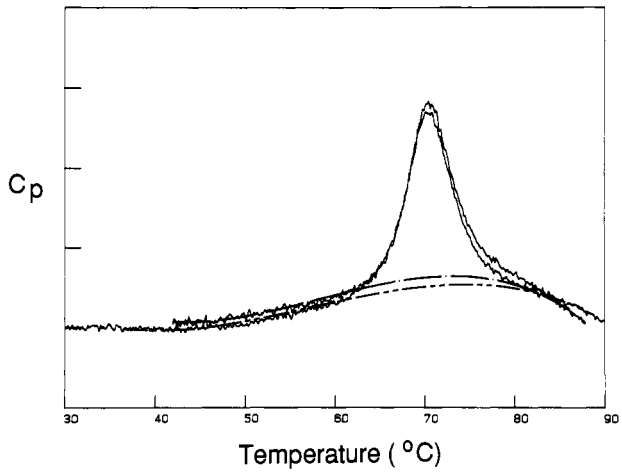

FIGURE 2: Unfiltered differential scanning calorimetric (DSC) curves of excess specific heat vs temperature for two preparations of the Cys-88/Ala-46/Ala-48 triple mutant. The base lines (fit with cubic splines as described in the text) are shown. After subtraction of these base lines, the T_m's coincide within 0.05 °C, and the areas agree within 1%.

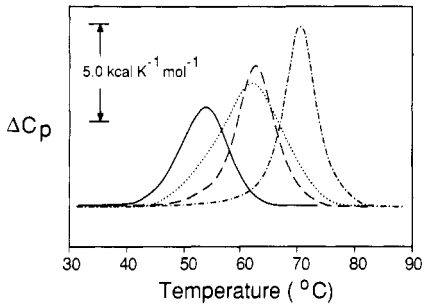

FIGURE 3: DSC curves of excess specific heat vs temperature for the wild-type amino-terminal domain of repressor (—), the Cys-88 mutant (– –), the Ala-46/Ala-48 double mutant (···), and the Cys-88/Ala-46/Ala-48 triple mutant (–·–). These data have been filtered, and the base lines have been subtracted as described in the text.

complete. Previously, Hecht et al. (1984) reported irreversible denaturation processes for the repressor, but their calorimetric experiments were performed with the entire repressor molecule and at much higher concentrations (4–14 mg/mL). Apparently, the irreversibility is related to the appearance of protein aggregates in the unfolded state, and their formation is dependent both on concentration and on the time that the protein is kept at temperatures above the T_m. It must be noted that even the transition of the entire repressor molecule is reversible when studied spectroscopically at low concentrations (<0.1 mg/mL) (Hecht et al., 1984).

Figure 3 shows the excess heat capacity versus temperature for the wild-type and mutant repressors. [The excess heat capacity (ΔC_p) curves were obtained after subtracting the base lines as described above.] As expected from previous studies, the wild-type protein denatured at 53.9 °C (Pabo et al., 1979; Hecht et al., 1984, 1986), the Cys-88 mutant denatured at 62.7 °C, and the Ala-46/Ala-48 double mutant denatured at 62.0 °C (Sauer et al., 1986; Hecht et al., 1986). The T_m of our triple mutant is 70.3 °C, an increase of 16 °C over the wild type. The calorimetric enthalpies and the calculated van't Hoff enthalpies are given in Table I. The calorimetric enthalpy of the wild-type repressor is similar to that measured by Hecht et al. (1986) in studies of the intact molecule (residues 1–236). However, our enthalpy and T_m for the Ala-46/Ala-48 double mutant are somewhat higher than those found with the intact molecule. The van't Hoff enthalpies were calculated by using the standard equation $\Delta H_{vH} = 4RT_m{}^2C_{p,max}/\Delta H$. In agreement with previous results, the $\Delta H_{vH}/\Delta H$ ratio is greater than 1 for the wild-type repressor, and this indicates the existence of intermolecular cooperation. The Cys-88 mutant and the Cys-88/Ala-46/Ala-48 triple mutant show similar behavior. The transition for the Ala-46/Ala-48 mutant appears less cooperative. A broader peak is observed in the DSC curve, and the calculated ΔH is actually smaller than the calorimetric ΔH.

Table I: Denaturation Properties of Wild-Type and Mutant Repressors[a]

protein	T_m (°C)	ΔH_{cal} (kcal mol^{-1})	ΔH_{vH} (kcal mol^{-1})
wild-type 1–92	53.9 ± 0.1	53.4 ± 5	79.8
Cys-88	62.7 ± 0.1	66.4 ± 7	95.4
Ala-46/Ala-48	62.0 ± 0.1	88.4 ± 10	62.4
Cys-88/Ala-46/Ala-48	70.3 ± 0.1	72.9 ± 4	116.3

[a] T_m is the temperature at which the denaturation of repressor is half-completed. ΔH_{cal} is the calorimetric enthalpy of denaturation, corresponding to the area under the DSC denaturation curves. The reported errors in ΔH reflect the uncertainties in the determination of the calorimetric base lines as discussed in the text. ΔH_{vH} is the van't Hoff enthalpy calculated from the denaturation curves.

The observed changes in transition temperatures reflect changes in the overall free energy of stabilization of the protein. With the data in Table I, it is possible to estimate the extra free energy of stabilization for each mutant at the transition midpoint of the wild-type repressor molecule, i.e., at the temperature for which $\Delta G = 0$ for the wild-type protein. According to the van't Hoff analysis of the data (Table I), the extra free energies of stabilization relative to that of the wild-type protein are 1.5 kcal/mol for the Ala-46/Ala-48 double mutant, 2.5 kcal/mol for the Cys-88 substitution, and 5.5 kcal/mol for the Cys-88/Ala-46/Ala-48 triple mutant. The value of 1.5 kcal/mol for the Ala-46/Ala-48 mutant is in good agreement with the value of 1.1 kcal/mol previously calculated by Hecht et al. (1986). A somewhat better comparison of the stabilities is to use a temperature at which all of the denaturation profiles overlap and to calculate the extra free energy from the degree of advancement of the transition. (This avoids the problem of extrapolating to the T_m of the wild-type protein). With 60 °C as the reference temperature, we obtain a value of 1.9 kcal/mol for the Ala-46/Ala-48 double mutant, 2.4 kcal/mol for the Cys-88 mutant, and 4.2 kcal/mol for the triple mutant. We note that the excess free energy of stabilization for the triple mutant roughly corresponds to the sum of the excess free energies of the Cys-88 mutant and of the Ala-46/Ala-48 double mutant.

The stabilizing effects of the Gly → Ala substitutions had been predicted by Hecht et al. (1986) based on their location within the third α helix of repressor. They reasoned that the pair of glycines found in helix 3 might destabilize that helix in the wild-type protein, since glycines are relatively uncommon in α helices [it is a "helix-breaker" by the rules of Chou and Fasman (1978)]. Replacing the glycine with another residue should stabilize the helix by reducing the conformational entropy of the unfolded state. Hecht et al. (1986) used alanine as a replacement because it frequently occurs in α helices (it is a strong "helix-forming" residue) and its small side chain reduces the possibility of introducing unfavorable van der Waals contacts.

The cysteine substitution at position 88 allows formation of an intermolecular disulfide bond. This disulfide had been predicted by a computer program (PROTEUS) that used a database of known disulfide bond conformations and searched for positions where new disulfide bonds might be added to

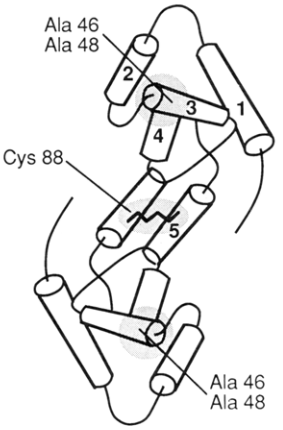

FIGURE 4: Sketch showing the N-terminal dimer of λ repressor, with cylinders used to represent α helices. Helices in one monomer are numbered, and the approximate positions of the mutations are indicated.

repressor (Pabo & Suchanek, 1986). The program suggested that a cysteine at residue 88, which is located within helix 5 and is near the dimer interface, could form a left-handed disulfide bond with a cysteine at residue 88 of the opposite subunit (Figure 4). Experimental analysis revealed that the Cys-88 mutant spontaneously formed a disulfide bond in vitro, bound to operator DNA more tightly than wild-type repressor, and was more stable than wild-type repressor to thermal or urea denaturation (Sauer et al., 1986). Presumably, the disulfide bond, like the glycine to alanine mutations, stabilizes repressor by reducing the entropy of the unfolded state. (An intermolecular cross-link could reduce the rotational, translational, and configurational entropy of the unfolded protein).

Can sets of mutations be combined to systematically increase the stability of a protein? Combining the Cys-88 and Ala-46/Ala-48 mutations, which change residues that are separated by 30 Å in the structure (Figure 4), clearly has yielded a more stable protein. We do not know how far this strategy can be extended, but genetic studies of kanamycin nucleotidyltransferase also indicated that several mutations could be combined to generate a more stable protein (Matsumura et al., 1986). A set of mutations have also been combined to generate a subtilisin variant with improved autolytic stability at alkaline pH (Cunningham & Wells, 1987). Our goal is to engineer a λ repressor that is stable to 100 °C and retains full DNA binding activity. Since a single mutation may stabilize a protein by only a few degrees, it clearly will be necessary to introduce multiple changes to engineer such a stable repressor. Our results, and the results of other laboratories, suggest that this approach is reasonable. If the stabilizing effects of mutations are roughly additive, it will be possible to test individual predictions or select individual mutations and then combine these to generate hyperstable proteins.

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