An Engineered Intersubunit Disulfide Enhances the Stability and DNA Binding of the N-Terminal Domain of λ Repressor†

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Received March 12, 1986; Revised Manuscript Received June 23, 1986

ABSTRACT: Site-directed mutagenesis has been used to replace Tyr-88 at the dimer interface of the N-terminal domain of λ repressor with cysteine. Computer model building had suggested that this substitution would allow formation of an intersubunit disulfide without disruption of the dimer structure [Pabo, C. O., & Suchanek, E. G. (1986) *Biochemistry* (preceding paper in this issue)]. We find that the Cys-88 protein forms a disulfide-bonded dimer that is very stable to reduction by dithiothreitol and has increased operator DNA binding activity. The covalent Cys^{88}–$Cys^{88'}$ dimer is also considerably more stable than the wild-type protein to thermal denaturation or urea denaturation. As a control, Tyr-85 was replaced with cysteine. A Cys^{85}–$Cys^{85'}$ disulfide cannot form without disrupting the wild-type structure, and we find that this disulfide bond reduces the DNA binding activity and stability of the N-terminal domain.

One of the major goals of the emerging field of protein engineering is the design of proteins with enhanced activity or stability. Our investigations have focused on the N-terminal, operator binding domain of the phage λ repressor. This domain binds operator DNA as a dimer, but the dimer is unstable in solution; monomers predominate at the concentrations typically used for binding studies (Pabo et al., 1979; Sauer et al., 1979). Since dimerization and operator binding are coupled, the apparent operator affinity should be increased by amino acid substitutions that stabilize the active, dimeric form of the protein.

The companion paper by Pabo and Suchanek (1986) describes a systematic computer search of the structure of the N-terminal domain (Pabo & Lewis, 1982) for residue positions where disulfide bonds could be added to stabilize the protein. This search identified a potential intersubunit disulfide bond, Cys^{88}–$Cys^{88'}$, that could be introduced by changing Tyr-88 to cysteine. Model building indicated that this disulfide could be accommodated in a favorable conformation with very small adjustments of the dimer interface. Figure 1 shows the dimer interface of the N-terminal domain and illustrates how the proposed disulfide bond could connect α-helix 5 of one monomer to α-helix 5 of the other.

In this paper, we describe the construction, purification, and properties of an N-terminal domain with cysteine, instead of the wild-type tyrosine, as residue 88. This altered protein spontaneously forms a covalent, disulfide-bonded dimer that has enhanced operator binding activity and is more stable to thermal or urea denaturation than the wild-type N-terminal fragment. A different disulfide bond, Cys^{85}–$Cys^{85'}$, which cannot form without disrupting the normal dimer interface, does not enhance the DNA binding activity or stability of the N-terminal domain.

† This work was supported by grants from the National Institutes of Health (AI-15706 and GM-31471), by a University Exploratory Research Grant from Procter and Gamble, and by a Junior Faculty Research Award from the American Cancer Society. R.S.S. was supported by a postdoctoral fellowship from the NIH.

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Materials and Methods

Constructions and Mutagenesis. Plasmid pLac1–102 encodes a protein fragment comprising the first 102 residues of λ repressor. To construct this plasmid, we used the 412 base pair *Eco*RI/*Rsa*I fragment, which contains the *lac*UV5 promoter and the N-terminal region of λ repressor, from pKB280 (Backman & Ptashne, 1978). This fragment was ligated into the *Eco*RI/*Hin*dIII backbone of pBR322 after filling in the 5′ overhang of the *Hin*dIII site to create a blunt end. The blunt ligation of these fragments creates an amber termination codon after the codon for residue 102 and thus directs synthesis of an N-terminal repressor fragment.

For oligonucleotide-directed mutagenesis, the small *Eco*RI/*Bam*HI fragment from pLac1–102 was cloned into the polylinker of phage M13mp8 (Messing & Vierer, 1982). In separate experiments, the Tyr-88 → Cys and Tyr-85 → Cys mutations were introduced into the repressor coding sequence in M13 by using antisense primers with single-base mismatches; 5′-CGCTTCACACATCTC-3′ and 5′-CATCTCGCAGATTTC-3′. Annealing, second-strand synthesis, ligation, transfection, and plaque hybridization were performed by standard procedures (Zoller & Smith, 1983; Carter et al., 1984). For each mutagenesis, several candidates were chosen by hybridization with labeled primers, sequenced by the dideoxy procedure (Sanger et al., 1977), and found when compared with wild type (Sauer, 1978) to contain only the expected Cys-88 or Cys-85 substitutions. These mutant N-terminal domains and their associated *lac* promoters were recloned into the *Eco*RI/*Bam*HI backbone of pBR322, generating mutant plasmids with the same structure as pLac1–102. The plasmid bearing the Cys-88 mutation was designated pRS30, and that bearing the Cys-85 change was designated pRS40.

Prior to protein purification, the wild-type and mutant 1–102 genes were cloned under transcriptional control of the hybrid *trp-lac* promoter (P_{tac}). For these constructions, the 482-residue *Msp*I fragment from pLac1–102 or mutant derivatives was cloned into the *Cla*I site of plasmid pEA300. This cloning strategy is completely analogous to that described by Amann et al. (1983) for intact λ repressor. Plasmids containing the

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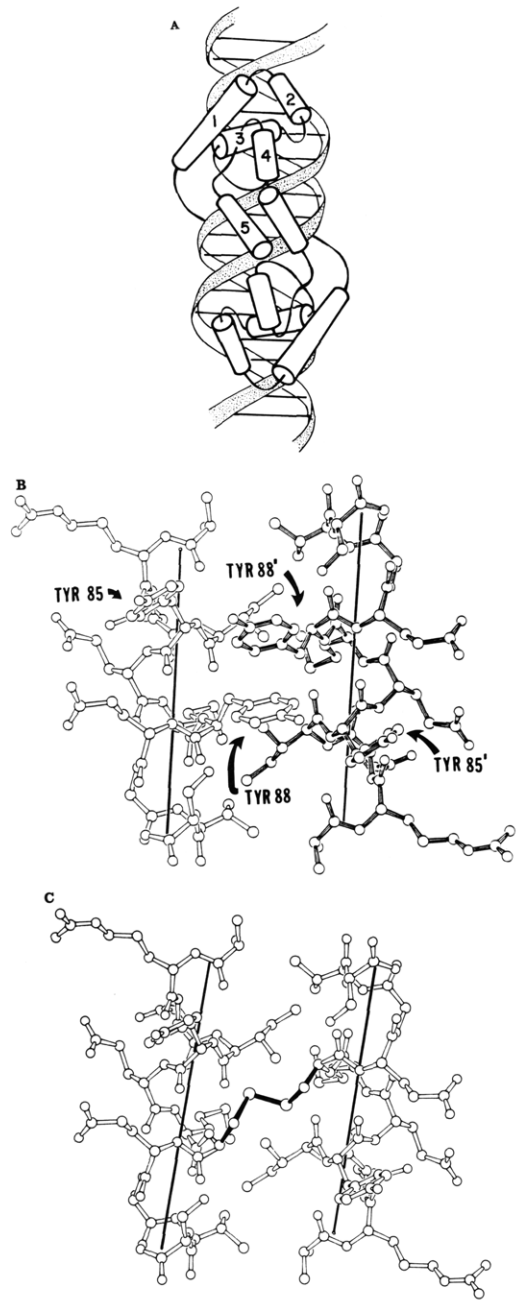

FIGURE 1: (A) Cartoon of the complex between the N-terminal domain dimer and operator DNA. (B) View of the wild-type helix 5/helix 5′ dimer interface. Note the Tyr-88/Tyr-88′ stacking interaction. (C) View of the proposed way in which the Cys^{88}–$Cys^{88'}$ disulfide bond could cross-link helix 5 and helix 5′.

tac promoter and 1–102 coding sequence were designated pKH110 for Cys-88, pKH111 for Cys-85, and pKH112 for wild type. These plasmids were routinely propagated in *Escherichia coli* strain W3110 *lac*I^QL8 (Brent & Ptashne, 1981) or in other strains which overproduce Lac repressor and thereby reduce transcription initiated at the *tac* promoter. Plasmids pKH110, pKH111, and pKH112 cannot be transformed into non-*lac*I^Q strains, and they kill *lac*I^Q strains in the presence of isopropyl β-D-thiogalactoside (IPTG). These observations suggest that overproduction of the 1–102 fragment of λ repressor is lethal. Lower level production seems to be tolerated since pLac1–102, pRS30, and pRS40 (in which transcription is driven by the *lac* promoter) can be propagated in strains without the *lac*I^Q allele.

Protein Purification. All steps of purification were monitored by sodium dodecyl sulfate (SDS) gel electrophoresis using 13.5% polyacrylamide gels and the buffer system of Laemmli (1970). Wild-type 1–102 and the Cys-85 and Cys-88 mutant proteins behaved similarly during purification except at the final gel filtration step.

Strain W3110 *lac*I^QL8 transformed with pKH110, pKH111, or pKH112 was grown at 37 °C in 10 L of LB broth supplemented with ampicillin (100 μg/mL). When A_{600} reached 1.0, IPTG (100 μg/mL) was added, and growth was continued for 3 h. The cells were harvested by centrifugation, resuspended in twice their weight of lysis buffer [100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8), 200 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM $CaCl_2$, 10 mM $MgCl_2$, 2 mM sodium azide, and 1.4 mM β-mercaptoethanol], and lysed by sonication. Phenylmethanesulfonyl fluoride (PMSF) (1 μL of a 100 mM stock in ethanol per milliliter of extract) was added immediately before sonication and once again during sonication. The crude lysate was diluted 5-fold in S buffer (10 mM Tris-HCl, pH 8, 2 mM $CaCl_2$, 2 mM sodium azide, 0.1 mM EDTA, 1.4 mM β-mercaptoethanol, and 5% glycerol) plus 200 mM KCl, 0.1% poly(ethylenimine) was added, precipitated material was removed by centrifugation, and the 1–102 fragment was then precipitated by addition of ammonium sulfate to saturation. The ammonium sulfate pellet was resuspended, dialyzed with Spectrapor 3 tubing into S buffer containing 50 mM KCl, and loaded on a 200-mL Bio-Rad Affigel Blue column. The column was washed with this buffer and eluted with a 2-L linear gradient from 50 to 750 mM KCl in buffer S. The 1–102 protein eluted at a salt concentration of approximately 500 mM. Fractions containing 1–102 were pooled, dialyzed into S buffer containing 50 mM KCl, and chromatographed on a 100-mL Bio-Rex 70 column using a 1-L linear gradient from 50 to 750 mM KCl in S buffer. The 1–102 fragments elute from this column at a salt concentration of approximately 500 mM. As a final purification step, the pooled Bio-Rex 70 fractions were concentrated by using an Amicon YM5 filter and chromatographed on a 3.2 × 55 cm column of Sephadex G-75 (superfine) in buffer S containing 100 mM KCl. The Cys-88 protein eluted from this column at a position near that expected for a dimer, the Cys-85 protein eluted between the dimer and monomer positions, and the wild-type fragment eluted near the position expected for a monomer.

G-75 chromatography was performed at room temperature; all other purification steps were carried out at 4 °C. The purifications yielded approximately 500 mg of wild-type protein, 500 mg of the Cys-85 fragment, and 250 mg of the Cys-88 fragment.

DNA Binding. DNA binding experiments were performed by using a 438 base pair (bp) *Eco*RI/*Nsi*I restriction fragment from pKB252 (Backman et al., 1978) which contains the λ O_R operator region. This fragment was end labeled by using DNA polymerase I large fragment and [α-^{32}P]dATP to fill in the *Eco*RI overhang. DNase protection experiments (Johnson et al., 1979) were performed at room temperature in a buffer containing 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 200 mM KCl, 100 μg/mL bovine serum albumin (BSA), 25 μg/mL salmon sperm DNA, and 50 μg/mL tRNA.

NMR Studies. Proton NMR experiments were performed at the 500-MHz facility of the Francis Bitter National Magnet Laboratory. For these studies, the proteins were exhaustively dialyzed against 50 mM ammonium bicarbonate and lyophilized. The powder was dissolved in the appropriate amount of NMR buffer [50 mM potassium phosphate (pD 7.4), 200

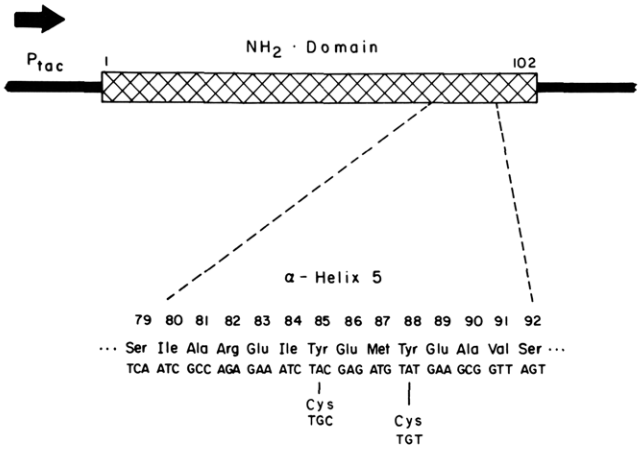

FIGURE 2: Schematic representation of the gene encoding the amino-terminal 102 residues of λ repressor. The protein and DNA sequences corresponding to α-helix 5 are shown as are the Tyr-85 → Cys and Tyr-88 → Cys site-directed mutations.

mM KCl, 1 mM sodium azide, 0.1 mM EDTA, and 99.98% D_2O] and placed in a 5-mm NMR tube.

Circular Dichroism. CD spectra were obtained with a Jasco J-500C spectropolarimeter. Proteins were dissolved at 30 μg/mL in CD buffer, which contains 10 mM potassium phosphate (pH 7.5), 50 mM KCl, and 0–8 M urea. Protein concentrations were calculated by assuming that a 1 mg/mL solution of wild-type 1–102 has $A_{280} = 0.54$ and that 1 mg/mL solutions of the Cys-85 and Cys-88 fragments have $A_{280} = 0.45$.

Oxidation and Reduction Experiments. To obtain the disulfide-bonded form of the Cys-85 protein, 20 mg of the protein was dissolved in 5 mL of A buffer [100 mM Tris-HCl (pH 8.7), 200 mM NaCl, and 1 mM EDTA], and 175 μL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2 mg/mL in 0.2 M potassium phosphate buffer (pH 7), was added. After 2 h at room temperature, the mixture was desalted on a Bio-Gel P2 column equilibrated in A buffer. The fractions containing protein were pooled, and additional reduced Cys-85 protein was added (3 mL of a 4 mg/mL solution in A buffer). This mixture was incubated for 24 h at room temperature and then chromatographed on a 3.2 × 55 cm column of Sephadex G-75 equilibrated in A buffer. The leading shoulder of the protein peak was pooled, concentrated, and rechromatographed. The leading 60–70% of the protein peak from the second G-75 column contained almost pure disulfide-bonded Cys-85 dimer.

The stabilities of the Cys^{85}–$Cys^{85'}$ and Cys^{88}–$Cys^{88'}$ disulfide bonds were assayed by treating the proteins with mixtures of oxidized (Sigma) and reduced dithiothreitol (BRL). All experiments were performed under a nitrogen atmosphere in A buffer at room temperature (22–24 °C). Incubations were for 0–48 h, and the reactions were quenched by addition of 0.3 volume of 1 M iodoacetic acid (in 1 N NaOH). After an additional 10 min, Laemmli sample buffer (65 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, and tracking dyes) was added, and the samples were loaded directly onto 13.5% SDS–polyacrylamide gels. The reactions appeared to be at equilibrium in that the populations of reduced and oxidized protein did not change between 24 and 48 h. Moreover, the populations obtained depended, as expected, on the ratio of oxidized and reduced dithiothreitol (DTT) as dictated by equilibrium thermodynamics.

RESULTS

Purification and Oligomeric Properties. To facilitate studies of the N-terminal domain of λ repressor, we constructed a plasmid that directs overproduction of a fragment corresponding to residues 1–102 of intact λ repressor. The Tyr-85 → Cys and Tyr-88 → Cys changes (Figure 2) were introduced into this vector by oligonucleotide-directed mutagenesis, and the wild-type, Cys-85, and Cys-88 proteins were purified (see Materials and Methods). β-Mercaptoethanol was added to all buffers during the purifications in an attempt to prevent oxidation of the cysteine-containing fragments to their disulfide-bonded forms. Nevertheless, 90% or more of the purified Cys-88 fragment was found to be in the disulfide-bonded form when assayed by SDS gel electrophoresis. This suggests that the Cys^{88}–$Cys^{88'}$ disulfide bond forms spontaneously, either in the cell or during purification. As shown in lane G of Figure 3, the purified Cys-88 fragment is completely converted to the Cys^{88}–$Cys^{88'}$ disulfide-bonded form after dialysis into a buffer that lacks reducing agents.

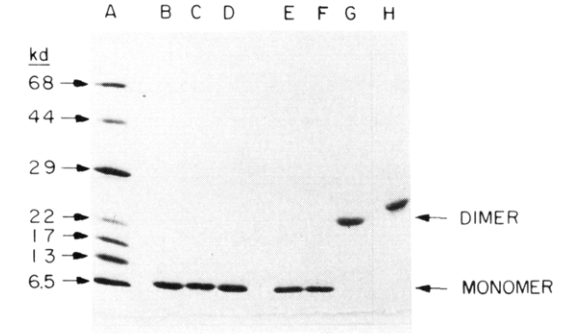

FIGURE 3: SDS gel electrophoretic analysis of the wild-type and cysteine-substituted 1–102 proteins. (A) Molecular weight standards; (B) reduced wild type; (C) reduced Cys-85; (D) reduced Cys-88; (E) unreduced wild type; (F) unreduced Cys-85; (G) unreduced Cys-88; (H) unreduced Cys^{85}–$Cys^{85'}$. Samples in lanes A–D were treated with 5% SDS–3% β-mercaptoethanol for 2 min at 90 °C prior to electrophoresis. Samples in lanes E–H were dialyzed into buffer A (100 mM Tris-HCl, pH 8.7, 200 mM NaCl, and 1 mM EDTA) and then loaded on SDS gels in sample buffer without β-mercaptoethanol.

The wild-type 1–102 fragment contains no cysteine and no disulfide bonds. This fragment undergoes a monomer–dimer transition with an apparent K_d of approximately 10^{-4} M (Weiss et al., 1986a). The purified Cys-85 protein formed only a small amount of disulfide-bonded dimer under conditions where the Cys-88 fragment was completely converted to dimer (cf. lanes F and G, Figure 3). However, in gel filtration experiments using identical protein concentrations, the Cys-85 fragments eluted at a higher apparent molecular weight than the wild-type 1–102 fragment, and in NMR experiments, the Cys-85 fragment was found to be dimeric at concentrations where the wild-type fragment was predominantly monomeric (data not shown). These data indicate that the Cys-85 protein forms a noncovalent dimer that is more stable than the wild-type dimer. The dimerization constant for Cys-85 appears to be near 10^{-5} M.

Stability to Reduction. A quantitative measure of the stability of an intersubunit disulfide bond can be obtained from the equilibrium constant for reduction by DTT:

$$R\text{–}SS\text{–}R + DTT \leftrightarrow 2R\text{–}SH + DTT_{ox}$$

$$K_{red} = [R\text{–}SH]^2[DTT_{ox}]/[R\text{–}SS\text{–}R][DTT]$$

For these studies, disulfide-bonded Cys^{85}–$Cys^{85'}$ dimers were obtained by activating the Cys-85 SH group by formation of a thionitrobenzoate–protein mixed disulfide (see Materials and Methods). The Cys^{85}–$Cys^{85'}$ and Cys^{88}–$Cys^{88'}$ dimers were treated with mixtures of oxidized (ox) and reduced (red) DTT, and the fraction of the protein in the monomeric form was

Table I: Operator DNA Binding

protein	$[R_{1/2}]$ (M)	K_1 (M)	K_2 (M)
wild type	5×10^{-7}	10^{-4}	2.5×10^{-9}
Cys^{88}–$Cys^{88'}$	6×10^{-8}		3.0×10^{-8}
Cys-85	7×10^{-7}	10^{-5}	4.9×10^{-8}
Cys^{85}–$Cys^{85'}$	1×10^{-5}		5.0×10^{-6}

[a] $[R_{1/2}]$ is the protein concentration (moles of monomer per liter) required for 50% protection of the λ O_R1 operator site. K_1 is the equilibrium dimerization constant for the wild-type and Cys-85 proteins. K_2 is the equilibrium dissociation constant for binding of the protein dimers to the O_R1 operator site. At the concentrations and conditions used, $[R_{1/2}]$ is approximately equal to the free monomer concentration for wild type and Cys-85. For these proteins, $K_1K_2 = [R_{1/2}]^2$.

established by SDS gel electrophoresis after alkylation of free sulfhydryl groups with iodoacetic acid. We have assumed that the mixed disulfide between DTT and the protein is relatively unstable and that the observed monomeric protein represents the fully reduced species. Under the experimental conditions used, K_{red} for the Cys^{88}–$Cys^{88'}$ protein was found to be about 0.9 mM, and K_{red} for the Cys^{85}–$Cys^{85'}$ protein was about 12 mM. This indicates that the Cys^{88}–$Cys^{88'}$ protein is more stable to reduction than the Cys^{85}–$Cys^{85'}$ protein. Under similar conditions, K_{red} for reduction of oxidized glutathione by DTT is approximately 10^3 M (Creighton & Goldenberg, 1984). Thus, the Cys^{88}–$Cys^{88'}$ and Cys^{85}–$Cys^{85'}$ disulfides are both considerably more stable than the disulfide in oxidized glutathione.

The properties of the Cys-88-substituted protein make it difficult to work with the protein in a fully reduced form at high protein concentrations. The Cys^{88}–$Cys^{88'}$ disulfide forms in the absence of thiol–disulfide exchange reagents and requires the continued presence of high concentrations of DTT for complete reduction. For these reasons, the studies reported below do not include the reduced Cys-88 species.

Operator DNA Binding. The binding of the wild-type and Cys-substituted proteins to the λ O_R1 operator site was assayed by DNase protection (Johnson et al., 1979). The concentrations of protein, $[R_{1/2}]$, required for 50% protection of the O_R1 operator site are listed in Table I. The Cys^{88}–$Cys^{88'}$ protein binds operator DNA most efficiently in that it protects the operator at the lowest protein concentration. To achieve comparable operator binding, approximately 10-fold higher concentrations of wild-type or Cys-85 fragments are required, and 200-fold higher concentrations of the Cys^{85}–$Cys^{85'}$ protein are required. In considering these data, it is important to remember that the Cys^{88}–$Cys^{88'}$ and Cys^{85}–$Cys^{85'}$ dimers bind operator DNA directly whereas the wild-type and Cys-85 proteins need to dimerize *and* bind the operator. Thus, binding of the wild-type and Cys-85 proteins involves the coupled equilibria:

$$2R \overset{K_1}{\leftrightarrow} R_2 + O \overset{K_2}{\leftrightarrow} R_2O$$

whereas binding of a disulfide-bonded dimer involves only the second reaction.

$$R_2 + O \overset{K_2}{\leftrightarrow} R_2O$$

Under conditions similar to those used for DNA binding, the K_1 dimerization constant is about 10^{-4} M for the wild type and 10^{-5} M for the Cys-85 fragment (Weiss et al., 1986a). These K_1 values have been used to calculate the K_2 operator affinity constants for wild type and Cys-85 which are shown in Table I. Comparison of the K_2 values in Table I shows that the wild-type dimer binds operator DNA with the highest affinity; the operator affinities of the Cys^{88}–$Cys^{88'}$ dimer and the noncovalent Cys-85 dimer are reduced 12-fold and 19-fold, respectively, and the affinity of the Cys^{85}–$Cys^{85'}$ dimer is reduced 2000-fold.

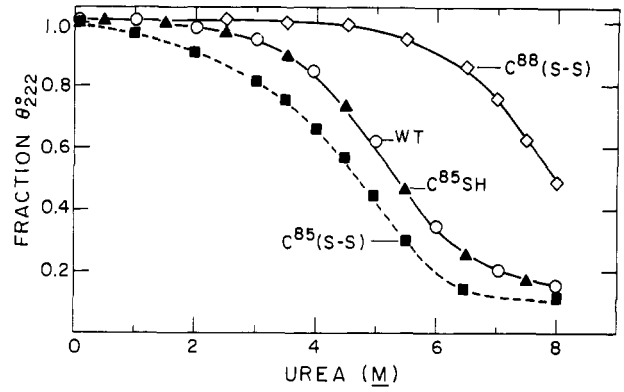

FIGURE 4: Denaturation by urea. All proteins were dissolved at 30 μg/mL in CD buffer at 22 °C. The ordinate axis is the molar ellipticity observed at 222 nm (θ_{222}) for a given urea concentration divided by the value observed in the absence of urea.

Stability to Urea Denaturation. The crystal structure of the wild-type N-terminal domain is predominantly α-helical (Pabo & Lewis, 1982), and circular dichroism (CD) can be used as a probe of its structure and stability (Hecht et al., 1984). The CD spectra for the wild-type, Cys-85, Cys^{85}–$Cys^{85'}$, and Cys^{88}–$Cys^{88'}$ proteins were similar (data not shown), but 5–10% changes in α-helical content probably would not have been detected in these experiments. Figure 4 shows CD denaturation curves, as a function of urea concentration, for each of the proteins. The wild-type and Cys-85 proteins have comparable urea stabilities, the Cys^{85}–$Cys^{85'}$ protein is slightly less stable than wild type, and the Cys^{88}–$Cys^{88'}$ protein is considerably more stable than wild type.

NMR Spectra and Thermal Stabilities. NMR provides another means of comparing the solution structures of the proteins. The aromatic regions of their ^1H NMR spectra are shown in Figure 5. The wild-type spectrum (A) has been completely assigned (Weiss et al., 1986b). Of special interest are the Tyr-22, Phe-51, and Phe-76 ring protons, which are buried in the hydrophobic core. Ring current calculations suggest that their unusual secondary shifts should be sensitive to structural displacements as small as 0.2 Å (Johnson & Bovey, 1958; Hoch et al., 1981; Weiss et al., 1986b). These resonances provide sensitive markers for the tertiary structure of the globular portion of the N-terminal domain, which is formed by α-helices 1–4 and contains the DNA binding surface of the protein. For the Cys-85 and Cys-88 proteins (spectra B–D of Figure 5), the resonances of Tyr-22, Phe-51, and Phe-76 are essentially the same as those of wild type. These data indicate that the tertiary structures of the mutant domains have not been affected by the sequence changes or disulfide bonds.

The resonances assigned to Tyr-60, Tyr-85, and Tyr-88 in the wild-type protein are expected to be influenced by dimer formation. In the dimer, Tyr-88 and Tyr-88′ are stacked; there are interactions between Tyr-85 of one monomer and Tyr-88 of the other and interactions between Tyr-60 and residues of the other subunit. These resonances provide a means of comparing the wild-type and mutant dimers. At the concentrations used for the experiments shown in Figure 5, the wild-type and Cys-85 proteins are noncovalent dimers. The Tyr-85 spin system is absent from the Cys-85 spectrum (Figure 5B) as expected. However, the Tyr-88 and Tyr-60 resonances are quite similar to those of wild type, suggesting that the

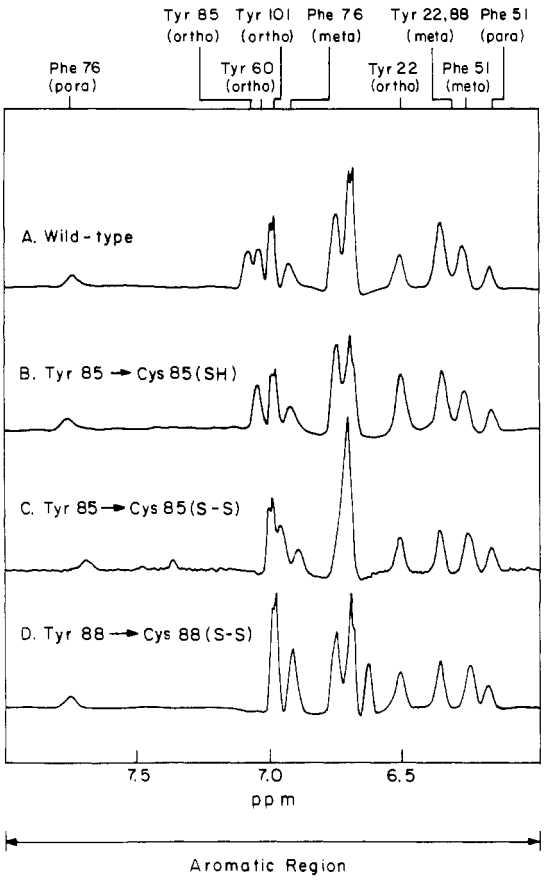

FIGURE 5: Aromatic NMR spectra at 500 MHz. Unless indicated, protein concentrations were 5 mM, and a convolution difference with parameters GM4, EM20, and subtraction factor 1.0 was applied before Fourier transformation. In all acquisitions, the residual HOD signal was presaturated for 1 s. (A) Wild type. For resonance assignments, see Weiss et al. (1986b). The spectrum is the sum of 200 transients with a recycle delay of 4 s. (B) Cys-85 protein. The spectrum is the sum of 216 transients with a recycle delay of 4 s. (C) Cys^{85}–$Cys^{85'}$ protein (concentration 2 mM). The spectrum is the sum of 1000 transients with a recycle delay of 2 s. Convolution difference parameters were GM5, EM30, and 1.0. (D) Cys^{88}–$Cys^{88'}$ protein. The spectrum is the sum of 604 transients with a recycle delay of 1.5 s.

wild-type and Cys-85 dimers have similar structures under these conditions.

Forming the covalent Cys^{85}–$Cys^{85'}$ dimer (Figure 5C) clearly affects the resonances of Tyr-60 and Tyr-88, and it seems likely that the Cys^{85}–$Cys^{85'}$ dimer differs significantly from the wild-type dimer. This is not surprising since the crystal structure of the wild-type protein shows that the C_α atoms of Tyr-85 and Tyr-85′ are not in positions that would allow formation of a disulfide bond. Formation of the Cys^{85}–$Cys^{85'}$ disulfide probably requires local unfolding and distortion of the structure or a rearrangement of the protein dimer contacts.

It is difficult to use the aromatic spectrum of Cys^{88}–$Cys^{88'}$ (Figure 5D) to evaluate the structure of this dimer since the Tyr-88 resonances normally provide a major marker for evaluating the dimer contacts. There are shifts in the Tyr-85 and Tyr-60 resonances, but these shifts could result from structural perturbations introduced by the Cys-88 disulfide bond or merely from the absence of the Tyr-88 ring current (Johnson & Bovey, 1958).

Thermal denaturation of the proteins was monitored by their aromatic NMR spectra. Spectral data are shown in Figure 6 for wild-type protein and the Cys^{88}–$Cys^{88'}$ protein. Figure 7 shows a plot of the integrated intensities of several resonances from each protein as a function of temperature. For wild type, the native resonances broaden and disappear between 48 and

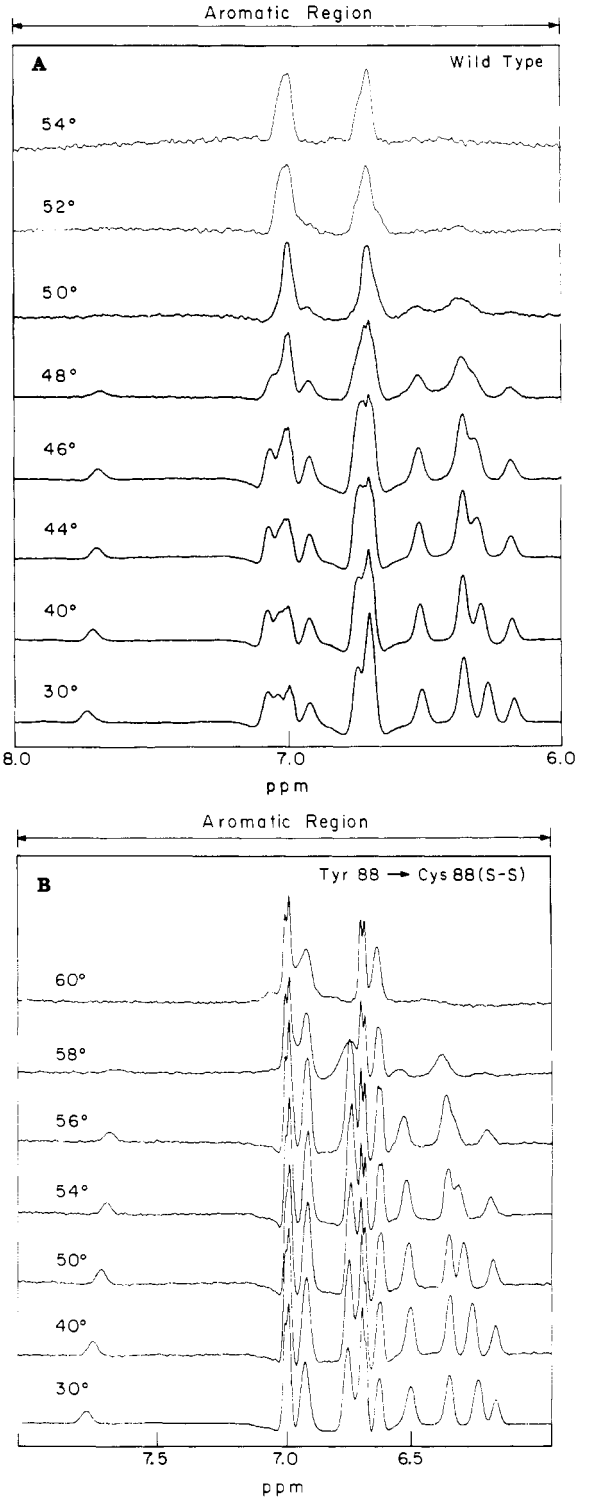

FIGURE 6: Aromatic NMR spectra as a function of temperature. The temperature of each sample was successively raised from the lowest to the highest temperature indicated. (A) Wild type. The spectrum was the sum of 104 transients with recycle delay of 2 s. Convolution difference parameters were GM4, EM20, and 1.0. (B) Cys^{88}–$Cys^{88'}$. Each spectrum is the sum of 100 transients with a recycle delay of 1.5 s. convolution difference parameters were GM4, EM20, and 1.0.

52 °C (Figures 6A and 7). In differential scanning calorimetry experiments, a wild-type fragment consisting of residues 1–92 has a T_m of 49 °C (Pabo et al., 1979), and the NMR experiments give similar estimates for the thermal stability of the wild-type N-terminal domain. The Cys^{88}–$Cys^{88'}$ protein denatures between 56 and 60 °C (Figures 6B and 7) and thus is more thermally stable than the wild-type protein. The Cys^{85}–$Cys^{85'}$ protein is less thermally stable than wild type,

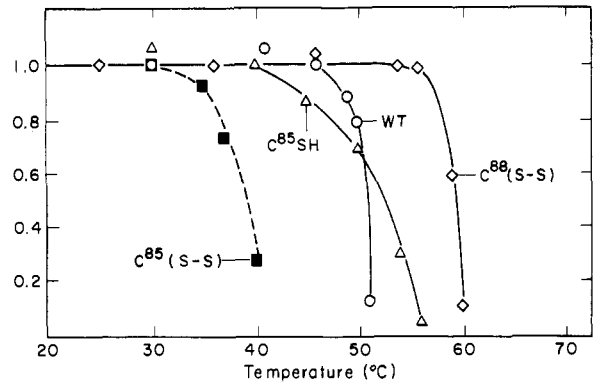

FIGURE 7: Thermal stability of the wild-type and cysteine-substituted proteins as monitored by aromatic NMR spectra. The ordinate axis represents the value of the integrated intensities of the four right-most native resonances (Tyr-22 ortho; Tyr-22/88 meta; Phe-51 meta; Phe-51 para) at a given temperature divided by this value at 20 °C.

and the reduced Cys-85 protein, which has a relatively broad denaturation transition, has a midpoint similar to that of wild type (Figure 7).

Discussion

The N-terminal domain of λ repressor binds operator DNA as a dimer. Relatively weak interactions between helix 5 and helix 5′ stabilize the dimer and hold the two monomers in the proper orientation for operator recognition. The experiments presented here were designed to test the prediction that changing Tyr-88 to cysteine would permit formation of a Cys^{88}–$Cys^{88'}$ disulfide bond without disrupting the wild-type N-terminal dimer (Pabo & Suchanek, 1986). Our results suggest that this prediction is correct.

The Cys^{88}–$Cys^{88'}$ disulfide formed spontaneously and was quite stable to reduction by dithiothreitol. This would be expected if the disulfide bond formed within the context of the normal helix 5/helix 5′ interactions, since these interactions would help to stabilize the disulfide against reduction. How similar are the wild-type dimer and the Cys^{88}–$Cys^{88'}$ dimer? The NMR spectra of the two proteins show that the tertiary structures of the globular portions of each monomer are very similar but the NMR spectra are less informative about the structure of the dimer interface. If the wild-type dimer and the Cys^{88}–$Cys^{88'}$ dimer had identical structures, then each dimer should have the same affinity for operator DNA. The Cys^{88}–$Cys^{88'}$ protein gives 50% operator protection at a concentration about 10-fold lower than that required for comparable protection by wild-type protein. We presume that this occurs because the Cys^{88}–$Cys^{88'}$ protein does not pay the energetic cost of forming a noncovalent dimer at low protein concentrations. However, the calculated operator affinity constant (K_2) for the wild-type dimer is about 12-fold stronger than that of the Cys^{88}–$Cys^{88'}$ protein (Table I). This affinity difference corresponds to a modest value of $\Delta\Delta G$ (1.5 kcal/mol), and thus the structural differences between the wild-type and Cys^{88}–$Cys^{88'}$ dimers are likely to be small. Crystallographic studies of the Cys^{88}–$Cys^{88'}$ protein are currently being pursued to identify possible structural differences between this protein and the wild-type dimer (E. G. Suchanek and C. O. Pabo, unpublished results).

The properties of proteins bearing the Tyr-85 → Cys substitution provide further evidence that it is not dimerization per se, but rather the structure of the dimer, that is of primary importance in determining operator affinity. For reasons that we do not yet understand, the reduced Cys-85 protein forms noncovalent dimers more readily than does the wild-type protein. However, it does not bind operator DNA more strongly. The operator affinity of the Cys-85 dimer is reduced 19-fold in comparison to the wild-type dimer, suggesting that changes may have occurred in the structure of the mutant dimer. The extremely weak DNA binding activity observed for the covalent Cys^{85}–$Cys^{85'}$ dimer illustrates this same point. Formation of this dimer probably requires significant changes in the wild-type structure, and the operator affinity of the Cys^{85}–$Cys^{85'}$ dimer is about 2000-fold weaker than that of wild type.

In evaluating the results described here and the effects of other N-terminal mutations (Hecht & Sauer, 1985; Nelson & Sauer, 1985), it is extremely important to know whether the dimer contacts in the repressor–operator complex are exactly the same as the dimer contacts in the protein crystal. If subtle shifts in the dimer contact are required for DNA binding, then one cannot optimize binding by designing a disulfide that stabilizes the dimer observed in the protein crystal. This issue is being addressed by structural investigations of a cocrystal that contains the N-terminal dimer and a 20 base pair duplex with the λ operator site (Jordan et al., 1985).

Acknowledgments

We thank Martin Karplus for advice and comments on the manuscript.

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Preparation and Properties of Porcine Relaxin Derivatives Shortened at the Amino Terminus of the A Chain†

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Received April 2, 1986; Revised Manuscript Received June 18, 1986

ABSTRACT: Porcine relaxins shortened at the N terminus of the A chain were produced after protection of all amino groups with the base-labile [[(methylsulfonyl)ethyl]oxy]carbonyl (Msc) protecting group. The first two amino acids were removed by cyanogen bromide digestion whereby simultaneously a free α-amino group was generated in position A3. The resulting des-Arg^{A1},Met^{A2}-$N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]relaxin was further shortened by preparative Edman degradation. The shortest derivative obtained was des-Arg^{A1},Met^{A2},Thr^{A3},Leu^{A4},Ser^{A5},Glu^{A6}-$N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]relaxin. The deprotection of the derivatives in alkaline media resulted in crude des-A(1–2)- to des-A(1–6)-relaxins, which were subsequently purified by gel filtration on Sephadex G-50 superfine followed by either ion exchange chromatography on CM-cellulose at pH 5.1 or high-performance liquid chromatography on reversed-phase columns. During the CNBr digest, a side product was isolated that was identified as the corresponding homoserine ([Hse^{A2}]relaxin) derivative. Shortened relaxin derivatives and [Hse^{A2}]relaxin were characterized by reversed-phase chromatography, electrophoresis, end-group determination, and amino acid composition. Circular dichroism studies revealed a distinct change in the structure of relaxins that were shortened by three and more amino acid residues. In the mouse interpubic ligament assay, des-A(1–2)-relaxin and [Hse^{A2}]relaxin were fully biologically active while the bioactivity of des-A-(1–3)-relaxin dropped to about 50%. Relaxins shortened by four and more amino acid residues were biologically inactive. The N-terminal region of the A chain of porcine relaxin appears to be important for the structural integrity of the hormone.

Relaxin, a hormone of pregnancy, causes the widening of the mammalian birth canal during late pregnancy and inhibits uterine contractions. Like insulin, the relaxin molecule consists of two polypeptide chains linked by inter- and intrachain disulfide bonds. The positions of the disulfide links are similar to those in insulin (Schwabe & McDonald, 1977).

Naturally occurring relaxins differ in their primary structure and in their biological potency over a wide range. For instance rat relaxin (John et al., 1981; Hudson et al., 1981) and pig relaxin (Schwabe et al., 1976, 1977; James et al., 1977) have a similarity of only 54%. However, pig relaxin is more potent than rat relaxin (Sherwood, 1979) in the mouse interpubic ligament assay. Shark relaxins (Gowan et al., 1981; Büllesbach et al., submitted for publication) differ from pig relaxin by about 48–50% and show low activity in the symphysis pubis relaxation assay as well as in the uterine relaxation assay (Gowan et al., 1981; Büllesbach et al., submitted for publication). Comparison of the primary sequences of all known relaxins (John et al., 1981; Schwabe et al., 1976, 1977; James et al., 1977; Gowan et al., 1981; Haley et al., 1982; Hudson et al., 1981, 1983, 1984) reveals that there is no invariant amino acid residue in the N-terminal region of the relaxin A chain (Figure 1), and we therefore decided to investigate the relevance of this portion of the relaxin molecule by chemically modifying native porcine relaxin.

Porcine relaxin (B29) can be prepared in relatively large quantities free of relaxin molecules that vary in length at the N terminus of the A chain (Büllesbach & Schwabe 1985a,b) and at the C terminus of the B chain (Büllesbach & Schwabe, 1985b; Niall et al., 1980; Walsh & Niall, 1980) by a combination of preparative carboxypeptidase A digestion and chromatographic procedures. Pure B29 relaxin thus obtained consists of the 22-residue A chain and a 29-residue B chain (Büllesbach & Schwabe, 1985b). It has been known that N-terminal elongation of porcine relaxin by one amino acid does not affect the biological potency (Schwabe, 1983; Büllesbach & Schwabe, 1985a). Schwabe and Braddon (1976) described the partial loss of biological activity in des-Arg^{A1},Met^{A2}-relaxin (porcine); however, there remained some uncertainty as to whether the loss of bioactivity was caused by the absence of the first two amino acids or by other side reactions. Tregear et al. (1983) reported the total synthesis of porcine relaxin chains and their random combination and that the combination mixture of A(4–22) and B(1–25) was bioactive in the uterine motility suppression assay. The same procedure was used to produce a [Nle^{A2}]B28 porcine relaxin that was also active in the same assay system (Tregear et al., 1981). Symphysis pubis elongation assay results were not reported. In this paper, we describe the systematic shortening of porcine relaxin molecules at the N terminus of the A chain and of [Hse^{A2}]relaxin, as well as some physical and biological

† This work was supported by NIH Grant HD-10540 and NSF Grant PCM-8302194 and in part by a Medical University of South Carolina Biomedical Research Support Grant, 1984.
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0006-2960/86/0425-5998$01.50/0