Domains in λ Cro Repressor. A Calorimetric Study†

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ABSTRACT: Thermodynamic properties of a mutant λ Cro repressor with Cys replacing Val[55] were studied calorimetrically. Formation of the S–S cross-link between neighboring Cys[55] residues in this dimeric molecule leads to stabilization of a structure formed by the C-terminal parts of the two polypeptide chains, which behave as a single cooperative domain upon protein denaturation by heating. This composite domain is very stable at neutral pH and disrupts at 110 °C. The S–S-cross-linked tryptic fragment (residues 22–66), which includes this C-terminal domain, has similar stability. The N-terminal parts of the polypeptide chains do not form any stable structure when isolated, but in S–S-cross-linked dimer, they form a single cooperative block which melts in an all-or-none way 9 °C higher than the un-cross-linked protein. The observed cooperatiion of the distant N-terminal parts in dimer raises questions regarding λ Cro repressor structure in solution.

According to crystallographic information, some DNA-binding proteins possess a very specific structural element called the "helix–turn–helix" motif. This region of the DNA-binding protein interacts directly with nucleic acid (Brenman & Matthews, 1989; Pabo & Sauer, 1984). The other important structural element in DNA-binding proteins, which are usually dimers, is a domain which is formed by the adjusted subunits and mediates their interaction essential for DNA binding (Pabo et al., 1979). The smallest member of this class of proteins is Cro repressor of phage λ. Its polypeptide chain consists of only 66 amino acid residues, a length which appears insufficient for formation of more than 1 domain. It is known, however, that this molecule is active only in the dimeric form (Johnson et al., 1978; Pakula & Sauer, 1989). The C-terminal parts of each polypeptide chain stack together, forming the antiparallel β-ribbon and providing the necessary intersubunit contacts (Ohlendorf et al., 1983).

A recent calorimetric study has shown that the thermal denaturation of the dimer of Cro repressor can be described as a two-state transition (Gitelson et al., 1991). This means that intersubunit interactions are so strong that the dimer forms a single cooperative block. In contrast to the wild-type protein, the mutant [Cys[55]]Cro, in which the two subunits are joined by a disulfide cross-link, denatures in two distinct stages. These stages could correspond to the melting of two different domains in the mutant molecule. It was concluded that the high-temperature transition corresponds to disruption of the cooperative structure which is formed by the C-terminal parts of two polypeptide chains. In this paper, we present experimental results which further substantiate this conclusion.

EXPERIMENTAL PROCEDURES

Wild-type λ Cro repressor and the mutant [Cys[55]]Cro, in which cysteine has been substituted for Val[55], were isolated as described (Gitelson et al., 1991).

CNBr-peptide(13–66) was obtained by CNBr hydrolysis of the polypeptide chain according to Beeley (1976). The tryptic fragment of [Cys[55]]Cro was obtained by treatment of the CNBr-peptide with trypsin. The fragments purified to homogeneity on a Mono S column using a 0–1.0 M gradient of KCl in 10 mM Tris-HCl, pH 7.9. Electrophoresis experiments (20% acrylamide in the presence of β-mercaptoethanol) have shown that the tryptic fragment consists of two polypeptide chains connected by an S–S bridge. The molecular mass of this fragment, estimated electrophoretically, is around 9.0 kDa. The peptide was identified by N-terminal amino acid analysis as described elsewhere (Chang et al., 1978).

Hydrolysis of the polypeptide chain of the mutant at the Cys[55] residue was carried out by 2-nitro-5-thiocyanatobenzoic acid, according to Jacobson (1973).

Protein concentrations were estimated spectrophotometrically using the extinction coefficients ($E^{1\%}_{276}$) 5.85, 4.30, and 4.76 for [Cys[55]]Cro repressor, the tryptic fragment, and the CNBr fragment, respectively.

The following molecular masses were used for thermodynamic calculations: 14.7 kDa for the dimer of either the wild or the mutant, 11.6 kDa for the CNBr fragment; and 8.5 kDa for the tryptic fragment.

Sedimentation velocity experiments were carried out on a Beckman Model E analytical ultracentrifuge; equilibrium ultracentrifugation was performed on a MOM 3170 (Hungary).

Calorimetric measurements were made using a DASM-4 scanning microcalorimeter which permits heating of aqueous solutions up to 130 °C under excess pressure (Privalov & Plotnikov, 1989). All calorimetric experiments were done at a heating rate of 1 K/min and a protein concentration of 2–4 mg/mL. The partial specific heat capacity of the proteins was determined according to the procedure described earlier (Privalov & Potekhin, 1986), using the following partial specific volumes: 0.734 $cm^3 \cdot g^{-1}$ for the wild-type repressor; 0.738 $cm^3 \cdot g^{-1}$ for the mutant; and 0.730 $cm^3 \cdot g^{-1}$ for either fragment. These values were determined by density measurements of the corresponding solutions using a DMA-2 (Anton Paar) densimeter.

A deconvolution analysis of the protein excess heat capacity function was performed using a modification (Filimonov et

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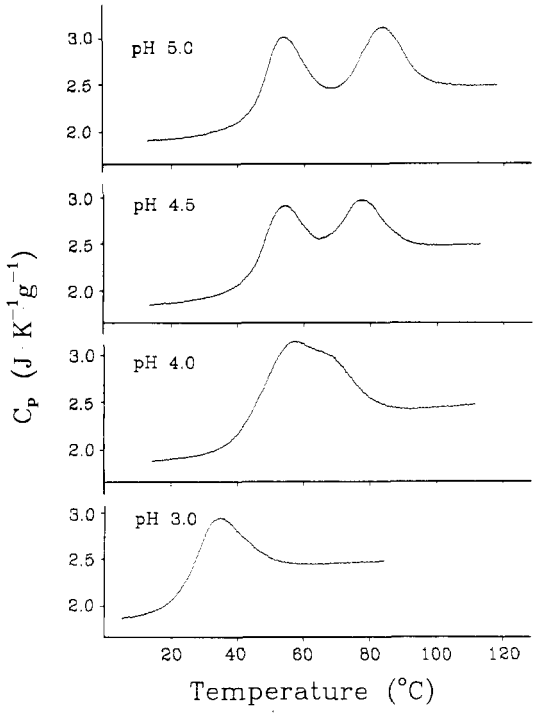

FIGURE 1: Temperature dependence of the [Cys^{55}]Cro repressor partial heat capacity in solution with various pHs (indicated on the curves).

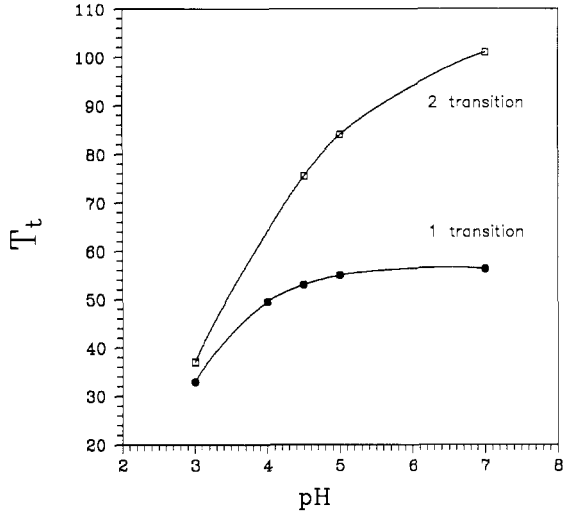

FIGURE 2: Transition temperature dependence on pH for the cooperative blocks in the [Cys^{55}]Cro repressor.

al., 1982) of the sequential procedure of Freire and Biltonen (1978).

RESULTS AND DISCUSSION

Figure 1 shows the temperature dependence of the partial heat capacity of [Cys^{55}]Cro at various pH values. Under all conditions, protein denaturation proceeds in at least two stages, since the thermograms display two clearly separated heat absorption peaks. The denaturation of the mutant protein is a reversible process, and at the repeated heating of the cooled solution, the heat absorption profile is reproduced completely. The observed heat absorption depends on the pH and ionic strength of the solution. With decreasing pH, both heat absorption peaks shift to lower temperatures, showing that the protein structure becomes less stable. It is remarkable that the temperature shifts of the two peaks are different

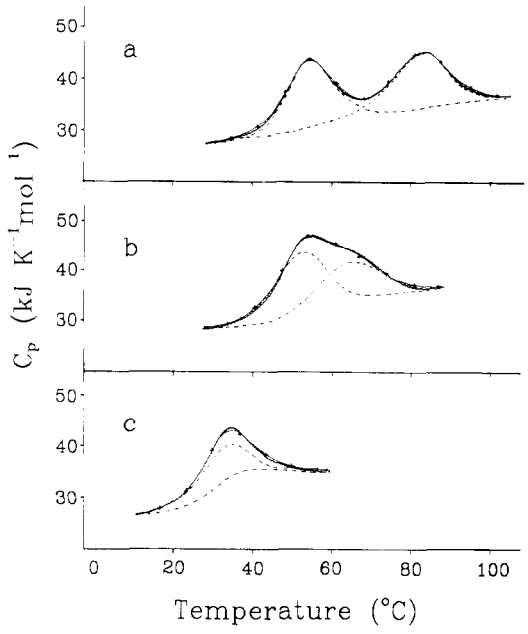

FIGURE 3: Deconvolution of the excess heat capacity function of the λ [Cys^{55}]Cro repressor in solution of 20 nM potassium phosphate buffer with different pHs: (a) pH 5.0; (b) pH 4.0; (c) pH 3.0. Crosses indicate the functions calculated using the transition parameters obtained by deconvolution analysis.

Table I: Temperatures and Enthalpies of Transitions in [Cys^{55}]Cro Repressor at Different pHs As Determined by Deconvolution Analysis of the Heat Absorption Curves

	first transition		second transition		
pH	T (K)	ΔH (kJ/mol)	T (K)	ΔH (kJ/mol)	ΔH^{tot} (kJ/mol)
3.0	310	162	306	50	212
4.0	326	205	339	170	375
4.5	327	204	350	199	403
5.0	327	211	355	205	416
7.0	328	219	374	253	473

(Figure 2); the temperature of the second transition changes much faster than that of the first one.

Deconvolution analysis of the excess heat capacity function of the [Cys^{55}]Cro repressor in solutions of different pH (Figure 3) shows that under all conditions the protein structure melts as if it consisted of two cooperative blocks which differ in stability. The thermodynamic parameters of the individual transitions obtained from the deconvolution analysis are given in Table I. The enthalpies of individual transitions plotted against transition temperatures (Figure 4) are expressed by two continuous functions whose slopes, $\partial\Delta_N^D H/\partial T_t$, are very close to the calorimetrically measured heat capacity increments of the two transitions: 2.9 ± 0.3 and 3.1 ± 0.3 kJ·K^{-1}·mol^{-1} for the first and second transition, respectively. The sum of these slopes is close to the calorimetrically measured heat capacity increment for denaturation of the mutant: $\Delta_N^D C_p$ = 6.4 ± 0.7 kJ·K^{-1}·mol^{-1}. The correspondence of these values is a strong argument for the reliability of the deconvolution analysis (Privalov & Potekhin, 1986).

The heat capacity changes for the two transitions are rather similar. However, the temperature of the second transition is more dependent on pH than that of the first one (see Figure 2). Because of that, at pH 3.0 both transitions occur at the same temperature, 32 °C. At this temperature, the enthalpy of the second transition should be significantly smaller than that of the first one. Therefore, the total denaturation enthalpy of the mutant is close to that of the wild-type repressor.

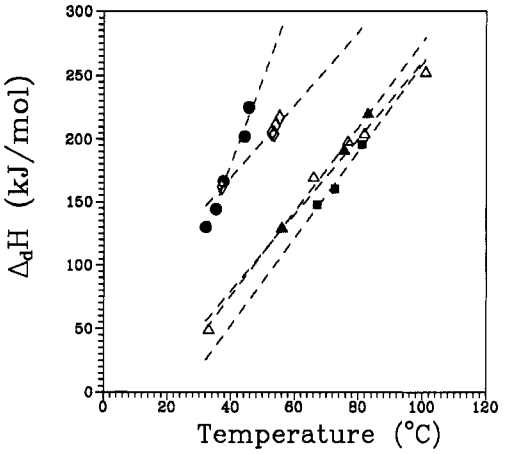

FIGURE 4: Temperature dependence of the transition enthalpy for two cooperative blocks in the [Cys^{55}]Cro repressor obtained from deconvolution analysis of the excess heat capacity function. (◊) First transition; (△) second transition. Filled symbols represent the teperature dependence of the calorimetric enthalpy of wild-type Cro repressor (●), CNBr fragment (▲), and tryptic fragment (■) of the [Cys^{55}]Cro repressor.

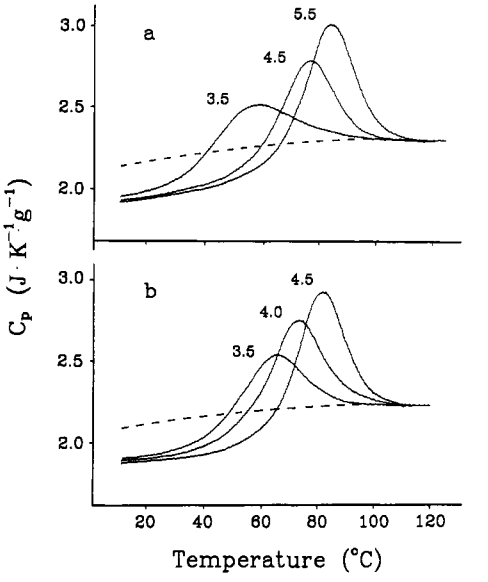

FIGURE 5: Temperature dependence of the partial heat capacity for the CNBr fragment (a) and the tryptic fragment (b) of the [Cys^{55}]-Cro repressor in solution at different pHs. The pH values are indicated on the curves. The dashed line shows the temperature dependence of the heat capacity for the CNBr fragment of the wild-type Cro repressor (a) and the (1–55) N-terminal fragment, pH 7.0, of [Cys^{55}-[Cro (b).

Notably, when the transition temperatures become identical, denaturation of the [Cys^{55}]Cro dimer proceeds as a two-state transition. The total denaturation heat capacity increment of the mutant is very close to the denaturation heat capacity increment of the wild-type Cro repressor, 6.4 ± 0.7 kJ·K^{-1}·mol^{-1}. This shows that the structure that melts in the second transition of the mutant also exists in the wild-type Cro repressor. The original assignment of the observed transitions to structural elements of the protein was further substantiated by calorimetric studies of isolated fragments of the Cro repressor.

Figure 5a presents melting curves of the (13–66) CNBr fragment of the mutant at different pH conditions. Removal of 12 amino acid residues from the N-terminal end of the polypeptide chain leads to the disappearance of the low-temperature transition on the melting curve (compare Figures 5a and 1). This result shows that the N-terminal part of the polypeptide chain is involved in formation of a structural domain and that its melting is associated with the first heat absorption peak. Removal of the N-terminal part does not significantly influence the stability of the C-terminal domain.

Calorimetric experiments on the (13–66)CNBr fragment of the wild-type repressor show (Figure 5a) that it does not form any stable cooperative structure in solution and that its heat capacity function is very similar to that of the completely unfolded polypeptide chain (Privalov et al., 1989). Thus, in the wild-type repressor, in contrast to the S–S-cross-linked mutant, the C-terminal domain is unstable without N-terminal parts of the chains.

In Table II are presented the calorimetric enthalpies determined from the area of the observed heat absorption peaks and van't Hoff enthalpies estimated from the sharpness of each heat absorption peak. The latter were determined assuming that the heat absorption peaks represent two-state transitions. It appears that the calorimetric and van't Hoff enthalpies are in rather good agreement, implying that heat denaturation of the fragment is approximated closely by a two-state transition.

The partial specific heat capacity of the CNBr fragment of the [Cys^{55}]Cro repressor at 20 °C is slightly higher than that of the native protein. This difference might be caused by partial unfolding of the N-terminal domain. We tried to specify the fragment containing the compact part (domain) using limited proteolysis.

Figure 6 shows the kinetics of tryptic proteolysis of the CNBr fragment. The relative mobilities of the products indicate that the molecular weight of the protein changes in a discrete way: in the early stage of the proteolytic reaction, there are two intermediate stable forms. The proteolytic fragment obtained after 20-min hydrolysis was stable to trypsin action for at least 1 h (Gitelson et al., 1991). N-Terminal sequence analysis of this fragment has shown that the first residue is an Asp.

Figure 5b presents the temperature dependence of the partial heat capacity of the tryptic fragment of the Cro repressor. The heat denaturation of the tryptic fragment displays one heat absorption peak, similar to that observed for the CNBr fragment. The transition temperature of this fragment under identical solution conditions is, however, slightly higher than that of the CNBr fragment (75 and 83 °C, respectively). Thus, the removal of the unstructured N-terminal part of the polypeptide chain leads to the stabilization of the remaining structure of the Cro repressor; i.e., there is negative cooperation between the folded central part of the dimeric molecule and the unfolded N-terminal parts of the polypeptide chains. On the other hand, as we showed above, the folded N-terminal parts cooperate positively with the central part.

The enthalpy values of both fragments are given in Table II. Comparison of the thermodynamic parameters of the transitions in the native protein and the tryptic fragment shows that the transition of the fragment is similar to the second transition of the native protein (see Tables I and II). The slope of the denaturation enthalpy function, $\partial\Delta_N^D H/\partial T_t$, for the tryptic fragment is close to that of the second transition in the native protein and to the experimentally measured heat capacity increment, $\Delta_N^D C_p$ = 2.8 ± 0.3 kJ·K^{-1}·mol^{-1}. This means that the dimer of the C-terminal fragment forms a hydrophobic core that is isolated from the core of the N-terminal domain.

Cutting the polypeptide chain at Cys^{55} according to Jacobson (1973), we obtained fragment 1–55, the residues of which constitute the N-terminal domain. The temperature depen-

Table II: Thermodynamic Characteristics of Heat Denaturation for the λ Cro Repressor and Its Fragments[a]

	λ Cro WT at pH				λ [Cys[55]]Cro at pH					CNBr fragment at pH			tryptic fragment at pH		
	5.18	5.48	6.00	7.00	3.0	4.0	4.5	5.0	7.0	3.5	4.5	5.5	3.5	4.0	4.5
T_m	35.5	38.0	44.0	46.2						56.8	75.4	82.7	67.4	73.3	81.1
ΔH_{cal}	146	163	201	221	199	349	383	408	458	129	178	220	148	162	195
ΔH_{vh}	156	170	213	219						108	174	212	124	150	183
ΔC_p	6.31	6.74	6.92	5.70	6.51	6.72	6.23	6.35		1.98	2.21	2.31	2.62	3.11	2.80
R	0.93	0.95	0.94	1.01						1.18	1.02	1.04	1.20	1.09	1.06

[a] T_d in degrees centigrade. ΔH_{cal} and ΔH_{vh} in kilojoules per mole. ΔC_p in kilojoules per mole per degree; $R = \Delta H_{cal}/\Delta H_{vh}$. Errors of ΔH_{cal} and ΔC_p determinations are 5% and 8%, respectively.

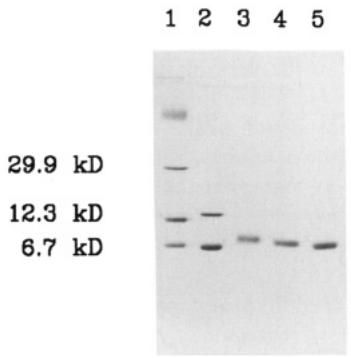

FIGURE 6: Kinetics of limited trypsin degradation of the CNBr fragment of the [Cys[55]]Cro repressor represented on the gel electrophoretic pattern by the mobility of the fragments obtained at different reaction times. Lanes of the 15–30% gradient Tris/glycine gel are the following: (1) markers—carboanhydrase B, molecular mass 29.9 kDa, cytochrome *c*, molecular mass 12.3 kDa, and aprotenin, molecular mass 6.7 kDa; (2) wild-type Cro repressor and [Cys]Cro mutant; (3) CNBr fragment of [Cys]Cro with trypsin, 100:1, at 10-min digestion; (4) 20-min digestion, and (5) 60-min digestion.

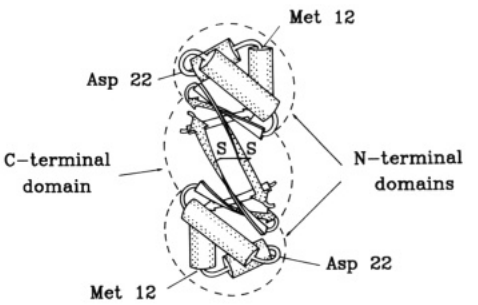

FIGURE 7: Schematic representation of the structural organization of the S–S-cross-linked dimer of the λ Cro repressor based on the X-ray structure of the λ Cro repressor (Anderson et al., 1981). The marked amino acids show sites of polypeptide chain hydrolysis at fragmentation.

dence of the partial heat capacity of this fragment under conditions of maximal stability is given in Figure 5b by the dashed line. In contrast to the native protein, the fragment does not show any heat absorption peak. Its heat capacity function is similar to that of a completely unfolded protein (Privalov et al., 1989). This means that the 1–55 fragment does not form a stable cooperative structure in solution under the considered conditions.

Thus, it follows that the stability of the N-terminal domain of the [Cys[55]]Cro repressor depends on the presence of the C-terminal domain. It is remarkable that the C-terminal domain influences the stability of the N-terminal domain even under conditions when its enthalpy of denaturation should be zero. The temperature dependencies of the transitions of the native [Cys[55]]Cro and of the C-terminal domain (Figure 4) indicate that at 25 °C the enthalpy of the C-terminal domain transition is zero. Therefore, the structure of the C-terminal domain is stabilized only entropically.

The sensitivity of the Cro repressor to degradation of the C-terminal segment has been shown by studying a deletion mutant (Hubbard et al., 1990). The removal of only five to six amino acid residues from the C-terminal domain of the wild-type Cro repressor results in complete disruption of the protein structure. This shows that the N-terminal domain can be stable only if the β-strand of the C-domain, which are involved in the formation of contacts between the monomers, are complete. The importance of these contacts was recently demonstrated by constructing a stable monomeric protein in which the β-strand has been substituted by a β-hairpin (Mossing & Sauer, 1990).

The main conclusion of the present study is that the structure of the dimeric molecule of the [Cys[55]]Cro repressor is organized into two cooperative blocks. One of these blocks, the central domain, is formed by the two C-terminal parts of both chains. The other one is formed by the N-terminal parts of the two polypeptide chains. One can assume that the wild-type Cro repressor has a similar molecular organization, but in that case, the stability of the C-terminal domain is significantly lower, and it melts simultaneously with the N-terminal parts.

As said, the N-terminal parts of the polypeptide chain of the Cro repressor do not form any cooperative structure when isolated. They form it only in the dimer. However, it is most surprising that in the dimer these two N-terminal parts form a single cooperative block which melts upon heating in an all-or-none way. This is clear from the perfect correspondence of the van't Hoff and calorimetric enthalpies of melting of a structure formed by these parts of polypeptide chains.

One could suppose that the N-terminal parts of polypeptide chains of the Cro repressor can fold individually into copact domains which are intrinsically unstable but are mutually stabilized in the dimer. However, this assumes strong positive interaction between these domains, as they are forming together a single cooperative block. The question is the following: How do these two peripheral domains, which, according to crystallographic information, are located rather far apart from each other (Figure 7), interact? This cannot be explained by assuming that we deal not with a dimeric but with a tetrameric molecule, in which N-terminal parts could be in contact. According to our equilibrium ultracentrifugation studies at 25 °C, the molecular mass of the λ Cro repressor is 17.2 kDa, which is close to that of the dimeric form (14.7 kDa). According to a sedimentation velocity analysis at this temperature, the sedimentation constant *s* is 1.24 S, which, for a compact globular protein, corresponds to a molecular mass of 15–17 kDa. Centrifugation in 2 M urea solution did not lead to any noticeable change in the molecular mass. Gel-filtration studies in the temperature range 25–50 °C showed no significant increase of molecular weight with increasing temperature, indicating that tetramerization does not occur upon heating.

This raises the question whether the λ Cro repressor dimer has the same structure in aqueous solution as in the crystal.

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