Two-stage thermal unfolding of [Cys55]-substituted Cro repressor of bacteriophage λ

G.I. Gitelson[1], Yu.V. Griko[1], A.V. Kurochkin[3], V.V. Rogov[1], V.P. Kutyshenko[2],
M.P. Kirpichnikov[3] and P.L. Privalov[1]

[1]Institute of Protein Research and [2]Institute of Biological Physics, Academy of Sciences of the USSR, Pushchino, Moscow Region
142292 and [3]V.A. Engelhardt Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow 117984, USSR

It has been shown by scanning calorimetry and ^{1}H NMR spectroscopy that thermal denaturation of mutant λ phage *cro* repressor in which Val55 was substituted for Cys, proceeds in 2 stages in contrast to the wild type protein. At neutral pH values, an additional cooperative transition has been observed at about 100°C. Calorimetric measurements on the mutant and its tryptic fragment lead to the conclusion that the two-stage character of thermal unfolding of the mutant is due to a disruption of an additional cooperative domain in the dimer molecule which is stabilized by the S–S crosslink.

1. INTRODUCTION

Bacteriophage λ *cro* repressor or Cro, a small globular protein consisting of 66 amino acid residues, is one of the best studied components of the λ phage transcription regulation system [1]. Its three-dimensional structure is known from X-ray crystallography [2] and is consistent with that of circular dichroism and NMR studies [3–8]. Cro forms sufficiently stable dimers by means of noncovalent interactions between the C-terminal parts of monomers. These dimers have a two-fold axis of symmetry passing between Val55 residues located one opposite the other in the antiparallel β-structure formed by the C-terminal part of the polypeptide chain [2].

According to circular dichroism data, thermal denaturation of Cro in solutions with neutral pH proceeds in one stage, simultaneously with the dissociation of the dimer at 44°C [3,9,10].

By means of protein engineering, a number of Cro mutants with various stabilities were obtained [10,11]. By substitution of the Val55 codon for Cys in the λ *cro* gene mutant [Cys55]Cro with two neighboring cystein residues in the dimer was obtained. The distance between the Cys residues enables the formation of the S–S bond between them upon oxidation [12].

In this paper we show that this S–S crosslink leads to the formation of a stable cooperative block of two C-terminal parts of the molecule in the dimer. As a result,

Correspondence address: P.L. Privalov, Institute of Protein Research, Academy of Sciences of the USSR, Pushchino, Moscow Region, USSR.

the temperature-induced denaturation of [Cys55]Cro with the crosslinked cystein proceeds in two stages in contrast to the wild-type protein.

2. MATERIALS AND METHODS

Cro and [Cys55]Cro were isolated as described in [13] with minor variations. *Escherichia coli* W3110lacIqL8 strain [14] was transformed by pJS306 plasmid (kindly provided by M.H. Caruthers, Colorado University, Boulder, USA) containing the synthetic λ *cro* gene, with an appropriate substitution in the case of [Cys55]Cro, under *tac* promoter control. Cells were grown up to a mid log phase, induced by addition of lactose to 50 mM and incubated for 3 h at 30°C. After disruption of cells by the French press and centrifugation, the super-
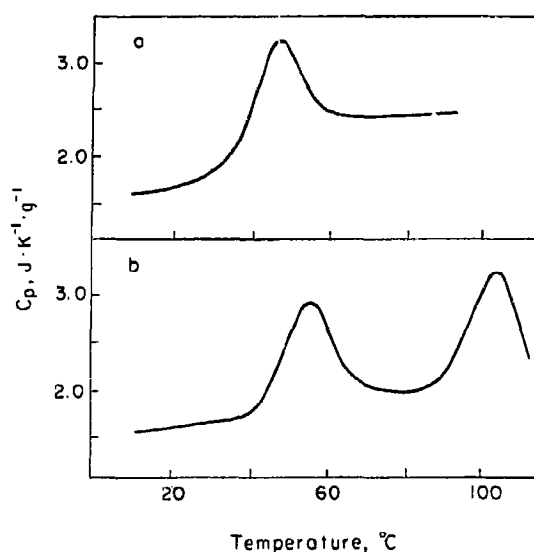

Fig. 1. Temperature dependence of the partial heat capacity of λ Cro-repressor (a) and [Cys55]Cro (b) in solution at pH 7.0.

natant was run through an anion exchange column. The purification procedure included consecutive chromatography on CM-Sephadex A-25, gel filtration through Sephacryl S-200 and cation exchange chromatography on CM-Trisacryl. Homogeneity of the proteins was checked by electrophoresis in the acidic urea system [15] and by HPLC.

Tryptic hydrolysis of λ Cro-repressor was carried out in solution of 50 mM Tris-HCl at 20°C with a substrate-to-enzyme molar ratio of

100:1. The hydrolysate was purified on the CM-Trisacryl column as indicated above. The peptides were identified by their N-terminal sequences, determined according to Chang et al. [16]. The protein concentration was measured using the Cro extinction coefficient, $A_{276}^{1\%} = 5.85$ [4], both for Cro and [Cys^{55}]Cro.

Calorimetric measurements were done on a DASM-4 scanning microcalorimeter (Bureau of Biological Instrumentation, Academy of Sciences of the USSR) at heating rates of 1.0 K/min using buffered
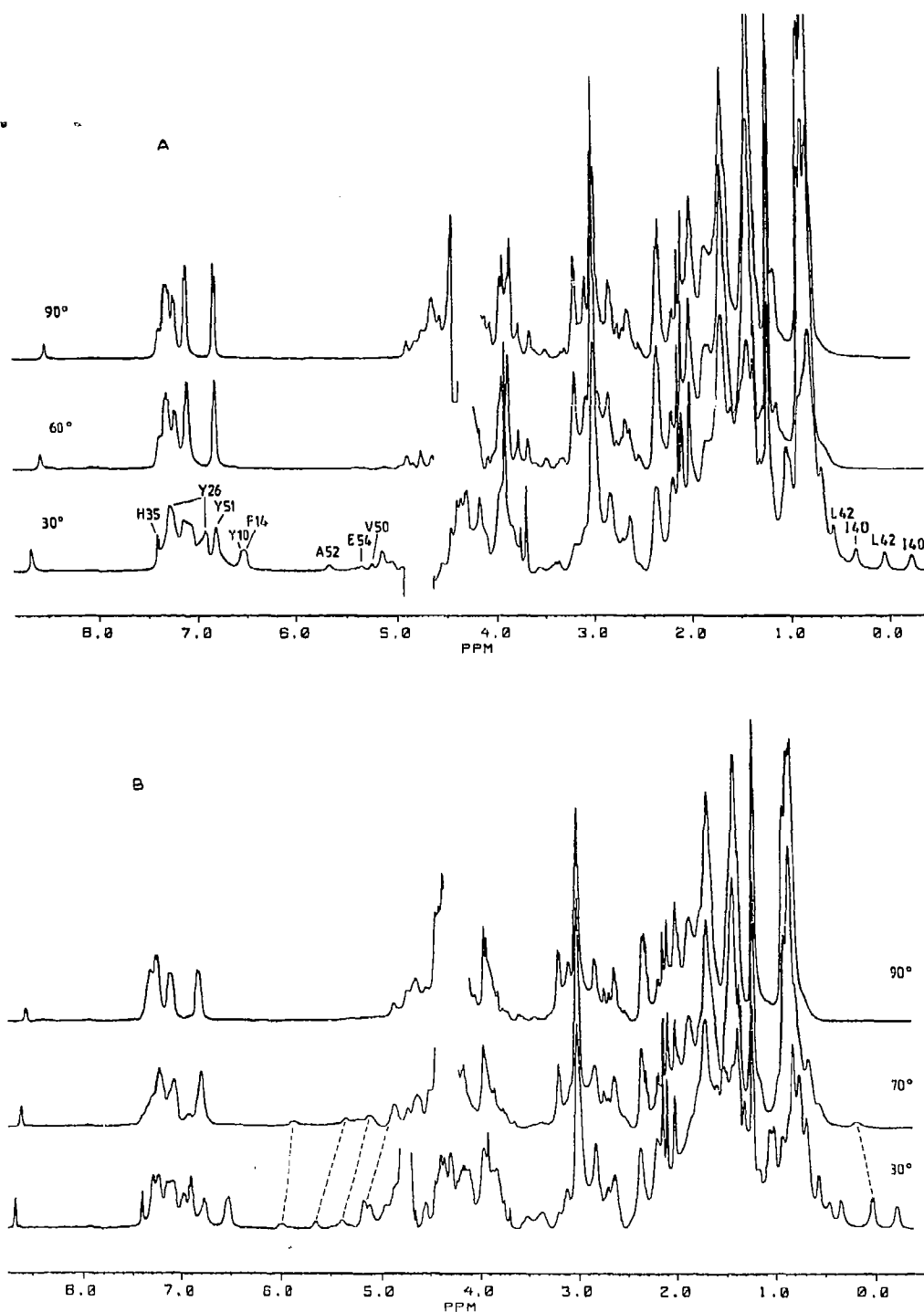

Fig. 2. ^{1}H NMR spectra at 400 MHz of λ Cro-repressor (A) and [Cys^{55}]Cro (B) at different temperatures. Protein concentration 1–1.5 mM, pH 4.7. Resonance assignment for Cro is given according to [5–8]. Dashed lines show the movement of some resonances with temperature.

solutions with a protein concentration of about 1.5 mg/ml in buffers containing 20 mM K-phosphate at neutral pH and 20 mM K-acetate at acidic pH. The partial heat capacity and calorimetric enthalpies for denaturation transition were obtained as in [17] using the calculated value 0.730 ml/g for the specific partial volume and 14.7 kDa for the molecular weight of the dimer.

For NMR studies, protein was dissolved at a concentration of 7–10 mg/ml in 99.9% D_2O with 200 mM $NaCD_3COO$, 0.1 mM EDTA, pH 4.7, without correction for the isotope substitution effect. ^1H NMR spectra were recorded at 400 MHz by a Brucker WM-400 spectrometer equipped with an Aspect 2000 data system using standard 5-mm probes. The temperature of the samples in the range of 21–92°C was controlled by a B-VT 1000 temperature controller with an accuracy of 1°C. Chemical shifts are presented relative to the sodium 4,4-dimethyl-4-silapenthane-1-sulfonate (DSS) methyl resonance.

3. RESULTS AND DISCUSSION

Fig. 1 represents the temperature dependence of partial heat capacities of λ Cro-repressor and [Cys^{55}]Cro mutant in neutral solution. The thermal denaturation of the wild-type λ Cro-repressor is characterized by one heat absorbtion peak with the melting temperature T_m = 46.4°C and calorimetric enthalpy of the process ΔH^{cal} = 225 kJ/mol of the dimer. In contrast to the wild-type λ Cro-repressor the melting curve for [Cys^{55}]Cro has two strongly pronounced peaks with T_m^1 = 54.5°C and T_m^2 = 102°C and calorimetric enthalpies ΔH^{cal} = 220 kJ/mol and ΔH^{cal} = 250 kJ/mol of the dimer, respectively. Both peaks are almost completely reproducible upon reheating of the same sample. The magnitude of these effects shows that each stage corresponds to some dramatic change in the protein structure.

NMR spectra of λ Cro-repressor and [Cys^{55}]Cro at 20°C and pH 4.7 display great similarity (Fig. 2): In the range from −0.3 to 0.6 ppm, all four resolved ring current shifted methyl resonances of Cro assigned to Ile^{40} and Leu^{42} are present in the [Cys^{55}]Cro spectrum too. Aromatic regions of the spectra (6.4–7.5 ppm) are al-

most identical. They only slightly differ in Tyr^{10} C$^\varepsilon$H and Phe^{14} C$^\delta$H signals at 6.52 ppm which are partially resolved in the Cro spectrum and are almost merged in [Cys^{55}]Cro. In the area of C$^\alpha$H signals from the residues involved in β-structure (5–6 ppm), the signals of Ala^{52} and Glu^{54} are also observed in the [Cys^{55}]Cro spectrum. At 5.25 ppm the well-resolved Val^{50} signal in the Cro spectrum is absent in the spectrum of the mutant and may merge either with Glu^{54} at 5.38 ppm or with Arg^4, Phe^{41}, Leu^{42}, Glu^{53} and Lys^{56} signals into the unresolved multiplet centered at 5.14 ppm.

Secondary chemical shifts are very sensitive to the micro-environment of individual groups and therefore the observed similarity of the Cro and [Cys^{55}]Cro spectra indicate unequivocally the similarity of their three-dimensional structures. Slight differences in the mutant structure from that of Cro can be expected in the region of Cys^{55} and of its neighboring residues.

When the temperature increases, the changes in the Cro and [Cys^{55}]Cro spectra are different. As in the case of calorimetric studies, Cro spectra demonstrate melting of the protein at lower temperatures than [Cys^{55}]Cro, and secondary chemical shifts reflecting the presence of the secondary and/or tertiary structure completely disappear at 64°C. In the [Cys^{55}]Cro spectrum no shifted signals of Ile^{40} methyls, of Tyr^{10} and Phe^{14} aromatic proton at 6.52 ppm are observed at 55–60°C. Some C$^\alpha$H signals are observed at 5–6 ppm over 80°C which evidences of the maintenance of some part of the initial β-structure. Even at 82°C the shifted signal formed by the Leu^{42} methyl group is observed at about 0.3 ppm (0.18 ppm at 70°C, see Fig. 2B).

Upon the temperature increasing up to 92°C, the [Cys^{55}]Cro spectrum inverts to a spectrum of an equivalent mixture of amino acids. This transformation oc-
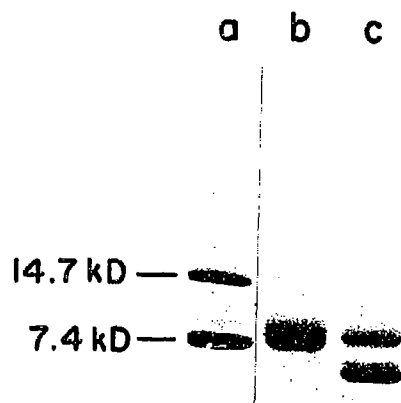

a   b   c

14.7 kD —

7.4 kD —

Fig. 3. Sodium dodecyl sulphate/polyacrylamide gel electrophoretic patterns of the wild-type λ Cro-repressor (7.4 kDa), [Cys^{55}]Cro (14.7 kDa) (a); trypsin digest of the non-reduced (b) and reduced (c) [Cys^{55}]Cro.
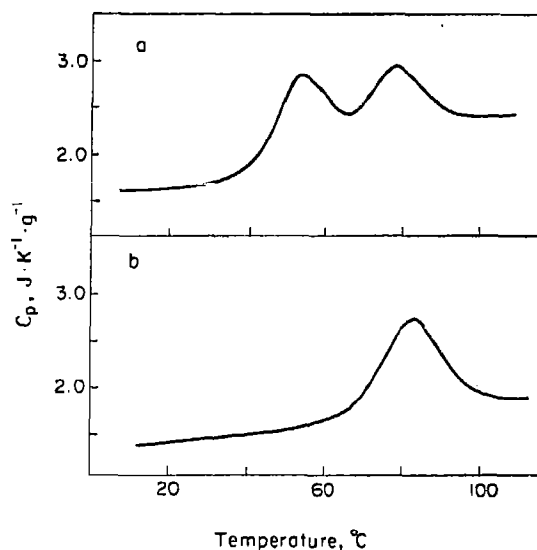

Temperature, °C

Fig. 4. Temperature dependence of the partial heat capacity of [Cys^{55}]Cro (a) and its C-terminal tryptic fragment (b) in solution at pH 4.5.

curs with the Cro spectrum at significantly lower temperatures.

The absence of new signals in the NMR spectra with unique chemical shifts typical for a new structure formation leads to the conclusion that the two-stage character of [Cys^{55}]Cro melting cannot be explained by the sequential transformation of the protein structure as a whole.

It is likely that the 2 heat absorption peaks correspond to the melting of different parts in the molecule. It looks as if the first heat absorption peak corresponds to the disruption of the structure formed by α-helices in the N-terminal part of the polypeptide chain, while the second high temperature peak represents melting of some structural block which is formed by the C-terminal parts of 2 polypeptide chains. If so, this stable cooperative block can be isolated by an appropriate cutting of the polypeptide chains.

The fragment of the [Cys^{55}]Cro (m.w. ≈ 9 kDa), has been obtained by limited tryptic hydrolysis of [Cys^{55}]Cro (Fig. 3). An addition of β-mercaptoethanol to the fragment leads to a decrease of the molecular weight (see Fig. 3c). This means that it is represented by C-terminal parts of the polypeptide chains of [Cys^{55}]Cro containing the S–S crosslink.

Fig. 4 represents the temperature dependence of the partial heat capacity of [Cys^{55}]Cro and of its proteolytic fragment. As seen, in contrast to the intact protein, the fragment under the same environmental conditions melts with one heat absorption peak with T_m close to T_m^2 of the second transition in [Cys^{55}]Cro. This indicates clearly that the structure formed by the covalently linked-together C-terminal segments of 2 monomers represents a single co-operative block.

The results presented allow us to conclude that the two-stage thermal unfolding specific for [Cys^{55}]Cro, in contrast to the wild-type λ Cro-repressor is due to stabilization of the block formed by C-terminal β-structural parts of the λ Cro-repressor by the S–S bond.

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