Studies of the structure of bacteriophage λ *cro* protein in solution

Globular structure of *cro* protein

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1. INTRODUCTION

cro Protein, or *cro*, is a repressor encoded by bacteriophage λ which specifically interacts with operator DNA and is required for lytic growth of the phage [1]. The small size of *cro* (M_r 7351) makes it a suitable model for studying specific DNA–protein interactions. A knowledge of the three-dimensional structure of the protein would help to gain a better insight into the character of such interaction.

The crystal structure of *cro* was studied in [2]. The secondary structure of *cro* and cI repressors of λ and 434 phages was predicted theoretically, and a model was proposed for the spatial organization of these repressors [3]. We have been investigating the structure of *cro* in solution. In [4], we reported resonance assignment in the aromatic region of ^{1}H NMR spectrum to particular types of amino acid residues. Here, based on data from NMR and UV spectroscopy, we show that *cro* in solution has a certain three-dimensional structure with different environments and accessibilities of amino acid residues. This also presents an assignment of resonances in the ^{1}H NMR spectrum of *cro*.

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Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; UV spectroscopy, ultraviolet spectroscopy; FID, free induction decay; NOE, nuclear Overhauser enhancement; EDTA, ethylendiaminetetraacetate

2. MATERIALS AND METHODS

cro was isolated from the overproducing *Escherichia coli* strain [5] using the procedure in [4].

^{1}H NMR spectra were recorded on a Bruker WH-360 spectrometer with a working frequency of 360 MHz. The temperature of the sample was 26°C. For improvement of the spectral resolution, the FID was subjected to convolutional [6] or Gaussian [7] transformation. The NOE was recorded as difference spectra [8]. The irradiation time for a particular resonance varied from 0.5–1 s. The values of chemical shifts are given relative to the resonance of sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate (TSP) with the necessary correction [9].

The pH was adjusted by adding 0.1 N ^{2}HCl or NaO^{2}H and measured with an Orion Research 601 A pH-meter using an Ingold combined glass electrodes.

UV-spectra were recorded with a Cary-118 (Varian) spectrophotometer in 1 cm cuvettes at 0.17 mM protein.

The molar extinction of *cro* ($\epsilon_{276} = 4330$) was determined with a Perkin-Elmer 240 CHN-analyzer.

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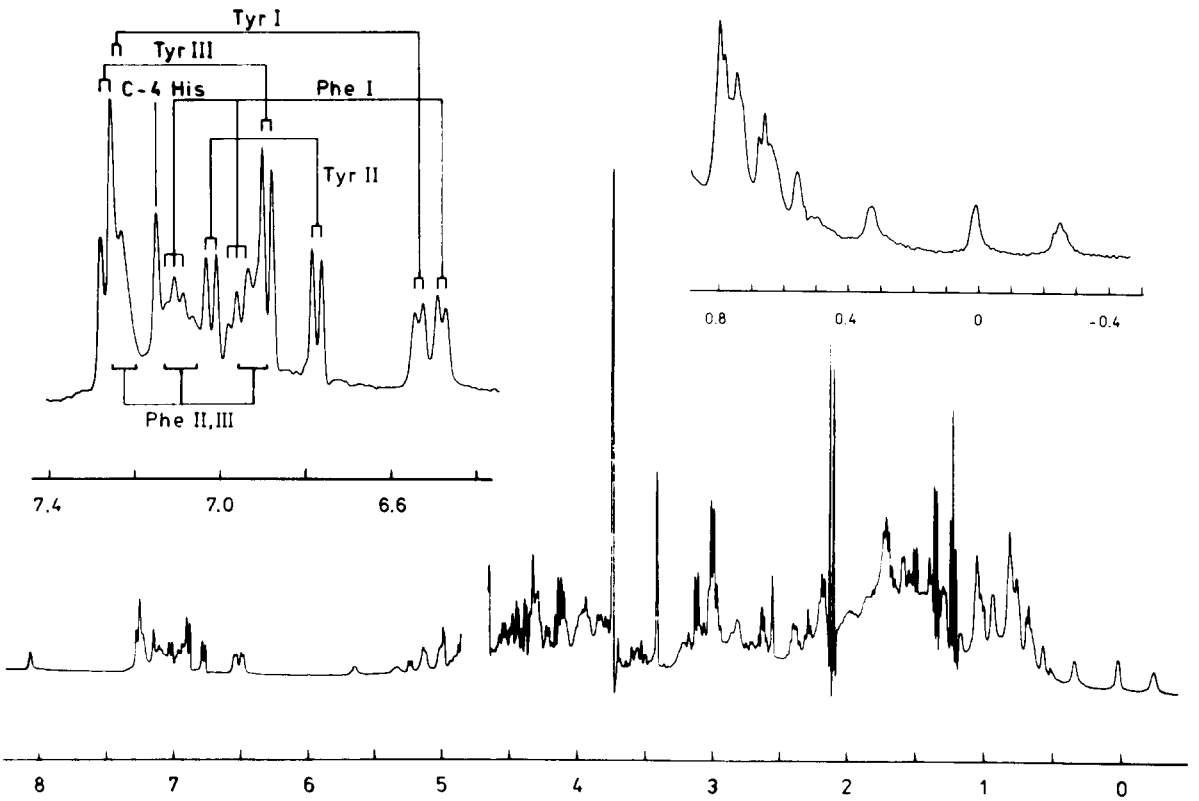

Fig. 1. ^1H NMR spectrum (360 MHz) of *cro* in ^2H_2O, 2 mM EDTA, pH 7.0 at 26°C: (a) the complete spectrum of the protein; (b) the aromatic region of the spectrum; (c) ring current-shifted methyl resonances in the aliphatic region of the spectrum.

3. RESULTS AND DISCUSSION

The ^1H NMR spectrum of *cro* with the resonance assignment in the aromatic region according to the type of amino acid residues [4] is shown in fig. 1. Some resonances of methyl groups in the side chains of aliphatic amino acid residues are shifted upfield in the spectrum of *cro* (from −0.25 to 0.7 ppm). Certain signals of $C^{\alpha}H$ groups have anomalous downfield shifts (to 5.7 ppm). This finding as well as a broad range of chemical shifts of the signals of aromatic protons are indicative of a complex globular structure of the protein. Heat denaturation makes the signals with anomalous chemical shifts either disappear or shift to positions typical of the ^1H NMR spectrum for a random-coil polypeptide chain in aqueous solution [10].

In the aliphatic region of the spectrum, the singlets with chemical shifts of 2.10 and 2.13 ppm most probably belong to the methyl protons of Met-1 and Met-12. The resonance at 2.10 ppm is narrower in spectra obtained without apodization (not shown). Therefore, assuming that the N-terminal amino acid is more mobile, we have assigned this resonance to Met-1. The assignment is supported by a change in the chemical shift of the second singlet belonging to Met-12 on denaturation, as a result of which it merges with the first one.

The titratable, at acid pH, doublet with a chemical shift of 1.35 ppm and a coupling constant of 7 Hz connected with the quartet at 4.13 ppm by spin-spin interaction was assigned by us to the $C^{\beta}H_3$ group of the C-terminal alanine (Ala-66).

Ring current-shifted methyl resonances correspond to three-proton signals in their intensity but have different widths (fig. 1b). In apodization

giving a width of ~6.5 Hz at a half-height of the line, the signals at 0.01, 0.33 and 0.56 ppm have a width of ~11 Hz while the signal at −0.25 ppm has a width of ~17 Hz. Therefore, the former three signals can be doublets whereas the latter one can be a triplet and belong solely to the $C^{\delta}H_3$ group of an isoleucine residue.

We have noted the anomalous values of chemical shifts for aromatic protons [4]. Their change at neutral and alkaline pH is shown in fig. 2.

Analysis of the titration curves for His-35 C-2 and C-4 proton resonances gives a pK of 6.60 and 6.59 (±0.03), respectively, with a Hill coefficient of 0.9. However, the pseudo first-order rate constant of deuterium exchange for C-2H is $1.8 \times 10^{-6}\ s^{-1}$ at pH 8.5 and 30°C when *cro* retains its native conformation [10,11]. This indicates that His-35 is accessible to the solvent [12] and does not interact with other ionizable groups in the protein. It is very likely that, just as in the crystal [2], His-35 in solution is located on the surface of a protein globule.

The localization of the histidine with pK 6.6 on the protein surface is consistent with the interpretation of data obtained for RNase A [13] and *P.brevicompactum* RNase [14].

Out of six doublets assigned to Tyr-10, Tyr-26 and Tyr-51, only two (Tyr III) are titrated within a pH range where *cro* retains its secondary structure and a large part of the tertiary structure [11]. The titration of Tyr II signals coincides with the globule unfolding whereas Tyr I signals do not change their chemical shift when the pH rises until the three-dimensional structure of *cro* is destroyed.

The accessibility of tyrosine residues in the *cro* molecule to a solvent was assessed by spectrophotometric titration (fig. 3). The increase in the absorbance at 295 nm with pH was analyzed in a model of three independently titratable groups, and the following pK values were obtained: 9.66, 11.27 and 11.70 (±0.04). Comparison with the titration curves indicates that the hydroxyl of Tyr III has a pK of 9.66. Hence, Tyr III is accessible to a solvent although it has a specific microenvironment which strongly affects the chemical shifts of its signals [4]. Tyr I and Tyr II are located inside the *cro* globule and deprotonation of their hydroxyls is related to alkaline denaturation of the protein.

A tyrosine residue entirely accessible to a solvent in linear tetrapeptides has a pK of 10.3 [9]. The lower pK in Tyr III can stem from the vicinity of a positively charged amino group.

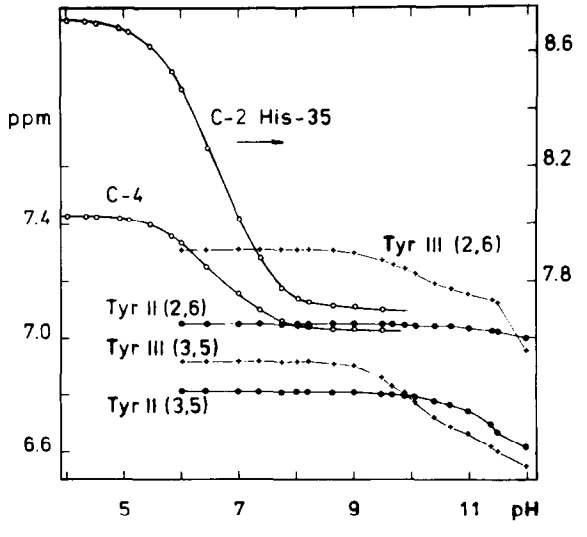

Fig. 2. Titration curves of *cro* aromatic protons in ^2H_2O, 0.2 M NaCl, 2 mM EDTA at 26°C (the right-hand scale of the ordinate is solely for His-35 C-2 protons).

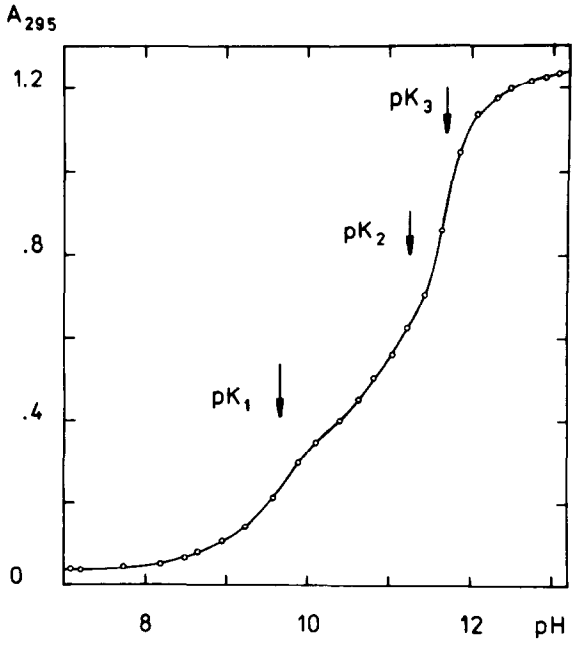

Fig. 3. Absorbance at 295 nm of a *cro* solution in H_2O, 0.2 M NaCl as a function of pH at 23°C.

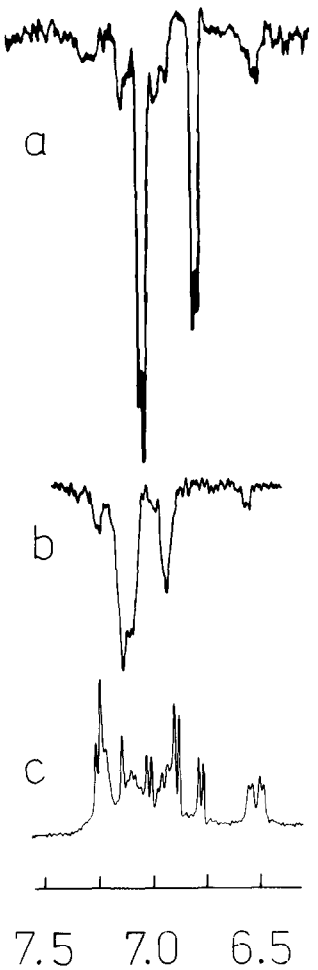

Fig. 4. NOE on *cro* aromatic protons (360 MHz): (a) convolution defference NOE spectrum with irradiation of Tyr II 2.6 protons at 7.02 ppm. The pre-irradiation time of the resonance is 1.0 s; (b) NOE in irradiation of the methyl resonance at −0.25 ppm. The pre-irradiation time of the resonance is 0.5 s, line broadening is 1 Hz; (c) the aromatic region of the *cro* spectrum.

As NOE measurements demonstrate, Tyr II and Phe I are the only aromatic amino acid residues interacting with one another (fig. 4a). When Tyr II 2.6 protons are irradiated, the NOE appears on aromatic protons assigned to Phe I. Assuming that the structure of *cro* in solution is close to a crystal one [2], Tyr-10 and Phe-14 located on one side of the neighbouring turns in the α-helical region Leu-7–Phe-14 can be such residues, and the distance between them must be ~5 Å.

The upfield shift of aliphatic methyl resonances is caused by their spatial proximity to aromatic cycles. For instance, when the $C^{\delta}H_3$-Ile methyl triplet at −0.25 ppm is irradiated, the NOE appears on Phe II and (to a lesser extent) Tyr I aromatic protons (fig. 4b).

The results are indicative of the unique spatial structure of *cro*. Comprehensive studies of the interactions between methyl and aromatic protons are currently underway.

Heat denaturation of *cro* is entirely reversible when the solution is heated to 60°C as demonstrated by NMR [10] and CD [11,15]. Such a readiness to restore the native three-dimensional structure at a low M_r and in the absence of disulfide bonds makes the *cro* repressor a suitable model for studying the processes of self-organization of protein molecules.

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