Photo CIDNP study on the complex formation of λ cro protein with O_R3

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A photo CIDNP spectrum of λ cro repressor protein showed that one of the three tyrosines and His 35 are quite accessible to the photosensitive dye. For the remaining two tyrosine residues one is slightly accessible, but the other is inaccessible. In comparison with the result of differential nitration at tyrosine side chains followed by the peptide analysis, it can be concluded that Tyr 26 is mostly exposed and Tyr 51 is slightly exposed on the surface of the cro dimer. On the addition of O_R3 17mer, His 35 and Tyr 26 are no longer accessible to the dye, which indicates that they are involved in interaction. However, a similar phenomenon was observed by adding CAP binding site 22mer. The interaction mechanism will be discussed.

Photo CIDNP *Cro protein* *Operator* *DNA-protein interaction* *Nitration* *NMR*

1. INTRODUCTION

Cro (tof) protein is a repressor protein which is responsible for the lytic growth of bacteriophage. It binds to right (O_R) and left operator (O_L) regions of phage genome competitively with cI repressor which is essential for lysogeny [1]. The crystal structure of λ cro protein was determined [2] and the complex of cro with the DNA fragment containing a part of O_R3 sequence, to which cro protein most strongly binds, has been obtained [3]. Combining the analysis of the crystal structure and the other data of genetics and chemical modification, a binding mechanism of cro protein with O_R3 was proposed [2,4]. In that model the helical region from residue Gln 27 to Ala 36 is thought to fit the major groove of the operator DNA in the B-form. However, there is no direct physicochemical evidence for the involvement of any specific amino acid residue of λ cro protein in the complex formation with O_R3. Here we employed photo CIDNP (chemically induced dynamic nuclear polarization) method [5] to clarify whether tyrosine and histidine residue of λ cro protein are involved in the O_R3 binding or not. In the proton resonance spectra of λ cro protein the signals due to three tyrosine residues were identified [6–8]. We could assign them to each specific tyrosine residue by differential nitration of tyrosines followed by peptide analysis [9]. Based on the assignments, the His 35 and Tyr 26 residues were shown to be involved in the interaction with O_R3.

2. MATERIALS AND METHODS

λ Cro protein was isolated from *E. coli* K-12 strain MH3 containing plasmid pKH 602 carrying the λ cro gene under control of *rec*A promotor. The plasmid pKH 602 was constructed by inserting the λ cro gene from pMY 30-2 into a *Bam*HI site of pKH 502 [10]. The *E. coli* cells containing the plasmid were grown to the log phase. Forty μg/ml nalidixic acid (an inducer of *rec*A promotor) was added to the media and the culture was incubated for 6 h. The cells were lysed by sonification followed by DEAE-cellulose column chromatog-

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raphy. Cro protein in the elute was purified by CM-Sephadex column chromatography (Pharmacia C-50) followed by Affigel Blue chromatography (Bio-rad). The yield was about 1 mg cro protein per g wet cell. The purity was more than 98% judged from SDS-acrylamide gel electrophoresis and HPLC.

Nitration of the tyrosyl residues in Cro was performed by adding tetranitromethane. Nitrated cro protein was digested by *Acromobacter* protease I at the lysine residue and separated by a reversed-phase column chromatography (Pharmacia PepRPC). A detail of the procedure is described in [9].

The synthesis of O_R3 17mer and CAP binding site 22mer of *E. coli lac* operator were described in [11]. The treatments of the DNA fragments before NMR measurements are the same to those mentioned in [12,13]. ^{1}H-photo CIDNP spectra were taken at 500 MHz on a JEOL GX-500s NMR spectrometer by using an especially designed probe with a 3-mm quartz rod to introduce a laser light. The cro protein was dissolved alone or with a DNA fragment into a D_2O solution with 20 mM phosphate buffer and some KCl at pD 7.0, to which 0.2 mM of a flavin dye (3-*N*-carboxylmethyllumiflavin) was added. The sample tube was irradiated in the probe for 0.1 s by 488 nm line (1w) from an NEC GLC-3300 argon ion laser prior to data acquisition. The irradiation time was tried to be as short as possible to avoid the effect of cross polarization. Alternating 'light' and 'dark' free induction decays were collected and subtraction yielded the photo CIDNP difference spectrum. A light–dark cycle took 40 s. All of the spectra were taken at 24°C.

3. RESULTS

The proton magnetic resonance spectrum of cro protein is shown in fig.1a. In the aromatic proton region the assignments of the C2 and C4 proton resonances from a single histidine residue at position 35 were established by pH titration [6]. The remaining resonances in the region are due to the three phenylalanine residues at positions 14, 41 and 58 and the three tyrosine residues at positions 10, 26 and 51. Although the pairs of 2,6 and 3,5 doublets of three tyrosines were identified by specific deuteration, decoupling and NOE ex-

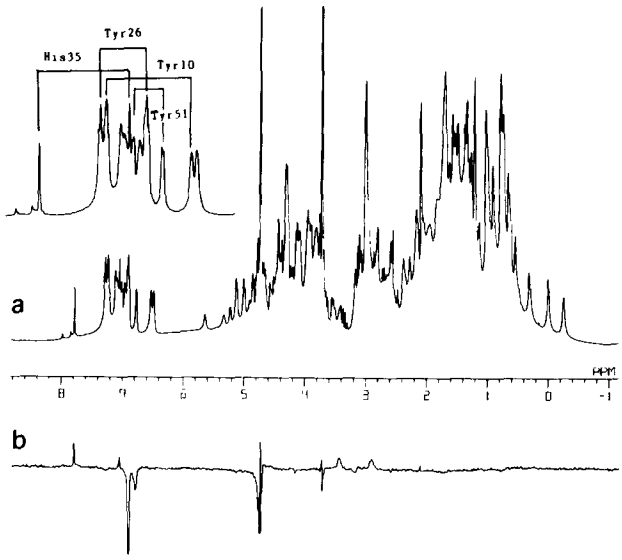

Fig.1. Photo CIDNP spectra of λ cro protein. (a) Dark spectrum and (b) photo CIDNP difference spectrum of 0.6 mM cro protein dimer in D_2O solution with 20 mM phosphate buffer and 0.3 M KCl at pD 7.0. 100 times accumulated.

periments [7,8], each pair has not yet been assigned to each specific tyrosine residue. We have assigned them by nitration followed by peptide analysis. On the mild treatment of the cro protein with tetranitromethane the intensity of the signal at 6.9 ppm decreased remarkably and that at 6.8 ppm lost intensity slightly. The doublet at 6.5 ppm did not change at all (fig.2a). The partially nitrated cro protein was digested by a protease at each lysine residue (except the Lys–Pro bond) and the resultant peptides were separated by reversed-phase column chromatography. About 70% of the peptide (residues 22–32) containing Tyr 26 has been nitrated and gave a new peak at a higher acetonitrile concentration region in a gradient elution pattern (fig.2b). The two long peptides (residues 40–62 and residues 40–63) containing Tyr 51 were nitrated by 30%, while the peptide (residues 9–18) containing Tyr 10 was almost inert to nitration. By comparison of the extent of the nitration and the decrease in intensity of the tyrosine resonances we could assign each tyrosine peak to specific tyrosine residue as shown in fig.1a [9].

In the photo CIDNP difference spectrum of cro protein itself strong absorption effects are ob-

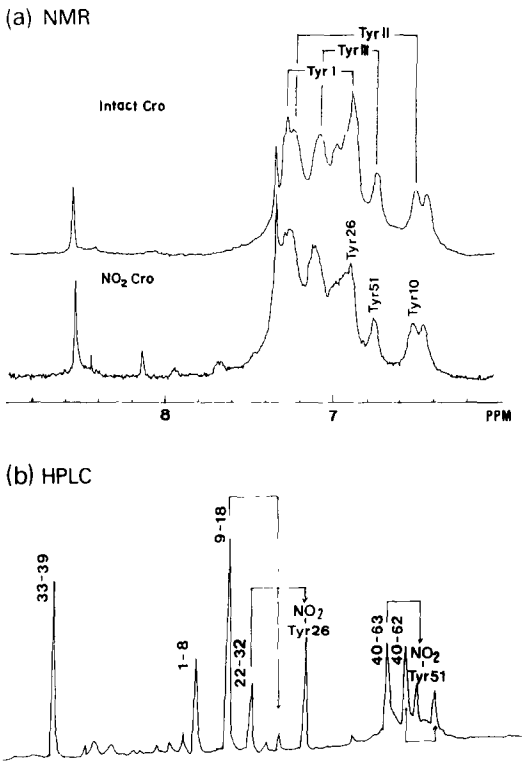

Fig.2. ^{1}H-NMR spectra and reversed-phase chromatography pattern of the partially nitrated cro protein. (a) 360 MHz proton NMR spectra of intact and partially nitrated cro protein (1.1 mM dimer concentration) in D_2O solution with 60 mM phosphate buffer and 0.3 M KCl at pD 6.0. (b) Reversed-phase column chromatography of the peptides obtained by digestion of the partially nitrated cro protein on a Pharmacia PepRPC column eluted with acetonitrile (0–45%)–water containing 0.1% TFA. The figures beside each peak indicate the residue number corresponding to each fragment.

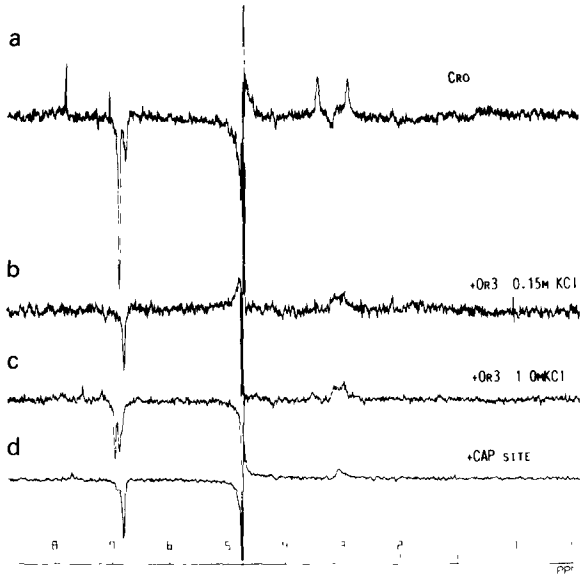

Fig.3. Photo CIDNP difference spectra of cro protein–DNA complexes. Each solution contains 0.28 mM cro protein dimer in 10 mM phosphate buffer at pD 7.0. (a) Cro protein itself in 0.15 M KCl solution, 16 times accumulated, (b) 1:1 cro/O_R3 in 0.15 M KCl, 16 times accumulated, (c) 1:1 cro/O_R3 in 1.0 M KCl, 100 times accumulated, and (d) 1:1 cro/CAP binding site 22mer in 0.15 M KCl solution, 400 times accumulated. Signal intensities are scaled relative to that of the methyl resonance at 2.0 ppm in the dark spectrum.

served for C2 and C4 proton signals of His 35 and remarkable emission effect on the 3,5 doublet of Tyr 26 (fig.1b). The 3,5 proton signal of Tyr 51 gives rise to a relatively weak but definite negative peak, although Tyr 10 does not at all. The absorption peaks at around 3 ppm are due to the C_β protons of the tyrosine residues. At such prolonged irradiation as for 0.5 s the 2,6 doublet of Tyr 26 at 7.3 ppm becomes polarized due to cross polarization. From the evidence, it can be said that His 35 and Tyr 26 are exposed on the surface of the cro dimer and Tyr 51 is slightly accessible to the dye. This result is quite consistent with the accessibility defined by the nitration experiment.

On the addition of the equimolar amount of O_R3 17mer (double strand O_R3 17mer/cro dimer = 1/1) we cannot see photo CIDNP signals for His 35 any more and only a weak negative enhancement is observed at 6.8 ppm (fig.3b). It can be readily concluded that the His 35 in the complex is completely shielded from the access of the dye. However, the origin of the negative peak at 6.8 ppm in fig.3b is not clear. From the signal position it is most probable that it arises from Tyr 51, though it slightly shifted downfield. There is another possibility that the Tyr 26 signal shifted upfield by the complex formation. When we compare the signal intensity in fig.3a,b taken under the same conditions, it is evident that the emission peak of Tyr 26 is significantly reduced in intensity. Therefore, without knowing the assignment of the 6.8 ppm

peak, it is safe to say that Tyr 26 is affected by the complex formation and remarkably shielded from the access of the flavin dye. With the increase of the salt concentration we can expect dissociation of the complex. As shown in fig.3c, the His 35 peaks and Tyr 26 peak appear at higher salt concentrations, although their peak positions are slightly different from those of cro itself.

To check whether the phenomenon is specific to the complex formation with O_R3, a deoxyoligonucleotide duplex of similar length was added to the cro protein solution. Here we used the 22mer duplex corresponding to the CAP binding site in *lac* promotor. As shown in fig.3d, its photo CIDNP spectrum is close to that in fig.3b, although a shoulder peak presumably due to Tyr 26 and a weak peak of His 35 are seen. It means that Tyr 26 and His 35 are also involved even in non-specific interactions, but the strength of binding seems a little weaker than with O_R3.

4. DISCUSSION

When we look at the crystal structure of the cro protein [2], His 35 lies in the helical region on the surface of the molecule. Tyr 26 locates at the junction of the two helical regions, α_2 from residues Gly 15 to Leu 23 and α_3 from Gln 27 to Ala 36 and its side chain seems also to be on the surface of the molecule. Therefore, the result of the present CIDNP experiment is quite consistent with the molecular structure of the cro dimer in solid. Tyr 10 and Tyr 51 locate, respectively, in a helix and β-sheet in the core of the dimer. From the crystal structure it is hard to anticipate whether there is a difference in the exposurability of both tyrosine residues, but the present result indicates that Tyr 51 is more accessible to the dye in solution.

Based on the crystal structure of λ cro protein, the interaction mechanism between the cro protein and O_R3 has been proposed [2,4]. The helical region α_3 from residue Gln 27 to Ala 36 is thought to fit the major groove of the B-form DNA and form-specific hydrogen bonds between some amino acid side chains and the parts of the base pairs exposed in the groove of O_R3. Another helical region α_2 and the C-terminal arm are assumed to contact the phosphate backbone. The interaction of the helical regions is thought to be common for DNA binding protein, since homology is noticed in the amino acid sequences of the repressors and cro proteins of several phages [14–16].

The present photo CIDNP experiment has shown that Tyr 26 and His 35 are involved in the complex formation with O_R3 and are shielded from the access by the dye, which gave validity to the above model. However, a similar phenomenon was observed for the non-specific interaction with the CAP binding site. The dissociation constant between the λ-DNA and cro protein was calculated to be $8–9 \times 10^{-9}$ M and that for the non-specific interaction with λ_{imm} DNA lacking the operator is smaller in the order of $10^{-3}–10^{-4}$ [17]. At such high concentrations as $10^{-3}–10^{-4}$ M used for NMR measurements most of the protein is in the complex form even in the non-specific manner. Also, the strength of the interaction depends on the condition and we could not find differences in the specific and non-specific interactions at lower temperatures [6]. In fact, one of the fluorotyrosine residues of the fluorinated cro protein was found to interact with calf thymus DNA [8], which implies that one of the tyrosine residues, probably Tyr 26, is used for the non-specific interaction with the DNA. A photo CIDNP experiment on the interaction of the N-terminal peptide of *lac* repressor with oligo-d(AT) also showed involvement of the tyrosine and histidine residues in the interaction [18]. The stabilization of non-specific interaction has been thought to come from mainly ionic interaction between the positively charged amino acid side chains and the ionized phosphate groups of DNA. However, experimental data hitherto obtained show us that shape complementarity with rough fitting also contributes much to the stability of sequence-independent interaction.

To get the picture of sequence-dependent interaction between O_R3 and cro protein in solution, more quantitative experiments are required. In the complex with CAP binding site DNA, small enhancements of His 35 signals are observed and in the photo CIDNP spectrum and their positions they are close to those of DNA free cro protein. On the other hand, in the photo CIDNP spectrum of the complex with O_R3, the His 35 signals are completely absent and even on the appearance at higher ionic strengths the position of the C2H of His 35 is a little higher than that of the free cro protein. Such small differences must be analysed

to discriminate between the sequence-dependent and -independent interaction.

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