Nuclear Magnetic Resonance Study on the Structure and Interaction of Cyclic AMP Receptor Protein and Its Mutants: A Deuterium-Labeling and Photo-CIDNP Study†

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ABSTRACT: The identification and assignment of the proton magnetic resonances of some aliphatic and aromatic amino acid residues of cyclic AMP receptor protein (CRP) are reported. The signals of the leucine and valine residues at around 0 ppm were identified on the basis of intermolecular nuclear Overhauser effects, deuterium labeling, and partial proteolytic digestion. On the addition of cAMP, methyl proton signals due to Val-49 and three leucine residues were detected as upfield-shifted signals at around −0.2 ppm. These signals can be used as indicators of the proper binding of cAMP because they are not observed on the addition of cGMP or 2′-deoxy-cAMP. They are also not observed on cAMP binding to mutant CRP*5 (Ser-62–Phe), which can only be activated by a high concentration of cAMP, but they are observed on cAMP binding to other mutant CRP*s (four species), which can be activated by lower concentrations of cAMP. The resonance of some aromatic protons, i.e., C-2H of two tryptophans, C-2H and C-4H of six histidines, and C-2,6H and C-3,5H of six tyrosine residues in CRP, were assigned by means of deuterium labeling and NOE measurements. The ^{1}H NMR spectrum of labeled CRP [Trp(ring-d_5), Phe(ring-d_5), and Tyr(3,5-d_2)] showed good resolution in the aromatic region. The addition of cAMP to this CRP in D_2O caused pronounced line broadening of resonances arising from the residues in the cAMP-binding domain, but the resonances of the DNA-binding domain were not affected. Several proton signals monitored in this study indicate that the contraction of the CRP molecule detected by small-angle X-ray scattering only occurs in the cAMP-binding domain and not in the DNA-binding domain. Photo-CIDNP experiments showed that His-199 is located on the surface of the CRP molecule and that access to this residue of the dye is completely prevented by the 22-mer DNA at the CRP-binding site. His-199 is located at the turn between two C-terminal β-strands. Therefore, it has become clear that the turn region between these two C-terminal β-strands is also involved in the interaction with promoter DNA.

Cyclic AMP receptor protein (CRP or CAP)[1] from *Escherichia coli* plays a key role in regulation of the expression of more than 20 genes of the bacterium. The binding of cAMP to CRP is accompanied by conformational changes and increases the affinity of CRP to specific DNA sequences near promoters, resulting in the enhancement of transcription (de Crombrugghe et al., 1984; Adhya & Garges, 1982).

There are two binding domains in the CRP monomer, one for the binding of the cAMP and the other for the binding of specific DNA sequences (Krakow & Pastan, 1973; Aiba & Krakow, 1981). The two domains of each CRP subunit are connected covalently by a stretch of polypeptides. The structure of CRP in complex with cAMP was revealed by an X-ray crystallographic study, at a resolution of 2.5 Å (Weber & Steitz, 1987) (Figure 1). The structure of CRP in the absence of cAMP and of the cAMP–CRP–DNA complex has not yet published.

By means of neutron scattering, Kumar et al. (1980) demonstrated that CRP undergoes a significant global structural change in the presence of cAMP. The radius of gyration is decreased, thus implying that CRP assumes a more symmetric or compact structure in the presence of cAMP. The neutron-scattering data are in good agreement with the result reported by Wu et al. (1974), who showed that cAMP induces a decrease in the rotational correlation time of CRP, as detected in fluorescence depolarization studies.

NMR has been used to investigate the structure of CRP and the effect of cAMP binding to the apoprotein (Clore & Gronenborn, 1982; Gronenborn & Clore, 1982; Lee et al., 1990). Due to its large molecular weight (dimer, 47 000), signal separation was worse and signal assignments were ambiguous. Here we employed deuterium labeling to simplify the spectrum and study the effect of cAMP binding on the structure of CRP.

Mutants of CRP may constitute a means of exploring the nature of the change in conformation of CRP caused by cAMP. We present evidence for allosteric conformational changes of CRP and mutant CRP*s induced by cAMP, as detected by ^{1}H NMR. We discuss the relationship of activity with the cAMP-induced conformational change of CRP.

CRP possesses a helix–turn–helix motif, which has been shown to be present in many bacterial DNA-binding proteins. It has been proposed that the helix–turn–helix motif of CRP interacts with DNA in such a way that amino acids located

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[1] Abbreviations: CRP, cyclic AMP receptor protein; NOE, nuclear Overhauser effect; CIDNP, chemically induced dynamic nuclear polarization; $NaDodSO_4$, sodium dodecyl sulfate; cAMP, adenosine 3′,5′-cyclic monophosphate.

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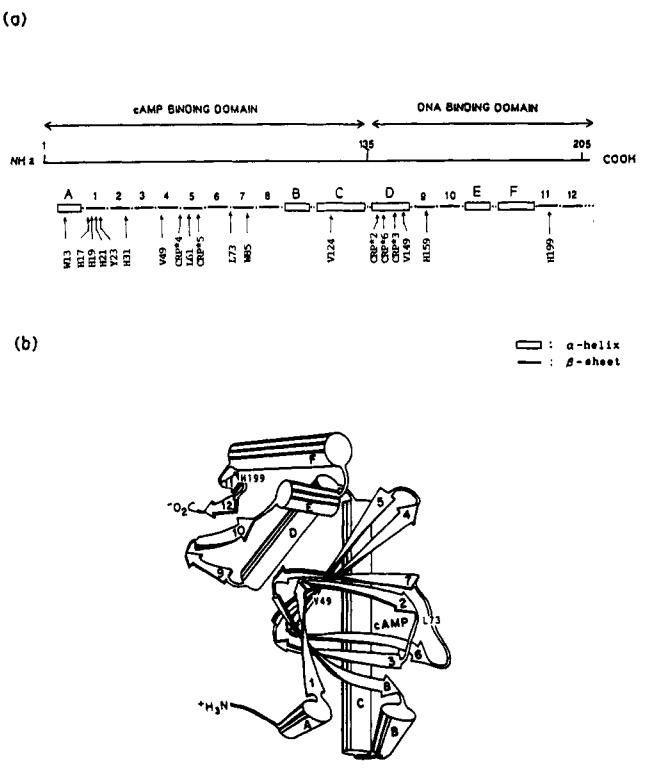

FIGURE 1: Structure of CRP. (a) Schematic representation of the primary and secondary structure of CRP. (b) Crystal structure of CRP determined by Weber and Steitz (1987).

in one of the helices come into contact with base pairs in the major groove of the DNA and thereby determine its specificity. This is helix F, which spans the region from Arg-180 to Lys-191. Recently it was shown that a mutation at position 195 (from Leu to Arg) confers a cAMP-independent function on CRP (Harman et al., 1988). Another mutant, at position 195 (from Leu to Pro), cannot ferment any sugars in either the presence or absence of cAMP, which indicates that this mutant is an inactive form of the protein (Harman et al., 1988). Position 195 is more carboxyl-proximal than recognition helix F of CRP.

We discuss the implication of the C-terminal residues of CRP in DNA binding on the basis of the CIDNP spectra. Almost no details of the interactions involved in the action of CRP at the molecular and atomic levels are known. Elucidation of the function of CRP depends on the establishment of a linkage among conformational changes, the binding of cAMP to CRP, and the interaction of CRP with DNA. This paper provides some information on this linkage.

Materials and Methods

Wild-type CRP and mutant CRP*s (Aiba et al., 1985) were purified from pp47 harboring pHA7 (Aiba et al., 1982) or its derivatives, respectively, by the method of Eilen et al. (1978). Their purity was greater than 99%, as judged on NaDodSO$_4$–polyacrylamide gel electrophoresis. αCRP, which lacks the carboxyl-terminal domain of CRP, was prepared by subtilisin digestion of the cAMP–CRP complex at position 116 (Eilen et al., 1978). The concentrations of CRP and αCRP were determined spectrophotometrically with $\epsilon_{278} = 4.1 \times 10^4$ M^{-1}·cm^{-1} and 2.6×10^4 M^{-1}·cm^{-1}, respectively, for the dimer (Takahashi et al., 1982).

The incorporation of deuterium-labeled leucine (methyl-d_3), valine (U-d_8), tryptophan (ring-d_5), tyrosine (ring-3,5-d_2), and phenylalanine (ring-d_5) into CRP was accomplished by culturing auxotrophic *E. coli* strains obtained from the *E. coli* Genetic Stock Center, National Genetic Institute. Cells were grown in 1 L of M9 medium containing 100 mg of Deuterium-labeled tryptophan, phenylalanine, and tyrosine. Deuterium-labeled leucine, valine, tryptophan, tyrosine, and phenylalanine were obtained from Cambridge Isotope Labs. cAMP and cGMP were obtained from Sigma.

The CRP-binding site 22-mer duplex in the *lac* gene of *E. coli* was synthesized by the phosphoro-*p*-anisidate method in the liquid phase (Lee et al., 1987). The sequence of the duplex is as follows.

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 21 22
5'-T A A T G T G A G T T A G C T C A C T C A A T-3'
3'-A T T A C A C T C A A T C G A G T G A G T T A-5'
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^{1}H NMR spectra were recorded on a JEOL GX 500 NMR spectrometer. For the NOE measurements, we employed a Bruker AM600 spectrometer at the Protein Engineering Research Institute. Samples for ^{1}H NMR in D_2O were prepared by freeze-drying. Sampling buffer contained 50 mM potassium phosphate and 0.2 M KCl. Chemical shift values are quoted in ppm downfield from the methyl proton resonance of 2,2′-dimethyl-2-silapentane-5-sulfonate and were measured relative to the internal standard dioxane. Only the values for the spectrum at pH 6.5 and 30 °C are quoted in order to avoid confusion due to the varying conditions. Spectra in H_2O were recorded by means of the 1−1 water suppression pulse technique (Kime & Moore, 1983; Clore et al., 1983). The nuclear Overhauser effect (NOE) was observed by means of interleaved difference spectroscopy with a presaturation pulse of 0.1–0.5 s and delays of 2 s between scans to permit relaxation of the system. CRP coordinates were obtained from the Brookhaven Protein Data Bank.

For the measurement of photo-CIDNP spectra, 0.1 mM 3-*N*-carboxymethyllumiflavin was added to the sample solution, and the tube was placed in a specially designed probe into which a laser beam was introduced through a quartz rod from an NEC GLC-3300 argon ion laser. A train of several continuous saturation pulses was followed by irradiation for 0.1 s with 488-nm light (1 W) prior to the detection pulse. A photo-CIDNP difference spectrum was obtained by subtracting a reference dark spectrum from a laser-irradiated spectrum. A total of 100 cycles were accumulated for each spectrum.

Results

Effects of cAMP, GMP, and 2′-Deoxy-cAMP Binding on the High-Field and Low-Field Regions of NMR Spectra. When cAMP was added to CRP in H_2O, new peaks appeared at −0.15 and −0.2 ppm (peaks L6 and V1) in the high-field region of the spectrum. A decrease in the intensity of the peaks at 0 and 0.1 ppm and an increase in the intensity of the peaks at 0.05 and −0.04 ppm (peaks L4 and L5) were also observed (Figure 2). Therefore, the exchange between the bound and free states is slower than the NMR time scale.

The large change in peak F in the low-field region is mostly completed on the addition of 1 mol equiv of cAMP, but the peaks in the high-field region are changed until 2 mol of cAMP has been added per mole of the CRP dimer. The addition of further cAMP did not cause any further change in the spectrum.

In the case of cGMP, the binding mode is also slow exchange on the NMR time scale. In the high-field and low-field regions, the features of the changes are not identical with those observed for cAMP binding. New peaks do not appear at −0.15 and −0.2 ppm on cGMP binding. The changes in the peaks in the high-field and low-field regions are completed

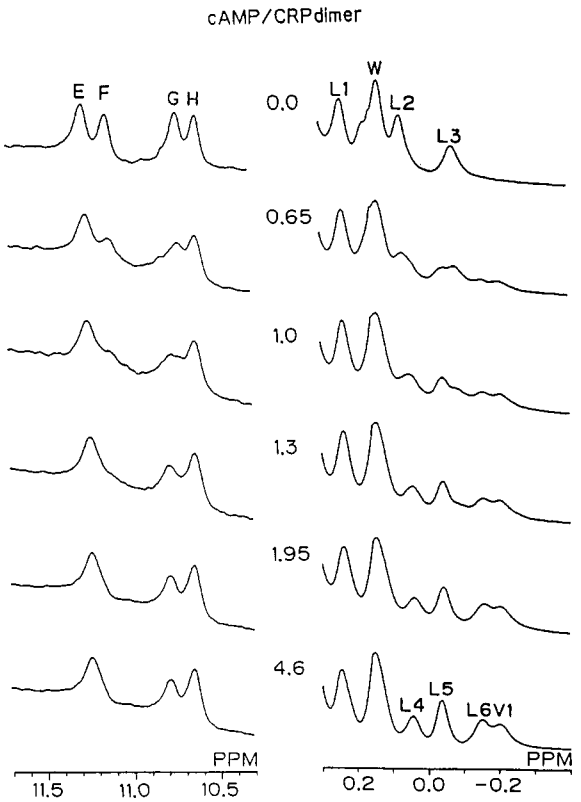

FIGURE 2: Effects of increasing concentrations of cAMP on the high-field and low-field resonances of CRP in H_2O, with different ratios of cAMP to CRP. The CRP concentration was 0.4 mM. The buffer contained 50 mM potassium phosphate and 0.2 M KCl (pH 6.5). The temperature was 30 °C.

after 2.5 mol of cGMP has been added per mole of the CRP dimer (data not shown). This can be explained by the weaker binding of cGMP than that of cAMP.

In the case of 2′-deoxy-cAMP, the addition of 2 mol of 2′-deoxy-cAMP did not cause any change in the high-field and low-field regions because of the low binding constant, even lower than that of cGMP. But the addition of a larger amount of 2′-deoxy-cAMP caused changes in the high-field and low-field regions. The features of the changes are not identical with those observed for cAMP binding. In the low-field region, peak F is not broadened but slightly low-field shifted. In the high-field region, new peaks do not appear at −0.15 and −0.2 ppm, which is similar to the case of cGMP binding (data not shown). The resonances at −0.15 and −0.2 ppm (peaks L6 and V1) only appeared on cAMP binding.

Assignments of the Resonance Peaks in the High-Field Region: Deuterium Labeling, Intermolecular NOE Measurement, and Proteolytic Digestion. In order to assign the peaks in the high-field region, a ^{1}H NMR spectra were also taken for Leu(methyl-d_3)CRP and Val(U-d_8)CRP (Figure 3). Resonances L1, L2, and L3 are absent in the spectrum of Leu(methyl-d_3)CRP (Figure 3, spectrum 2a), which consequently indicates that resonances L1, L2, and L3 are methyl proton resonances of Leu residues. In the case of the cAMP–CRP complex spectrum, resonances L4, L5, and L6 are absent in the spectrum of Leu(methyl-d_3)CRP (Figure 3, spectrum 2b), and resonance V1 is absent in the spectrum of Val(U-d_8)CRP (Figure 3, spectrum 3b). Therefore, resonances L4, L5, and L6 are methyl proton resonances of Leu residues, and resonance V1 is a methyl proton resonance of the Val residue.

For detailed assignments of three Leu and Val residues, saturation of the resonances for peaks L4, L5, L6, and V1 was

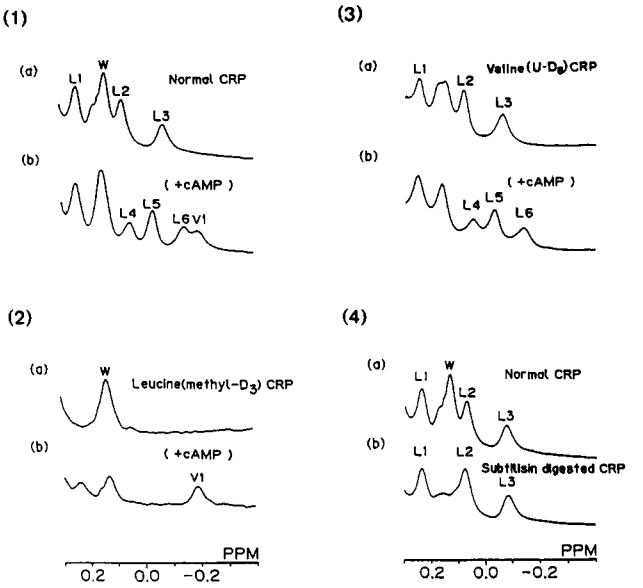

FIGURE 3: The high-field regions of the ^{1}H NMR spectra of CRP and the cAMP–CRP complex. (1) CRP (a) and the cAMP–CRP complex (b). (2) Leu(methyl-d_3)CRP (a) and the cAMP–labeled CRP complex (b). (3) Val(U-d_8)CRP (a) and the cAMP–labeled CRP complex (b). (U means uniformly labeled.) (4) CRP (a) and enzyme(subtilisin)-digested αCRP (b).

carried out in turn under low-power condition (Figure 4). On irradiation of peak V1, small NOEs were induced on the peaks of cAMP (Figure 4, spectrum 1). This NOE seems to be a direct NOE, but not spin diffusion, since this NOE shows the most rapid NOE buildup and is noticeable even at 100 ms. But the NOE at 8.8 ppm seems to be spin diffusion since in appeared on 500-ms irradiation (it did not appear on 250-ms irradiation). In order to prevent spillover onto the V1 peak, saturation of the resonances L4, L5, and L6 was carried out on Val(U-d_8)CRP (Figure 4, spectrum 2). No NOE on the peaks of cAMP could be detected on the irradiation of peaks L4, L5, and L6, though some NOE peaks are seen for the aromatic proton signals of the side chains. This indicates that the residue that gives resonance V1 is located closer to cAMP than those that give resonances L4, L5, and L6. The NOE pattern in the high-field region is typical of that of the methyl groups of valine (or leucine) residues.

To substantiate the assignments of high-field methyl resonances L1, L2, and L3, CRP was partially digested with subtilisin. Figure 3, spectrum 4, shows the high-field regions of the ^{1}H NMR sepctra of CRP and digested CRP. The L1, L2, and L3 resonances seen in the spectrum of intact CRP are present and resonance W is absent in the spectrum of digested CRP. Therefore, we can deduce that the residues responsible for resonances L1, L2, and L3 are located in the amino-terminal domain of CRP and the residue responsible for signal W is located in the carboxyl-terminal domain of CRP. Tentative assignments of the signals in the low- and high-field regions are given in Table I.

Single Amino Acid Substituted Mutant CRPs. We have studied five cAMP-independent mutants of CRP (Aiba et al., 1985). All five mutations involve single-base changes. The amino acid substitutions in the mutant proteins were in two specific regions, i.e., those encoding amino acid residues 53–62 and 141–148 of the CRP gene. The first region may participate in cAMP binding, while the second region appears to be the interdomain interaction region between the N-terminal cAMP-binding and C-terminal DNA-binding domains. Mutants CRP*2 (Gly-141–Asp), -3 (Leu-148–Arg), -4 (Asp-53–His), and -6 (Arg-142–Cys) have an increased affinity for

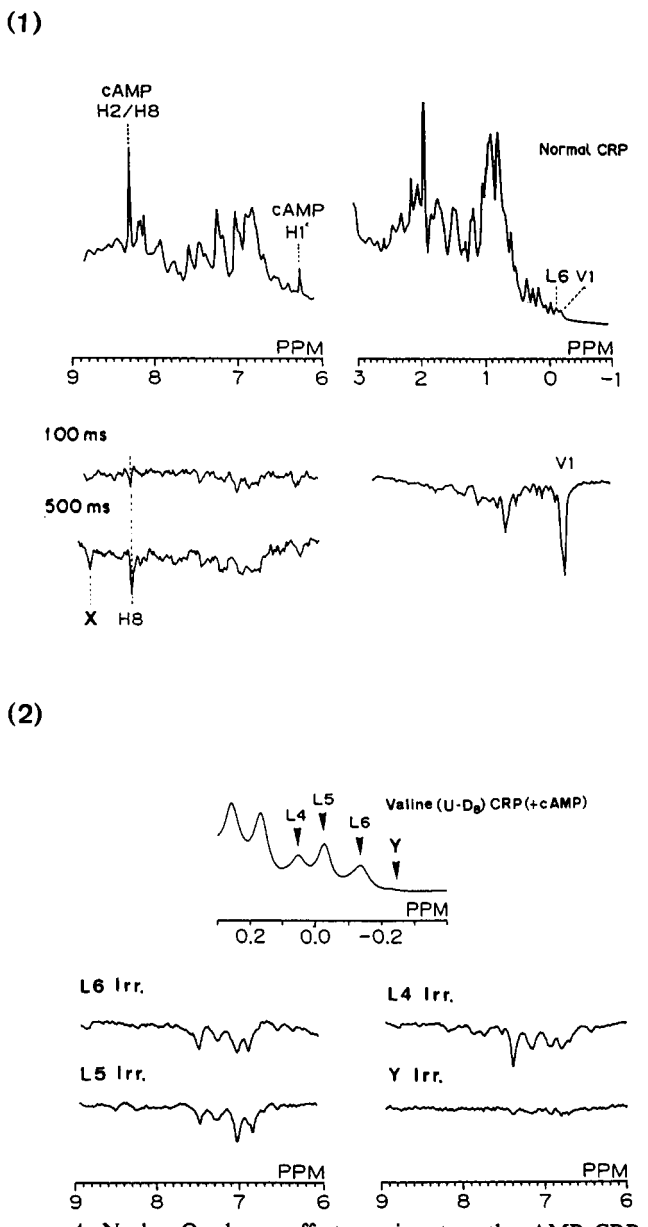

FIGURE 4: Nuclear Overhauser effect experiments on the cAMP–CRP complex in H_2O. (1) Reference spectrum (top) and NOE difference spectra (off-resonance minus on-resonance). V1 resonances (−0.2 ppm) are saturated for 100 and 500 ms, respectively. (2) Reference spectrum (top) and NOE difference spectra of cAMP–Val(U-d_8)CRP. Resonances L6, L5, L4, and reference position Y were respectively saturated for 500 ms. The CRP concentration was 0.4 mM and the cAMP concentration was 1 mM. The temperature was 30 °C.

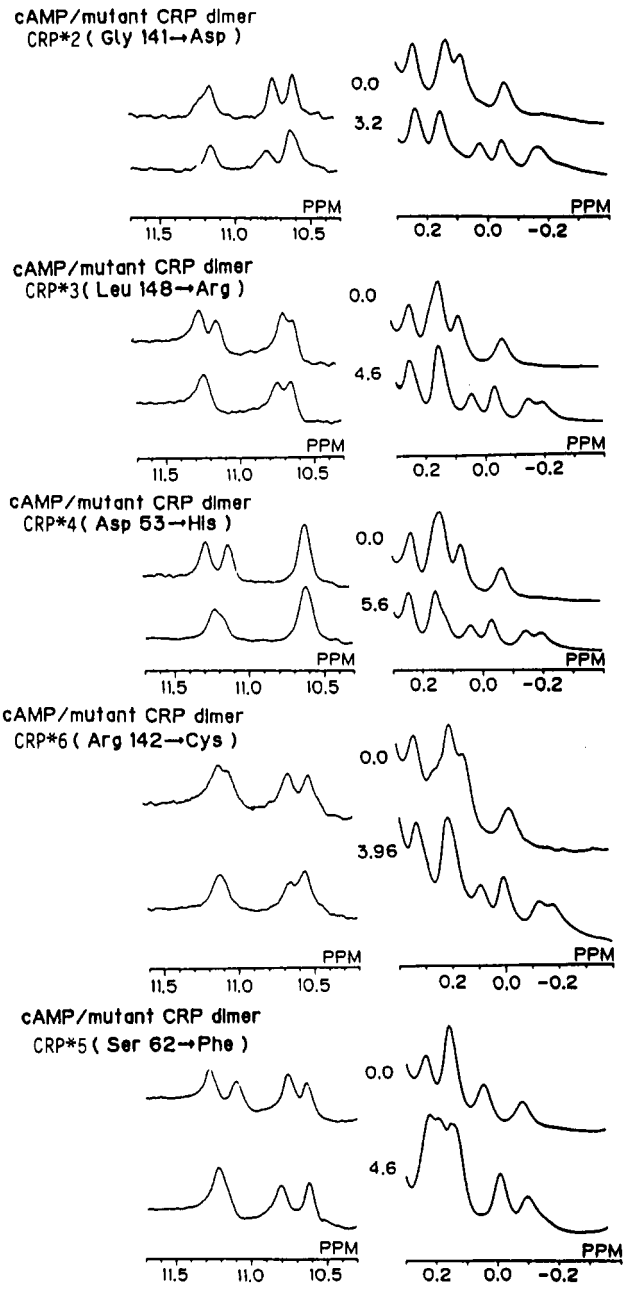

FIGURE 5: Effects of increasing concentrations of cAMP on the high-field and low-field resonances of mutants CRP*2, -3, -4, -5, and -6 in H_2O, with different ratios of cAMP to mutants CRP*2, -3, -4, -5, and -6. The buffer contained 50 mM potassium phosphate and 0.2 M KCl. The temperature was 30 °C.

cAMP, while CRP*5 (Ser-62–Phe) can only be activated by a high concentration of cAMP (Aiba et al., 1985; unpublished results).

Figure 5 shows the spectra of the mutant proteins. The mutated regions of CRP*2, CRP*3, and CRP*6 are in the D α-helix (Figure 1), which is located at the amino-proximal boundary of the DNA-binding domain. In the high-field region, these spectra are similar to that of native CRP. However, in the low-field region, there are some differences in the spectra between the mutant CRP*s and native CRP. Resonances F ang G originate from the residues in the cAMP-binding domain, as mentioned above. The resonances of mutants CRP*2, 3, and 6 are different from those of native CRP. Therefore, it can be considered that the amino acid substitutions in the D α-helix of the DNA-binding domain change the conformation of the cAMP-binding domain, and thus the changes of peaks F and G occur. When cAMP is added, the spectral changes of the mutant CRP*s are similar to those of native CRP. Significant broadening of peak F and minor broadening of peak G occurred, and new peaks appeared at −0.15 and −0.2 ppm.

In mutants CRP*4 and CRP*5, the mutated regions are in the β4- and β5-sheets of the cAMP-binding domain. The spectrum of mutant CRP*4 is different from that of native CRP in the low-field region. The G peak (Trp-85 residue) is slightly shifted to higher field. When cAMP was added, the spectral changes of this mutant CRP were similar to those of native CRP. New peaks appeared at −0.15 and −0.2 ppm in the high-field region. The spectrum of mutant CRP*5 is identical with that of native CRP in the low-field and high-field regions, but when cAMP was added, the spectral changes of this mutant CRP were different from those of native CRP. New peaks did not appear at −0.15 and −0.2 ppm in the high-field region.

Table I: Peak Positions and Tentative Assignments of the Isolated Peaks in the Lower and Higher Field Regions of the Spectrum of CRP

peak no.		chemical shift (ppm)		
free	bound	free	bound	assignment
E		11.25	11.25	His-159 NH[a]
F		11.15		unknown NH
G		10.75	10.80	Trp-85 NH[a]
H		10.65	10.65	Trp-13 NH[a]
L1	L4	0.25	0.05	Leu-61, -73, -124 CH_3
L2	L5	0.07	−0.04	
L3	L6	−0.80	−0.15	
	V1		−0.20	Val-49 CH_3
W		0.13	0.13	CH_3 group in DNA-binding domain

[a] From Lee et al. (1990).

Assignments of the Resonances in the Aromatic Region: Deuterium Labeling and NOE Measurements. CRP contains six histidine, two tryptophan, six tyrosine, and five phenylalanine residues in one subunit. To assign the resonances of the two Trp residues, a ^1H NMR spectrum was obtained for Trp(ring-d_5)-labeled CRP (Figure 6, spectrum 2). Resonances G′ and H′ are absent from the spectrum. Therefore, these two singlets were assigned to C-2H resonances of Trp residues.

For simplication of the spectrum in the aromatic region, the ^1H NMR spectrum was obtained for the labeled CRP, which was deuterated at all the aromatic rings of Phe and Trp residues. The spectrum is shown in Figure 6, spectrum 3, in comparison with the corresponding spectrum for the nondeuterated CRP (Figure 6, spectrum 1). A large number of proton resonances between 6.2 and 7.7 ppm disappeared in Figure 6, spectrum 3, showing that the remaining signals are due to His and Tyr residues. The ^1H NMR spectrum in Figure 6, spectrum 3 shows good resolution in the aromatic region.

The ortho and meta protons of Tyr residues are separated by 0.248 nm, a distance that should lead to strong NOE enhancements. In order to assign the resonances of the aromatic protons of the six Tyr residues, saturation of the resonances designated as S′, T′, U′, V′, and V was carried out in turn. Saturation of the resonances designated as Q, Q′, R, R′, S, T, and U was also carried out in turn (data not shown). From the results of NOE experiments, all resonances originating from each Tyr residue in the aromatic region could be identified, as shown in Figure 6, spectrum 3. In order to discriminate between the 3,5 and 2,6 resonances of Tyr residues, ^1H NMR was also obtained for Trp(ring-d_5)-, Phe(ring-d_5)-, and Tyr(ring-3,5-d_2)-labeled CRP. Since the remaining signals were due to His(C-2H,C-4H) and Tyr(C-2,6H), we can assign the C-2,6H resonances of the six Tyr residues (Figure 6, spectrum 4).

Effects of cAMP Binding and cGMP Binding on the Aromatic Region of NMR Spectra. Between 6.2 and 7.7 ppm in Figure 6, spectrum 4, well-resolved isolated peaks can be observed. The addition of cAMP to this CRP in D_2O caused pronounced broadening of resonances A, C, D, P, A′, C′, D′, and P′, but resonances B, E, B′, and E′ were not affected by cAMP binding. The addition of cGMP to CRP caused line broadening of resonances D, P, D′, and P′, but resonances A, B, C, E, A′, B′, C′, and E′ were not affected. We previously assigned peak B to His-199 and peak E to His-159, which are in the DNA-binding domain (Lee et al., 1990). Peaks A, C, D, and P are due to the residues in the cAMP-binding domain. The percentage intensity decrease of peaks by cAMP binding and the chemical shift of assigned histidine residues are listed in Table II.

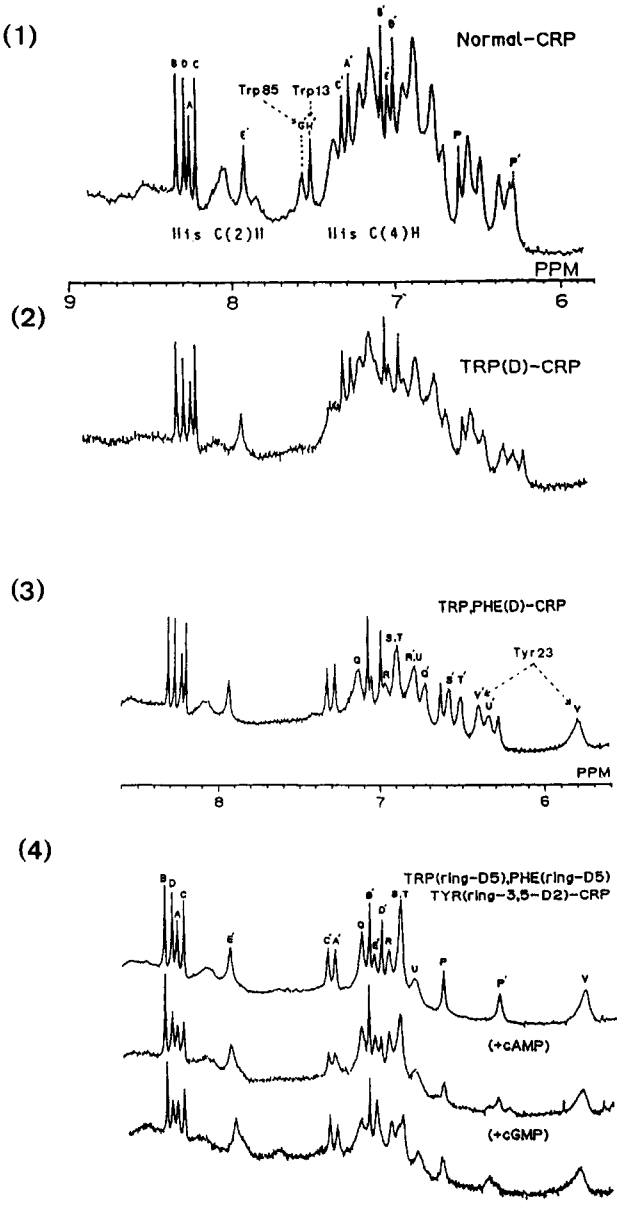

FIGURE 6: Comparison of the aromatic regions in the ^1H NMR spectra. (1) Normal CRP. (2) Trp(ring-d_5)-labeled CRP. (3) Trp(ring-d_5)- and Phe(ring-d_5)-labeled CRP. (4) Trp(ring-d_5)-, Phe(ring-d_5)-, and Tyr(ring-3,5-d_2)-labeled CRP, the cAMP–labeled CRP complex, and the cGMP–labeled CRP complex. The buffer contained 50 mM potassium phosphate and 0.2 M KCl. The ratio of cAMP and cGMP to CRP was 1. The temperature was 30 °C.

Photo-CIDNP Spectra of CRP. As shown in Figure 7, the photo-CIDNP difference spectrum of the CRP protein exhibits three sharp positive peaks due to the C-2 and C-4 protons of two histidines and strong negative broad peaks probably due to the 3,5 protons of tyrosine residues. Peaks B and B′ were assigned previously as the C2 and C4 protons of the His-199 residue (Lee et al., 1990; Clore & Gronenborn, 1982). Peak C is assigned as C2 of the His 17 (or 19) residue. The CIDNP data for CRP indicate that only two of the six histidines in the CRP subunit are located on the surface of CRP and His-199 is the most exposed. On the addition of cAMP, the photo-CIDNP signal of the His-199 residue does not change, and the photo-CIDNP signal C (of His-17 or -19 residue) becomes a little broadened (Figure 7). A weak broad negative peak (probably due to tyrosine residues) newly appeared at 6.6 ppm. Therefore, some conformational change occurred on cAMP binding. On mixing of the cAMP–CRP complex with the CRP-binding site 22-mer, the photo-CIDNP signals

Table II: Peak Positions and Tentative Assignments of Histidine and Some Aromatic Amino Acid Residues in CRP and the Decrease of Peak Intensity on the Binding of cAMP

peak no.	chemical shift (ppm)	intensity dec (%)	assignment	domain
A	8.92	32	His-31{C2H	
A′	7.29	38	{C4H	
C	8.21	48	His-71{C2H	
C′	7.34	36	(or19){C4H	
D	8.28	40	His-19{C2H	cAMP binding
D′	7.00	40	(or17){C4H	domain
G′	7.57		Trp-85 C2H	
H′	7.52		Trp-13 C2H	
P	6.65	53	His-21{C2H	
P′	6.29	45	{C4H	
V	5.80	35	Tyr-23{C2,6H	
V′	6.41		{C3,5H	
B	8.32	0	His-199{C2H	
B′	7.09	0	{C4H	DNA binding
E	7.94	7	His-159{C2H	domain
E′	7.06	0	{C4H	

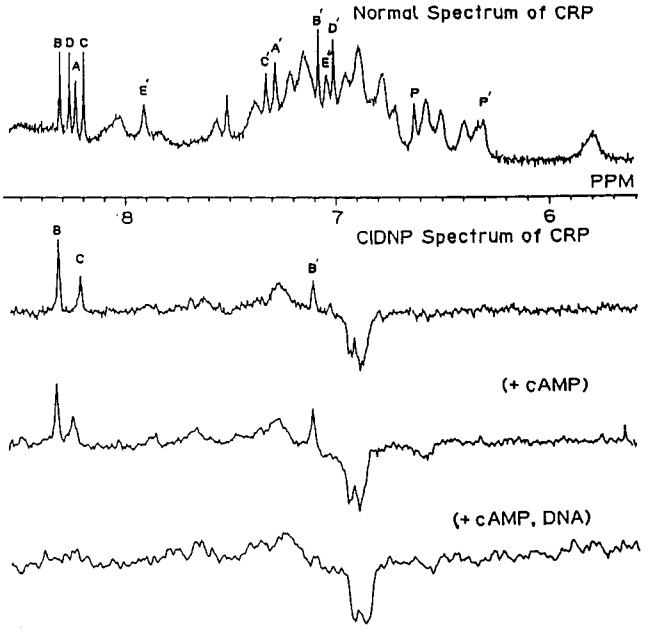

FIGURE 7: Photo-CIDNP spectra of CRP, the cAMP–CRP complex and cAMP–CRP–DNA complex. The buffer contained 50 mM potassium phosphate and 0.2 M KCl. The temperature was 30 °C. The ratio of cAMP–CRP to DNA was 1.

of two histidine residues disappeared, but the tyrosine signals were not affected (Figure 7).

Discussion

On the addition of cAMP, new peaks appeared at around −0.2 ppm. Irradiation of the V1 peak (−0.2 ppm) induced an NOE on the peak due to cAMP. This can be explained by the fact that the newly appearing peak of CRP is shifted due to the ring current effect of the adenine moiety of cAMP. There are several hydrophobic side chains in close proximity to the adenine ring, as shown in the crystal structure of CRP (Figure 8). They are Val-49 and Leu-61 on one side of the adenine ring and Leu-73 and Leu-124 of the other subunit on the other side of the ring (Weber & Steitz, 1987). The methyl protons of Val-49 are within a distance of <5 Å from H8 of cAMP. Thus, an NOE at H8 on irradiation at the methyl protons would be expected. The candidate residue for the peak at −0.2 ppm (V1) is Val-49. The L4, L5, and L6 peaks are considered to be ring-current-shifted methyl peaks of three leucine residues (Leu-61, Leu-73, and Leu-124). Since cAMP is bound in the syn conformation, H8 and H1′ are very close

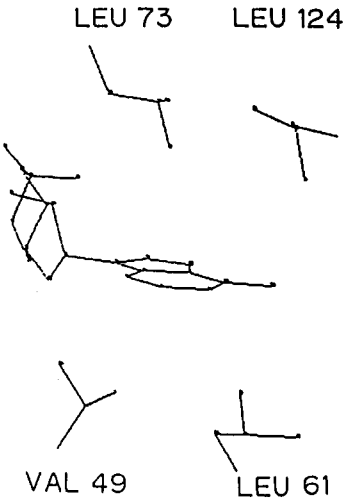

FIGURE 8: Relative locations of a cAMP molecule and the nearest hydrophobic amino acid side chains in the crystal structure (Weber & Steitz, 1987).

to and oriented toward each other (Gronenborn & Clore, 1982). Irradiation of the H1′ peak of cAMP induced an NOE on the H8 peak of cAMP (data not shown). Thus, the NOE at 8.3 ppm in Figure 4 is confirmed to be due to H8 of cAMP.

The pronounced broadening of peak F in the low-field region is mostly completed on the addition of 1 mol equiv of cAMP, but the changes of peaks in the high-field region are completed on the addition of 2 mol equiv of cAMP. This may be explained by assuming that the binding of 1 mol of cAMP to one subunit of CRP mostly changed the CRP structure and the binding of the second mole of cAMP to the other subunit of CRP did not affect the CRP structure. The second cAMP molecule may go smoothly into the cAMP-binding pocket. Therefore, the changes of peaks in the high-field region occurred due to the ring-current effect of cAMP until both the pockets are filled by two molecules of cAMP. Takahashi et al. (1989) recently presented evidence that the allosteric activation of CRP is induced upon binding of the first mole of cAMP.

On the addition of cGMP, such upfield-shifted peaks could not be observed. Nevertheless, cGMP really binds to CRP, as demonstrated by the shifts of signals at around 11 ppm. The guanine moiety of cGMP does not seem to fit the pocket at the binding site of CRP well. The bound guanine moiety of cGMP seems to take on the anti conformation, as judged from the absence of NOE between H8 and H1′ (Gronenborn & Clore, 1982). On the addition of 2′-deoxy-cAMP, such upfield-shifted peaks could not be observed. Therefore, 2′-deoxy-cAMP also does not seem to fit the pocket at the binding site of CRP well. These lines of evidence may explain the lack of activation of CRP by cGMP and 2′-deoxy-cAMP. Therefore, the newly appearing high-field peaks at around −0.2 ppm on the addition of cAMP can be used as good indicators of appropriate fitting of cAMP and potential as to the induction of activation.

On enzyme digestion of the carboxyl-terminal domain, the peak at 0.13 ppm (W peak), which may be assigned to a methyl group, was eliminated from the spectrum. This peak, on the other hand, remained unchanged on the addition of cAMP. This means that the structural change induced by the binding of cAMP is not transmitted to the C-terminal DNA-binding domain. The signals due to the C-terminal domain and those due to other parts are additive in the spectrum, indicating the absence of a strong interaction between the domains.

Figure 5 clearly indicates that the conformations of unliganded CRP* mutants differ from that of wild-type CRP. Comparison of the NMR spectra of CRP and mutant CRP*s (five species) complexed with cAMP showed many small changes associated with the cAMP-binding domains but no changes in the DNA-binding domains. When cAMP was added, high-field-shifted peaks L4, L5, L6, and V1 appeared in the spectra of mutants CRP*2, -3, -4, and -6. However, these high-field-shifted peaks did not appear in the spectrum of mutant CRP*5 (Ser-62–Phe) on cAMP binding. Ser 62 is in close proximity to the adenine moiety of cAMP in the cAMP–CRP complex in crystals. The aromatic ring of Phe (at position 62) in mutant CRP*5 may hinder the formation of a proper hydrogen bond between CRP and cAMP and thus hinder proper fitting of cAMP into the cAMP-binding pocket. Consequently, high-field-shifted peaks L4, L5, L6, and V1 can be used as indicators for the proper binding of cAMP and may also be important indicators of the activation of CRP.

We could assign the C-2,6H and C-3,5H resonances of the six Tyr residues in CRP. Only one of the six Tyr residues showed a different NMR spectral pattern as compared with the other five Tyr residues. The spectral pattern of this Tyr residue (peaks V and V′ in Figure 6, spectrum 3) is not a typical one of a Tyr residue in a mobile random-coil peptide chain, the C-3,5H resonance appearing at the higher field than the C-2,6H resonance. The chemical shift of this Tyr residue is more high-field-shifted than those of the other five Tyr residues. One of the six His residues in CRP (peaks P and P′ in Figure 6, spectrum 4) also showed a different NMR spectral pattern as compared with the other five His residues. The chemical shift of this His residue is more high-field-shifted than those of the other five His residues. These high-field shifts of the resonances of one His (peaks P and P′) and one Tyr (peaks V and V′) are considered to be mainly due to the ring-current effect of their aromatic side chains. We noted that His-21 and Tyr-23 are in the same β1-sheet and are in close proximity to each other in the crystal structure of CRP. Therefore, we tentatively assign peak P to His-21 and peak V to Tyr-23. Resonances P and P′ showed a pK_a of 6.1. Since we previously assigned peak B to His-199, peak A to His-31, and peak E to His-159 (Lee et al., 1990; Clore & Gronenborn, 1982), peaks C and D can be assigned to His-17 and His-19 (or His-19 and His-17), respectively.

When cAMP was added, peaks A, C, D, P, A′, C′, D′, and P′ of the residues in the cAMP-binding domain became notably broadened, but peaks B, E, B′, and E′ of the residues in the DNA-binding domain were not broadened. A small-angle X-ray scattering study by Kumar et al. (1980) showed compaction of the CRP molecule when cAMP was bound. The results of this NMR experiment suggest that such compaction of the CRP molecule by cAMP binding occurs only in the cAMP-binding domain and does not occur in the DNA-binding domain. This finding is quite consistent with the evidence obtained for the methyl proton resonances in the high-field region. Both facts imply that there is not so strong an interaction between the cAMP-binding domain and the DNA-binding domain and that the structural rigidity caused by cAMP binding is not strongly transmitted to the DNA-binding domain. However, there is also evidence that the binding of cAMP to apo-CRP increases the affinity to DNA. On the addition of cAMP, the two cAMP-binding domains of the two subunits of CRP seem to become realigned, coming closer to each other. As a consequence, the relative orientation of the DNA-binding domains is affected and may lead to a change in the affinity to DNA. It is clear that the mode of intersubunit interaction is affected by cAMP binding, because some tyrosine residues become exposed on the surface, as detected by photo-CIDNP.

Photo-CIDNP experiments showed that His-199 is located on the surface of the CRP molecule and that access to this residue by a dye is completely prevented by the CRP-binding site 22-mer DNA. His-199 is located at the turn region between the β11- and β12-sheets (Figure 1). This region is more C-terminal proximal than the F α-helix, which had been thought to be the only DNA contact region (Figure 1). The turn region between the β11- and β12-sheets had not been known to be directly implicated in specific DNA binding, although it is located in the DNA-binding domain of CRP.

The N-terminal portion of endonuclease V and the positively charged flexible N-terminal arms of the λ repressor and *Eco*RI restriction enzymes are known to participate in DNA binding. These arms wrap around the DNA and aid correct positioning of the proteins on their targets (Dowd & Lloyd, 1989; Pabo et al., 1982; Leighton & Lu, 1987; Weiss et al., 1984; McClarin et al., 1986). Lys-201 in the turn region has a positive charge, which can establish an electrostatic interaction of this nature between this turn region of CRP and DNA; the positive charge at the Lys-201 side chain would interact with the negatively charged phosphate backbone. The predicted contacts through Lys-201 require substantial DNA bending/kinking (Ebright et al., 1989), though we could not detect such a distortion for a short DNA fragment like the 22-mer. Gel retardation assays provide evidence of bending of the promoter DNA in the specific complex with CRP (Liu-Johnson et al., 1986). Further work in our laboratory is being directed toward establishing the role of Lys-201 in the CRP–DNA complex and toward developing a model for the allosteric change caused by the binding of cAMP and DNA.

Registry No. cAMP, 60-92-4; His, 71-00-1.

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A ^{1}H NMR Probe for Mobility in the Reactive Center Loops of Serpins: Spin-Echo Studies of Native and Modified Forms of Ovalbumin and α_1-Proteinase Inhibitor†

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ABSTRACT: It has recently been proposed that the expression of inhibitory activity in serine protease inhibitors (serpins) is a function of the mobility of the extended α-helical reactive center loop [Stein, P. E., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. J., & Carrell, R. W. (1990) *Nature 347*, 99-102]. We have employed solution ^{1}H NMR methods, including the Carr-Purcell-Meiboom-Gill (CPMG) and Hahn spin-echo pulse sequences, to try to identify such regions by virtue of their anticipated longer T_2 relaxation times in two of the best characterized members of the serpin superfamily, ovalbumin and α_1-proteinase inhibitor. The CPMG spectra of native ovalbumin reveal the presence of long-lived resonances from the methyl protons of alanine residues and the CH_3 protons of leucine or valine residues as well as the acetyl and ring methine protons of the carbohydrate moieties. Following reaction of ovalbumin with subtilisin Carlsberg to generate plakalbumin [where excision from within the reactive center loop homologue of a hexa- or heptapeptide, with sequence (E)-A-G-V-D-A-A, occurs], its CPMG spectrum retained almost all of the originally present long-lived resonances. Concurrent with the retention of these mobile resonances in plakalbumin is the appearance of two additional resonances consistent with the formation of new C and N termini. On the basis of the proposed mobility of the reactive center loop, it had been expected that removal of the alanine-rich hexapeptide would result in loss of some or all of the long-lived alanine methyl resonances. Similar CPMG experiments were conducted with native, proteolytically (papain) modified, and N-chlorosuccinimide-oxidized α_1-proteinase inhibitor, where a very similar set of long-lived (mobile) resonances was identified, which persisted within each form of the inhibitor. The results lead us to conclude that the extended α-helical loop of serpins is not unusually mobile. These findings and their significance are discussed in terms of the instances of segmental internal motion (i.e., mobility and flexibility) within polypeptide domains demonstrated by NMR spectroscopy, and also with regard to the recently proposed ovalbumin-based model for the mobile reactive center loops of serpins.

The elucidation of the X-ray crystallographic structure of cleaved (Wright et al., 1990) and native ovalbumin (Stein et al., 1990) has further stimulated an already active interest (Huber & Carrell, 1989) in serpin structure and function. This pair of native and proteolytically modified structures serves as a critical point of reference in our attempts to understand structure-function relationships within the serpin superfamily.

In the structure of native ovalbumin, the reactive center loop was found to exist as an extended α-helix in which the Debye-Waller B factors for residues in the helix relative to residues in the protein core show some degree of elevation. This was interpreted as an indication of a flexible, mobile structure for the α-helix (Stein et al., 1990). Since a computer-simulated reconstruction of the cleaved α_1-proteinase inhibitor (α_1-PI)[1] reactive center loop also indicates the presence of an α-helix, it has been proposed that such a flexible structural element may play a critical role in the general process of recognition of the reactive site of serpins by target proteases and their successful inhibition.

One method that has proved to be extremely useful and sensitive for probing discrete domains of proteins that possess

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[1] Abbreviations: CPMG, Carr-Purcell-Meiboom-Gill; α_1-PI, α_1-proteinase inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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