Comparison of cAMP Receptor Protein (CRP) and a cAMP-Independent Form of CRP by Raman Spectroscopy and DNA Binding

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Received January 4, 1991

ABSTRACT: The secondary structures of the cAMP receptor protein (CRP), a complex of CRP and cAMP, and a cAMP-independent receptor protein mutant (CRP*141gln) were examined by using Raman spectroscopy. Spectra were obtained from CRP and CRP*141gln dissolved in 0.3 M NaCl and 30 mM sodium phosphate at protein concentrations of 30–40 mg/mL. CRP and CRP·cAMP$_1$ were compared at lower protein concentrations (10–12 mg/mL) in a solvent of 0.35 M NaCl and 20 mM sodium phosphate. Raman analysis indicates that CRP structural changes induced by one bound cAMP or by the Gly to Gln mutation at residue 141 are small. Spectra of the three CRP samples are essentially identical from 400 to 1900 cm^{-1}. This result differs from the Raman spectroscopy study of CRP and CRP·cAMP$_2$ cocrystals [DeGrazia et al. (1990) *Biochemistry 29*, 3557]. The latter work showed spectral differences between CRP and CRP·cAMP$_2$ consistent with alterations in the protein conformation. These studies indicate that CRP and CRP·cAMP$_1$ in solution are similar in structure and differ from CRP·cAMP$_2$ cocrystals. Protease digestion and a DNA binding assay were also employed to characterize the wild-type and mutant proteins. CRP*141gln exhibited the same conformational characteristics of previously reported cAMP-independent mutant proteins. It was sensitive to proteolytic attack in the absence of cAMP, or upon addition of cGMP. In the absence of cAMP, both wild-type and mutant CRPs bound noncooperatively to a 62 bp *lac* promoter DNA. The equilibrium constants were $\approx 10^6$ M^{-1} in 0.1 M Na$^+$. CRP*141gln had a 2–4-fold higher affinity for the 62 bp DNA than CRP. Both proteins showed cooperative binding with 75 and 84 bp DNAs lacking *crp* sites.

The binding of a transcription regulatory protein to specific DNA sites is often controlled by a small molecule that binds to the protein. The cAMP receptor protein (CRP or CAP) and its cofactor, cyclic adenosine monophosphate (cAMP), exemplify this type of control system (Adhya & Garges, 1982; deCrombrugghe, 1984). In the absence of cAMP, CRP binds to DNA with low affinity. When cAMP forms a complex with CRP, the protein binds with high affinity to specific sites near a number of promoters in *Escherichia coli* and modulates transcription initiation.

Studies by Heyduk and Lee (1989) indicate that there are at least three conformational states of the protein. These states correspond to free CRP, CRP·cAMP$_1$, and CRP·cAMP$_2$. Results from several investigations indicate that CRP·cAMP$_1$ forms a high-affinity complex with promoter sites. Only one cAMP per protein is required for the high-affinity CRP–DNA complex (Garner & Revzin, 1982; Fried & Crothers, 1984). The association constant of CRP·cAMP$_1$ to the *lac* DNA promoter is significantly greater than either CRP alone (Fried & Crothers, 1984) or CRP·cAMP$_2$ (Heyduk & Lee, 1990).

CRP is isolated as a dimer of identical polypeptide subunits. X-ray diffraction studies on a crystal of CRP·cAMP$_2$ show that each subunit is folded into two domains separated by an eight amino acid "hinge" (McKay et al., 1982; Weber & Steitz, 1987). Crystal structures of CRP alone or with one bound cAMP are not yet available. It has been suggested that cAMP alters the orientation of the two subunits or causes the domains within a subunit to change their orientation (McKay et al., 1982; Gargas & Adhya, 1985).

A Raman spectroscopy study showed that CRP·cAMP$_2$ cocrystals and CRP in solution have differences in several spectral regions sensitive to polypeptide secondary structure (DeGrazia et al., 1990). The data were consistent with the interpretation that CRP with two bound cAMPs has less α-helix and more β-strand than free CRP. In the current work, Raman spectroscopy was employed to compare the structural characteristics of CRP, CRP·cAMP$_1$, and a cAMP-independent form of the protein, CRP*141gln (Kim, Garges, and Adhya, unpublished results). The latter CRP* has glutamine replacing glycine at residue 141. Protease digestion and DNA binding studies were also used to compare the in vitro properties of CRP*141gln and CRP.

The Raman analysis indicates that the structural change(s) to CRP induced by one bound cAMP or by the CRP* mutation is (are) small. Spectra of CRP, CRP·cAMP$_1$, and CRP*141gln are essentially identical. Results from DNA binding studies show that both wild-type and mutant CRPs exhibit noncooperative binding to a 62 bp *lac* promoter DNA in the absence of cAMP. This binding mode was recently described using different conditions (Hudson et al., 1990). CRP without cAMP forms a weak but noncooperative complex at *crp* sites. The highly cooperative binding of CRP to nonspecific genomic DNA (Garner & Revzin 1979) was observed for two short DNA fragments lacking *crp* sites.

Materials and Methods

Wild-type CRP was isolated from an *E. coli* strain containing the plasmid pHA7 (Aiba et al., 1982). CRP*141gln was isolated from *E. coli* strain HB101 which contained the plasmid pHA7 modified in its *crp* gene. A mutation in codon 141 (GGC to CAG) resulted in a CRP* phenotype in vivo

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0006-2960/91/0430-5076$02.50/0

(Kim, Garges, and Adhya, unpublished results). The wild-type glycine residue is replaced by glutamine.

Both wild-type and mutant proteins were purified by using previously described procedures (DeGrazia et al., 1985, 1990). The wild-type and mutant CRPs were approximately 95% pure as judged by electrophoresis on 12.5% polyacrylamide gels with 0.1% SDS. Concentrations were determined spectroscopically by using an extinction coefficient of 3.5×10^4 at 280 nm (Fried & Crothers, 1984). The proteins were stored in 0.2 M NaCl, 56 mM potassium phosphate, 1 mM EDTA, and 10–20% glycerol.

The conformations of the two proteins were characterized by subtilisin digestion (Harman et al., 1986). Protein samples were dialyzed into a buffer containing 30 mM Tris·HCl, pH 8.0 at 25 °C, 2.5 mM $MgCl_2$, 0.1 mM EDTA, and 100 mM KCl. Both mutant and wild-type CRPs were digested for 90 min in a reaction that contained a 75 to 1 weight to weight ratio of protein to subtilisin. The concentration of CRP used in each 25-μL reaction was 3.8 μM. When cAMP or cGMP were added, they were at concentrations of 100 or 200 μM, respectively. The reaction was stopped by the addition of phenylmethanesulfonyl fluoride (PMSF) to 1 mM. The digested CRP protein samples were run on a 12.5% PAGE gel with 0.1% SDS.

The gel retardation assay (Garner & Revzin, 1981; Fried & Crothers, 1981; DeGrazia et al., 1985) was used to characterize CRP binding to DNA fragments. Three DNAs were employed. One was a 62 bp fragment containing the major *crp* binding site of the *lac* promoter (DeGrazia et al., 1985). The center of the *crp* site is 21 bp from one end of the fragment. The other two DNAs were a 75 bp *Hin*fI fragment from pBR322 and a 84 bp *Hae*III DNA from pVH51 (gift of S. Abhiraman). Neither of these DNAs contains *crp* binding sites. Twenty-microliter reaction volumes contained 0.26 μM DNA with different molar ratios of CRP. The solvent was 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM DTT, and 0.1 M NaCl. After CRP and DNA were mixed at 25 °C, the solutions were equilibrated for 30 min. Two microliters of 30% glycerol was added, and the samples were loaded onto a 7.5% polyacrylamide gel [46:1 acrylamide:bis(acrylamide)]. The gel buffer contained 0.089 M Tris–borate and 1 mM Na_2 EDTA. Samples were electrophoresed for 3 h at 120 V and the DNAs visualized by staining with ethidium bromide. Free DNA band images in negatives used for densitometer scans were linearly related to DNA concentration.

CRP samples were prepared for Raman spectroscopy studies by dialyzing 1–3 mg with 0.35 M NaCl and 20 mM sodium phosphate (pH 6.5). They were concentrated by centrifugation through a Centricon-30 filter (Amicon Inc.) to approximately 10–12 mg/mL. CRP at this concentration was employed to compare spectra of CRP and CRP·$cAMP_1$ complexes. The latter were formed by adding 2 μL of a concentrated cAMP solution to 20 μL of CRP solution.

Samples used to compare wild-type CRP with CRP*141gln were prepared in a similar way but with a different buffer. A pH 8.0 phosphate buffer with 0.15 M NaCl was used for dialysis. The protein concentrations were increased after Centricon centrifugation using a Speedvac centrifuge (Savant Inc.). The final buffer was estimated as 0.3 M NaCl and 30 mM sodium phosphate (pH 8.0). Protein concentrations were estimated to be 30–40 mg/mL.

Raman spectra were obtained from gellike solutions loaded into 2-mm i.d. quartz capillary cells. An argon ion laser with the laser line at 514.5 nm was employed as the light source. A description of the instrumentation has been previously given

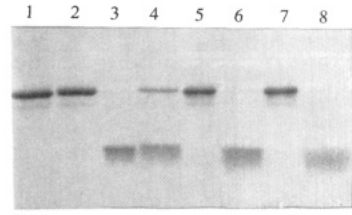

FIGURE 1: Gel electrophoresis results of subtilisin digestion of wild-type and mutant CRPs in the presence or absence of cyclic nucleotides. Concentrations and digestion conditions are described under Materials and Methods. Lanes 1 and 2 contained untreated CRP and CRP*141gln, respectively. Lanes 3–8 were treated with subtilisin: (3) CRP + cAMP; (4) CRP*141gln + cAMP; (5) CRP + cGMP; (6) CRP*141gln + cGMP; (7) CRP; (8) CRP*141gln.

(Wartell & Harrell, 1986). Each spectrum is the sum of four or more scans. Each scan is recorded at 1 cm^{-1} intervals with a 2-s integration time. The laser power at the cell was approximately 200 mW. All spectra were recorded at room temperature.

Secondary structure analysis of the amide I region (1630–1750 cm^{-1}) was carried out by using the method developed by Williams (1983, 1986). A difference spectrum between wild-type and mutant CRP, or wild-type CRP and CRP·$cAMP_1$, was obtained after subtraction of background curves from each spectrum. Background curves were generated by using a nonlinear least-squares fitting program (Wartell & Harrell, 1986). Overlapping regions were examined from 600 to 1500 cm^{-1}. The sum of the background curve and simulated Raman peaks was fit to the spectra from 600 to 1150 cm^{-1} and from 900 to 1500 cm^{-1}. The generated background curves were then subtracted from actual spectra. The peak at 1441 cm^{-1} served as an intensity standard in the subtraction of two protein spectra.

Results

*Proteolytic Digestion of CRP and CRP*141gln.* Protease digestion can distinguish between two conformations of wild-type CRP (Eilen & Krakow, 1977; Harman et al., 1986). CRP is resistant to proteolysis in the absence of cAMP. The addition of cAMP causes CRP to undergo a conformational transition that enables subtilisin to digest it to a stable core fragment of approximately 13 000 Da (Tsugita et al., 1982). Harman et al. (1986) showed that three different CRP*s were digested in the absence of cAMP. The mutations occurred at residue 144, residues 127 and 170, and residues 127, 170, and 195. These CRP*s apparently adopt a structure with characteristics similar to the wild-type CRP·cAMP complex. Figure 1 shows the subtilisin digestion of wild-type CRP and CRP*141gln in the presence and absence of cAMP and cGMP. As expected, CRP is digested only in the presence of cAMP. CRP*141gln is digested in the absence as well as the presence of cAMP. This property is consistent with its in vivo characterization as a cAMP-independent CRP (Kim, Garges, and Adhya, unpublished results). Lanes 5 and 6 of Figure 1 also show that CRP*141gln is digested in the presence of cGMP while wild-type CRP is not. This characteristic is also observed with other CRP*s (Harman et al., 1986).

*Binding of CRP and CRP*141gln to DNA.* The gel electrophoresis retardation assay was used to characterize the binding of CRP and CRP*141gln to several DNAs. Figures 2 and 3 show titrations of the 62 bp *lac* DNA with CRP and CRP*141gln, respectively. The reaction mixtures placed in lane 1 of each figure contained 100 μM cAMP. The reaction mixtures placed in the remaining lanes did not contain cAMP. In the absence of cAMP (lanes 2–8, Figure 2; lanes 2–9, Figure 3), two bands are observed corresponding to free DNA and

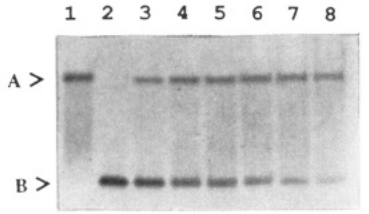

FIGURE 2: Binding of wild-type CRP to the 62 bp *lac* DNA using the gel retardation assay. Each lane contains 0.26 μM DNA. Free DNA band is indicated by B> and CRP–DNA complex by A>. Only lane 1 has cAMP (100 μM). Molar ratios of CRP to DNA for lanes 1–8 are 4:1, 0, 2:1, 4:1, 6:1, 8:1, 12:1, and 16:1, respectively.

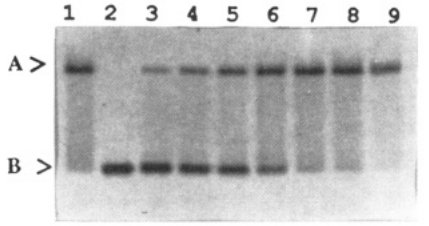

FIGURE 3: Binding of CRP*141gln to the 62 bp *lac* DNA using the gel retardation assay. Free DNA band is indicated by B> and CRP–DNA complex by A>. Each lane contains 0.26 μM DNA. Only lane 1 has cAMP (100 μM). Molar ratios of CrP*141gln to DNA for lanes 1–9 are 4:1, 0, 1:1, 2:1, 3:1, 4:1, 6:1, 8:1, and 16:1, respectively.

a protein-DNA complex. In the absence of cAMP, CRP has a noncooperative binding mode to fragments containing *crp* sites. This result is similar to the recent observation by Hudson et al. (1990) obtained under different solvent conditions. Unlike the latter work, no other bands were observed up to the highest protein:DNA ratio employed (16:1).

We noted that the retardation of the complex of *lac* DNA and CRP in the absence of cAMP (lanes 3–8, Figures 2, and lanes 3–9, Figure 3) was always slightly less than for the complex observed with cAMP (lane 1). This difference may be attributed to conformational differences between CRP–DNA complexes in the presence and absence of cAMP. The complex between CRP and cAMP and the *lac* promoter site induces substantial DNA bending (Wu & Crothers, 1984; Liu-Johnson et al., 1986; Buc et al., 1986). DNA bending is not evident in the absence of cAMP (Dripps & Wartell, 1987). The effect of a bend locus on the mobility of a fragment is expected to be small when the locus is at the end of a DNA (Wu & Crothers, 1984; Zinkel & Crothers, 1990). This is the situation for the *crp* site in the 62 bp *lac* fragment.

Binding constants of CRP and CRP*141gln for the 62 bp *lac* DNA in the absence of cAMP were estimated from titration experiments (e.g., Figures 2 and 3). Each protein was assumed to bind to an isolated DNA site. The association constant, K, was set equal to $[C]/[D][P]$ with [C], [D], and [P] the concentrations of the CRP·DNA complex, the free DNA, and the free CRP dimer, respectively. The concentrations of bound and free DNA were determined from the total DNA added and densitometer scans of the free DNA bands. The free protein concentration was determined from $[P] = \alpha[P_T] - [C]$ with α the percentage of total protein ($[P_T]$) active for site-specific binding. On the basis of titrations of the 62 bp *lac* DNA with CRP and cAMP, a value of $\alpha = 33\%$ was employed. Values of K for wild-type and mutant CRPs were determined from plots of log ($[C]/[P]$) vs log [D] (Fried & Crothers, 1984). On the basis of three repeated experiments per protein, K for the wild-type CRP is 3.7×10^5 M^{-1} ± 30%. For CRP*141gln, it is 1.3×10^6 M^{-1} ± 20%.

The 75 and 84 bp DNA fragments were each titrated with CRP and CRP*141gln under conditions identical with Figure 3. The only observable complexes above the free DNA bands were of such high molecular weights that they barely entered the gels (not shown). Similar results were previously observed using heterogeneous genomic DNA (Takahashi et al., 1979; Saxe & Revzin, 1979). This cooperative CRP binding mode is now demonstrated for homogeneous DNA fragments lacking *crp* sites. Hudson et al. (1990) suggested that special genomic sequences may be needed to nucleate this cooperative binding mode. Unless both of the above fragments contain special sequences, the above results do not support this hypothesis. A comparison of their sequences revealed two common five base pair stretches, AGCGG and ATCAG, uncorrelated in their relative locations. No common stretches longer than five base pairs were found.

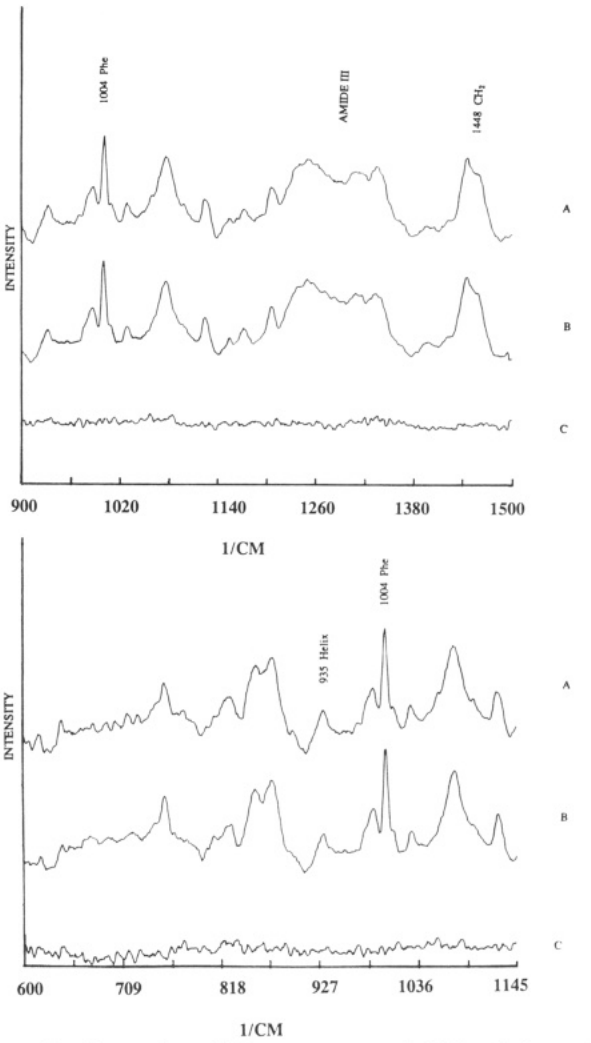

FIGURE 4: Comparison of Raman spectra of CRP solution and CRP·cAMP$_1$ solution from 600 to 1145 cm^{-1}, and from 900 to 1500 cm^{-1}. (A) CRP·cAMP$_1$; (B) CRP; (C) difference spectrum of (A) minus (B).

Raman Spectra. Previous Raman spectroscopy studies on CRP in solution and CRP·cAMP$_2$ microcrystals attempted to obtain solution spectra of CRP and cAMP (DeGrazia et al., 1990). These efforts were unsuccessful due to precipitation problems at the high concentrations used. By decreasing the CRP concentration and changing the ionic strength, this problem was overcome (see Materials and Methods). A clear gellike solution was obtained with CRP and cAMP concentrations of about 0.28 and 0.32 mM, respectively. On the basis of the binding constants of cAMP to CRP in 0.4 M ionic strength (Takahashi et al., 1981), 76% of the CRP is in a CRP·cAMP$_1$ complex, 16% is free CRP, and 8% is CRP·

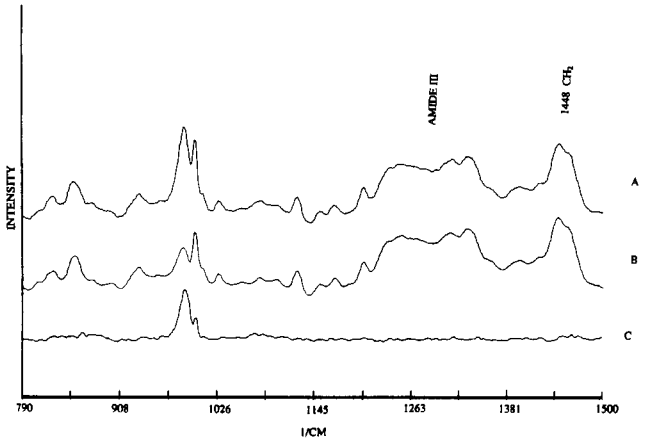

FIGURE 5: Comparison of Raman spectra of solutions of CRP*141gln and CRP from 790 to 1500 cm^{-1}. (A) CRP*141gln; (B) CRP; (C) difference spectrum of (A) minus (B).

Table 1: Results of Secondary Structure Analysis of the Amide I Region

	% α-helix	% β-strand	% reverse turn	% random coil
CRP	44	28	18	10
CRP*141gln	41	31	17	11
CRP·$cAMP_1$	43	29	18	10

$cAMP_2$. Since CRP·$cAMP_1$ is the dominant form, we refer to this solution as CRP·$cAMP_1$. Figure 4 compares Raman spectra of this solution and a solution of free CRP. Two overlapping regions are shown. The difference spectra indicate that the conformational change to CRP induced by the binding of one cAMP cannot be detected by Raman spectroscopy.

A comparison of the spectrum of CRP*141gln with that from wild-type CRP is shown in Figure 5. With the exception of the bands around 980 cm^{-1}, the region from 790 to 1500 cm^{-1} shows no differences. The changes around 980 cm^{-1} are probably due to pH-sensitive changes of the solvent's phosphate vibrations. The PO_4^{2-} vibrational bands are sensitive to variations of pH between 6 and 8 as well as molarity changes. Since the two CRP samples were prepared independently, it is likely that small differences in the solvents account for the 980 cm^{-1} region. No other differences were observed from 600 to 1500 cm^{-1}.

The region from 2450 to 2750 cm^{-1} is known to contain a cysteine S–H stretching vibration that is sensitive to the local environment of this group (Tu, 1986). Since cysteine cross-linking experiments (Eilen & Krakow, 1977) indicate that one of the three cysteines per subunit is altered upon cAMP binding (Cys-178), we examined this Raman band for the CRP samples. Raman spectra from CRP solutions showed one band at about 2540 cm^{-1} (not shown). Only minor differences in the widths of the S–H bands were observed.

Analysis of the Amide I Region. The secondary structure distributions of the three CRP samples were estimated from analysis of the amide I region (Williams, 1986). Table I lists the values determined from the solutions of CRP, CRP*141gln, and CRP·$cAMP_1$. Distributions are the same within the error (±4%) expected for the analysis. They are the same as values obtained in a previous Raman study of CRP from this lab (DeGrazia et al., 1990).

Discussion

Distinct protein conformational states exist for free CRP and CRP·$cAMP_1$. Probes that can distinguish these states are protease digestion (Krakow & Pastan, 1973; Heyduk & Lee, 1989), fluorescence of tryptophan or attached fluorescent probes (Wu et al., 1974; Heyduk & Lee, 1989), and chemical modification of cysteine residues (Eilen & Krakow, 1977; Hyduk & Lee, 1989). The Raman data presented in this work indicate that the structural differences between CRP and CRP·$cAMP_1$ are small. No significant changes are observed in the secondary structure distribution or conformationally sensitive Raman bands of CRP.

As expected from its in vivo properties, the protein CRP*141gln has a higher affinity for the *lac* promoter DNA than wild-type CRP. The binding constant of this CRP* for the *lac* DNA is 2–4-fold higher than for wild-type CRP. The mutant protein also mimics the protease digestion properties of CRP·$cAMP_1$ as observed with several other CRP*s (Harman et al., 1986, 1988). The Raman spectrum of this protein is essentially identical with wild-type CRP. This observation lends further support to the conclusion that only small conformational changes are needed to convert free CRP to its functionally active form.

It is of interest to compare the results described in this work with the Raman analysis of CRP and CRP·$cAMP_2$ (DeGrazia et al., 1990). Unlike the above spectra, several differences were observed between the spectra of CRP and CRP·$cAMP_2$. They indicated these two forms differed in secondary structure. Overall, the Raman data imply that one bound cAMP produces a small structural change in CRP whereas binding of the second ligand induces a more substantial change.

The above conclusion is consistent with other studies. The effect of cAMP concentration on conformational probes such as protease digestion and fluorescence indicated that CRP, CRP·$cAMP_1$, and CRP·$cAMP_2$ assume different protein conformations (Heyduk & Lee, 1989). ^1H NMR experiments by Gronenborn and Clore (1982) showed virtually no change in the NMR spectrum of CRP upon the binding of one cAMP. A slow conformational change was detected subsequent to the binding of one cAMP and was complete only when both cAMP binding sites were almost completely saturated. Although X-ray scattering indicates that one bound cAMP reduces the radius of gyration of CRP (Kumar et al., 1973), this does not appear to be reflected in the Raman or NMR spectra.

The noncooperative binding between CRP and the 62 bp *lac* fragment in the absence of cAMP corroborates results recently reported by Hudson et al. (1990). Unlike the latter work, which observed a ladder of bands at high CRP:DNA ratios, only a single complex between CRP and the 62 bp *lac* DNA was observed. This difference may be due to the higher ionic strength we employed in both the reaction buffer and the gel buffer (≈0.1 M vs. 0.0225 M), or the reduced sensitivity of ethidium staining relative to autoradiography. The current studies do not provide evidence for the idea suggested by Hudson et al. (1990) that special sequences are required for cooperative CRP binding. Both nonspecific DNA fragments examined bound CRP in a cooperative manner. Although two five base pair stretches are common to both DNAs, these sequences are dissimilar to poly[d(A-T)]·poly[d(A-T)] and poly[d(I-C)]·poly[d(I-C)] which bind CRP in a cooperative manner (Saxe & Revzin, 1979).

Our results suggest that CRP without cAMP forms a weak but specific complex at the *crp* site. This complex is able to inhibit cooperative aggregation of other CRP in a manner similar to the strong CRP·$cAMP_1$–DNA complex. The equilibrium binding constant of the CRP–*lac* DNA complex, 3.7×10^5 M^{-1}, may not be high enough to maintain its stability during a DNase I footprinting experiment. This may account for the apparent failure of CRP to protect the *crp* site from

DNase I digestion (Hudson et al., 1990).

ACKNOWLEDGMENTS

We thank Sankar Adhya and Susan Garges for discussions and for their critical reading of the manuscript. We also thank Tom Maier for assistance in maintaining the Raman system.

Registry No. Gly, 56-40-6; cAMP, 60-92-4.

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