Conformational Transitions of Cyclic Adenosine Monophosphate Receptor Protein of *Escherichia coli*. A Fluorescent Probe Study†

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ABSTRACT: cAMP receptor protein (CRP) of *Escherichia coli* has been labeled covalently with two fluorescent reagents, *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate (1,5-I-AENS) and dansyl chloride. Both AENS-CRP and dansyl-CRP are fully active in binding cAMP and promoting transcription of the *gal* operon. When cAMP is added to a solution of AENS-CRP, there is an increase in fluorescence intensity and a blue shift of the emission maximum of AENS-CRP, indicating a conformational transition of the protein. This conformational change is induced only by cAMP and its biologically active analogs (tubercidin 3′,5′-monophosphate and N^6,$O^{2'}$-dibutyryl-cAMP), but not by cyclic nucleotides (such as cGMP and 1,N^6-etheno-cAMP) which are competitive inhibitors of cAMP. Thus, the observed conformational change of CRP induced by cAMP may be related to the cAMP-dependent gene transcription. In addition, another specific conformational change of CRP occurs when CRP interacts with the *lac* operon in the presence of cAMP. This is demonstrated by a cAMP-sensitive blue shift and a quenching of the emission spectrum of AENS-CRP upon binding to λ*h80dlac* DNA but not to λ*h80* DNA. Nanosecond fluorescence depolarization studies of dansyl-CRP reveal that addition of cAMP does not elicit association or dissociation of CRP.

Expression of the lactose operon and several other inducible operons of *Escherichia coli* requires adenosine 3′,5′-monophosphate (cAMP) and a cAMP receptor protein (CRP)[1] (Pastan and Perlman, 1970; Riggs *et al.*, 1971). Studies with purified CRP (Riggs *et al.*, 1971; Anderson *et al.*, 1971) in *in vitro* transcription systems have shown that CRP regulates the *lac* (de Crombrugghe *et al.*, 1971) and *gal* operons (Nissley *et al.*, 1971), presumably by binding at the promotor site of the genome and stimulating the specific initiation of mRNA synthesis. However, the molecular mechanism of such regulation is still unclear. Moreover, no specificity of the binding of CRP to DNA containing the *lac* operon, or to any other specific DNA, has thus far been demonstrated.

The affinity of CRP for DNA is dependent on cAMP. It has been proposed that cAMP may elicit a conformational change in CRP necessary for DNA binding (Anderson *et al.*, 1971). This hypothesis was supported by the observation that cAMP increased the susceptibility of CRP to digestion by proteolytic enzymes (Krakow and Pastan, 1973). In this report we will present direct physical evidence that a conformational change does occur in a fluorescent-labeled CRP upon binding of cAMP. The labeled CRP is fully active in binding cAMP and promoting cAMP-dependent *gal* transcription. The conformational change observed is induced only by cAMP and its biologically active analogs, but not by other nucleotides. In addition, a different conformational change of CRP occurs when it interacts with the *lac* operon-containing DNA in the presence of cAMP. Nanosecond fluorescence depolarization measurements indicate that cAMP does not induce association or dissociation of CRP.

Materials and Methods

Chemicals. cAMP and [^{3}H]cAMP (28 Ci/mmol) were obtained from Schwarz BioResearch. Tubercidin 3′,5′-monophosphate was a kind gift of Dr. G. B. Whitfield, Jr., Upjohn Co. ε-cAMP, AMP, ADP, ATP, GTP, CTP, and UTP were from P-L Biochemicals. Other cyclic nucleotides, *N*-tris(hydroxymethyl)methylglycine (Tricine), and 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) were purchased from Sigma. *N*-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulfonate (1,5-I-AENS) was synthesized as described by Hudson and Weber (1973).

cAMP Receptor Protein, RNA Polymerase, and DNA. The cAMP receptor protein (CRP) was purified from *E. coli* B by a modified procedure of Anderson *et al.* (1971). The procedure involved disruption of cells with a Manton-Gaulin laboratory homogenizer, centrifugation to remove cell debris, batchwise treatment of the crude extract with DEAE-cellulose, and chromatography of the eluate on phosphocellulose. Two phosphocellulose columns were used, one at pH 6.8 and the other at pH 7.7. The CRP eluted by a linear salt gradient showed a peak at 0.5 M KCl at pH 6.8 and at 0.26 M KCl at pH 7.7. After ammonium sulfate precipitation (at 60%), the protein was further purified by passage through a DEAE-Sephadex column (at pH 7.7). Such purified CRP preparation possessed a specific activity of 6300 units/mg of protein (one unit is defined as that amount of CRP required to bind 1 pmol

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[1] Abbreviations used are: CRP, cAMP receptor protein; NTP, nucleoside triphosphate; 1,5-I-AENS, *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate; AENS, *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate group; cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; cTuMP, tubercidin 3′,5′-monophosphate; ε-cAMP, 1,N^6-ethenoadenosine 3′,5′-monophosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; Nbs_2 5,5′-dithiobis(2-nitrobenzoic acid); PP_i, inorganic pyrophosphate; *lac*, the lactose operon; *gal*, the galactose operon; SDS, sodium dodecyl sulfate.

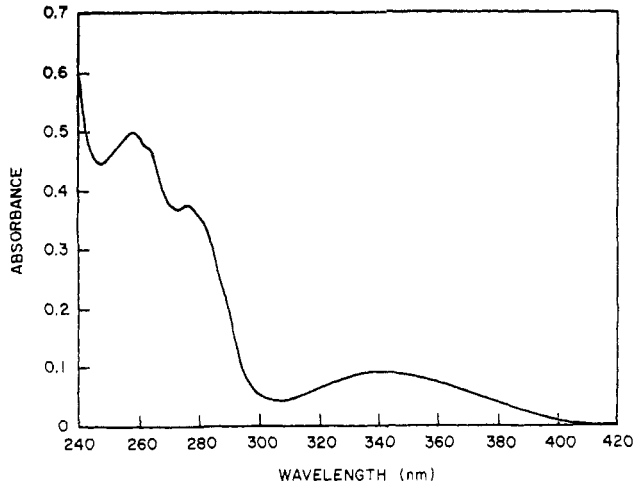

FIGURE 1: Absorption spectrum of AENS-CRP (1.1×10^{-5} M) in 0.05 M Tris-HCl (pH 8), 0.15 M KCl, 0.01 M $MgCl_2$, and 0.1 mM dithiothreitol (buffer C). The molar ratio of dye to protein is 1.4:1.

of [^{3}H]cAMP). SDS polyacrylamide gel electrophoresis (Weber and Osborn, 1969) showed that the CRP prepared as above was at least 98% pure. RNA polymerase was prepared from *E. coli* B as described previously (Wu and Wu, 1973). The enzyme had a specific activity of 1000–2000 units/mg of protein and was judged to be at least 95% pure by SDS polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The λ*h80dlac* and λ*h80* DNA were generous gifts from Dr. H. F. Kung. T7 DNA was prepared by Dr. L. Yarbrough and λ*pgal25* DNA was extracted from purified λ*pgal25c-I847Sam7* phage as described by Nissley *et al.* (1971). The phage was prepared by heat induction of a lysogenic strain (M-9) which was a gift from Dr. I. Pastan.

cAMP Binding Assay and in Vitro Transcription of Gal Operon. The cAMP binding activity of CRP was assayed by the procedure of Anderson *et al.* (1971), in which the CRP-bound [^{3}H]cAMP was precipitated with cold saturated ammonium sulfate. The reaction mixture (0.1 ml) contained 10^{-6} M [^{3}H]cAMP (7000 cpm/pmol), 0.01 M Tris-HCl (pH 8), 0.01 M AMP, 0.01 M $MgCl_2$, and 1–5 μg of CRP. After standing for 5 min in ice, 0.4 ml of cold saturated ammonium sulfate was added. The precipitate was collected by centrifugation, dissolved in 0.5 ml of water, and counted in a liquid scintillation counter with Bray's solution. The ability of CRP to stimulate transcription of the *gal* operon was determined by a modification of the procedure of Nissley *et al.* (1971). The reaction mixture (0.25 ml) contained 25 mM Tris-HCl (pH 8), 0.15 M KCl, 0.01 M $MgCl_2$, 0.2 mM dithiothreitol, 0.1 mM [^{3}H]GTP (8000 cpm/nmol), 0.15 mM ATP, UTP, and CTP, 0.1 mM cAMP, 10 μg of λ*pgal25* DNA, and 3 μg of RNA polymerase, in either the presence or absence of the indicated amount of CRP. After preincubation of the mixture without enzyme for 3 min at 37°, the reaction was initiated by addition of RNA polymerase and terminated after 10 min at 37° by addition of 0.2 ml of 0.1 M PP_i and 1 ml of cold 5% trichloroacetic acid. The resulting precipitate was collected on glass fiber filters (Whatman GF/C, 2.4 cm), washed with cold 1% trichloroacetic acid, dried, and counted with a toluene-based scintillation fluid. The addition of CRP and cAMP evoked a 2.2-fold stimulation in total RNA synthesis in this assay. This increase in total RNA synthesis is due to the read through into λ genes from the specific cAMP–CRP-sensitive site in the *gal* region (Nissley *et al.*, 1971), and thus provides a simple means of measuring the stimulatory effect of CRP and cAMP on *gal* transcription.

Fluorescent Labeling of CRP and Sulfhydryl Group Analysis. CRP labeled with 1,5-I-AENS was prepared by addition of the fluorescent labeling reagent to a solution of CRP in 0.02 M Tris-HCl (pH 8), 0.2 M KCl, 0.1 mM EDTA, and 5% glycerol. The molar ratio of 1,5-I-AENS to CRP ranged from 10 to 40. The reaction mixture was incubated for 1 hr at room temperature and overnight at 4° in the dark. The free dye was then removed by passing the mixture through a Sephadex G-25 column followed by extensive dialysis against 0.05 M Tris-HCl (pH 8), 0.15 M KCl, 0.01 M $MgCl_2$, and 0.1 mM dithiothreitol (buffer C). The stoichiometry of labeling was determined either by using tritiated 1,5-I-AENS or by measuring the absorbance of the bound AENS ($\epsilon_{340\ nm}$ 6000 M^{-1} cm^{-1}) (Hudson and Weber, 1973). Sulfhydryl groups of CRP before and after fluorescent labeling were determined with Nbs_2 according to the method of Ellman (1959).

CRP labeled with dansyl chloride was prepared as follows. CRP (1–2 mg) was first dialyzed against 0.02 M Tricine buffer (pH 8.5) containing 0.15 M KCl, 0.1 mM EDTA, and 5% glycerol. A suspension of 10% dansyl chloride on Celite in Tricine buffer was then reacted with dialyzed CRP (molar ratio of dye to CRP was 50:1) for 5 min at 25°, and the reaction mixture was immediately centrifuged. The supernatant was passed through a Sephadex G-25 column to remove the free dye. Protein concentrations were determined by the methods of Bücher (1947) and Lowry *et al.* (1951).

Absorption and Fluorescence Spectroscopy. Absorption spectra were measured with a Cary 118-C recording spectrophotometer. A cell path length of 1 cm was used. Fluorescence measurements were performed on a Hitachi Perkin-Elmer MPF-3 spectrofluorometer. Peak absorbance of samples were less than 0.05 to obviate inner filter effects. All spectral measurements were made at 22°.

Nanosecond Fluorescence Depolarization Measurements. The kinetics of fluorescence emission in the nanosecond time range were measured by the single photon counting technique (Lami *et al.*, 196′) using an Ortec 9200 nanosecond fluorescence spectrometer. The sample was repetitively excited by light pulses which had a full width at half-maximum of approximately 1 nsec. The exciting light was filtered by a Corning 7-60 filter and polarized in the y direction by a Polaroid HNB sheet polarizer. The emitted light was detected through a Kodak Wratten 65(A) filter at 90° to the incident light. The y and x components of the fluorescence ($F_y(t)$ and $F_x(t)$) were obtained by rotating a sheet polarizer through 90° on the emission side. These data were recorded on a multichannel analyzer, which was interfaced to a PDP-11 digital computer. The time-dependent emission anisotropy, $A(t)$, as a function of time (Perrin, 1934; Jablonski, 1961)

$$A(t) = [F_y(t) - F_x(t)]/[F_y(t) + 2F_x(t)] \quad (1)$$

was calculated by computer analysis of the data.

Results

Fluorescent Labeling of cAMP Receptor Protein. When CRP was incubated with the fluorescent reagent, 1,5-I-AENS, at pH 8, 1.4 mol of the dye was bound per mol of the protein (mol wt 45,000). The stoichiometry of the labeling was determined by the absorption spectrum of the labeled CRP (AENS–CRP) as shown in Figure 1, and by the incorporation of [^{3}H]AENS. The results obtained by these two methods were essentially the same. Prolonged incubation (24 hr) at 4° with

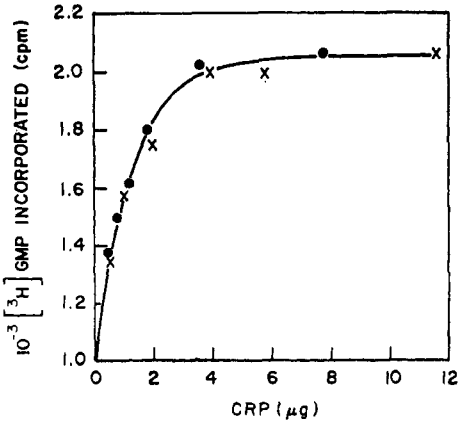

FIGURE 2: Effect of CRP and AENS-CRP concentrations on the synthesis of RNA with λ*pgal25* DNA as template. Stimulation of total transcription was determined as described under Materials and Methods with the indicated amounts of CRP or AENS-CRP. Activity is reported as total cpm of trichloroacetic acid precipitable [^{3}H]RNA product: (×) CRP; (●) AENS-CRP.

a large excess (more than 50-fold) of fluorescent reagent did not significantly increase the stoichiometry of the labeling.

CRP was also labeled with dansyl chloride using the Celite method (Rinderknecht, 1960). The dansyl-CRP obtained has a molar ratio of dye to protein of 1. SDS polyacrylamide gel electrophoresis (Weber and Osborn, 1969) showed that the AENS and dansyl groups were covalently attached to the protein. A Nbs_2 titration of an unmodified CRP at pH 8 yielded 1.5 reactive sulfhydryl residues per CRP molecule. AENS–CRP had less than 0.1 sulfhydryl residue available for reaction with Nbs_2, while dansyl-CRP still possessed 1.2 reactive sulfhydryl groups. Thus, AENS was covalently attached to the sulfhydryl residues of CRP, whereas dansyl chloride reacted primarily at a different site.

The insertion of the fluorescent probes did not significantly alter the biological activity of CRP. The dissociation constant of the cAMP–CRP complex was the same for unlabeled and labeled CRP (both dansyl- and AENS–CRP) as measured by ammonium sulfate precipitation assay (and by fluorescence titration as described below). Moreover, as shown in Figure 2, AENS–CRP was equally as active in stimulating *in vitro gal* transcription as unlabeled CRP. Similar properties were observed for dansyl-CRP.

Fluorescence Properties of AENS–CRP. The fluorescence emission spectra of AENS–CRP in the presence and absence of cAMP are given in Figure 3. When 1×10^{-4} M cAMP was added to a solution of AENS–CRP, there was a 30% increase in fluorescence intensity. In addition, the emission maximum was shifted from 480 to 470 nm. The fluorescence enhancement and blue shift indicate a conformational transition of AENS–CRP upon binding of cAMP.

The effect of different concentrations of cAMP on the fluorescence intensity of AENS–CRP is shown in Figure 4. The fluorescence intensity at 470 nm increased with increasing cAMP concentration. The concentration of cAMP required for half-maximum fluorescence enhancement was 5×10^{-6} M. This value is in good agreement with the dissociation constant of the cAMP–CRP complex determined by equilibrium dialysis (Anderson *et al.*, 1971). The titration curve shown in Figure 4 was carried out in the presence of 10 mM $MgCl_2$. Fluorescence enhancement was not observed in the absence of $MgCl_2$. On the other hand, when cAMP binding to CRP was measured by the $(NH_4)_2SO_4$ precipitation assay (Riggs *et al.*, 1971), the binding was not dependent on divalent metals.

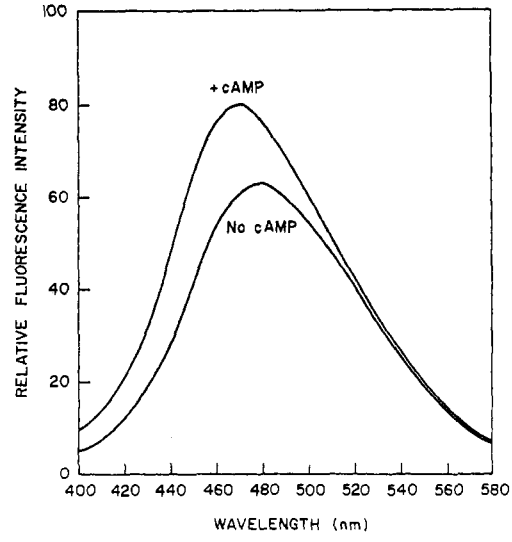

FIGURE 3: Effect of cAMP on the fluorescence emission spectrum (uncorrected) of AENS-CRP. The solution contained 5.3×10^{-7} M AENS-CRP in buffer C either in the presence or absence of 1×10^{-4} M cAMP. The excitation wavelength was 340 nm.

Effect of Other Cyclic and Noncyclic Nucleotides. A number of the cAMP derivatives have been shown to inhibit cAMP binding to CRP and to decrease cAMP–CRP-dependent transcription (Anderson *et al.*, 1972). However, only tubercidin 3′,5′-monophosphate (cTuMP) was reported (Nissley *et al.*, 1971) to be effective in stimulating *gal* transcription. In order to determine the specificity of the cAMP-induced conformational transition of CRP described above, various cAMP analogs and purine nucleotides were tested with regard to their ability to change the spectroscopic properties of AENS–CRP. The results obtained are summarized in Table I. Of all analogs and nucleotides studied, only cTuMP and N^6,$O^{2'}$-dibutyryl-cAMP were able to mimic cAMP action in producing both fluorescence enhancement and blue shift of the emission spectra of AENS–CRP. In contrast, cyclic 3′,5′-GMP (cGMP) and 1,N^6-etheno-cAMP (ε-cAMP) decreased the fluorescence intensity with no apparent shift in the wavelength maximum. Noncyclic purine nucleotides and 2′,3′ analogs of cAMP have little effect on the fluorescence properties of AENS–CRP.

Table II shows the effects of various cAMP analogs on the

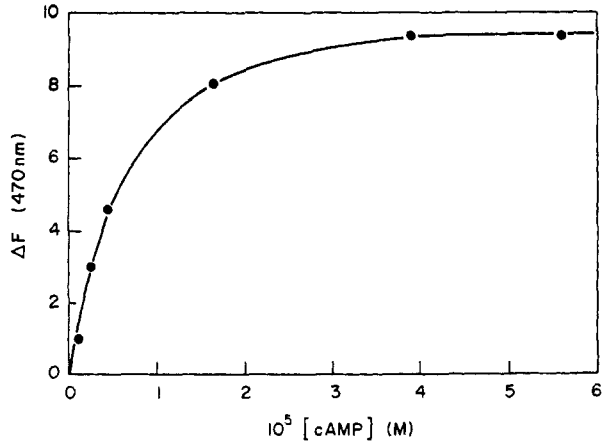

FIGURE 4: Fluorimetric titration of AENS-CRP with cAMP. The solution used was as described in Figure 3. The concentrations of cAMP were varied from 1.2×10^{-6} to 5.6×10^{-5} M. ΔF represents the increase in relative fluorescence intensity at 470 nm.

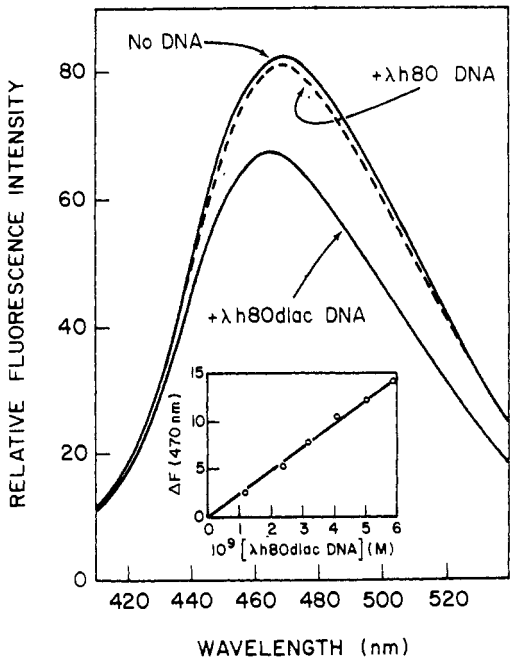

FIGURE 5: Effect of λ*h80dlac* DNA and λ*h80* DNA on the fluorescence emission spectrum of AENS-CRP in the presence of cAMP. The solution (0.5 ml) contained 5.3×10^{-7} M AENS-CRP and 1×10^{-4} M cAMP in buffer C in the absence or presence of 6×10^{-9} M of λ*h80dlac* DNA or λ*h80* DNA as indicated in the figure. The decrease in relative fluorescence intensity at 470 nm (ΔF) as a function of the λ*h80dlac* concentration is shown in the inserted plot. The excitation wavelength was 340 nm.

synthesis of RNA with λ*pgal* DNA as template. The observations that cTuMP stimulates the *gal* transcription and cGMP is a competitive inhibitor of cAMP are consistent with previous reports (Nissley *et al.*, 1971; Emmer *et al.*, 1970). In addition, we have found that $N^6,O^{2'}$-dibutyryl-cAMP could replace cAMP in promoting *gal* transcription and ε-cAMP acted as an inhibitor of the cAMP–CRP-dependent *gal* transcription.

Interactions with DNA Containing lac Operon. In the absence of cAMP, addition of λ*h80dlac* DNA showed no significant change in the fluorescence properties of AENS–CRP. In the presence of cAMP (1×10^{-4} M), however, λ*h80dlac* DNA (6×10^{-9} M) produced quenching (18%) of fluorescence intensity and a blue shift (5 nm) of the emission maximum (Figure 5). No such changes were observed when either λ*h80* DNA or T7 DNA (not shown) was added in the presence or absence of cAMP.

Thus, again, we have observed a specific cAMP-dependent conformational transition of CRP upon binding to *lac* operon-containing DNA. Unfortunately, neither the binding constant nor the stoichiometry of the CRP-λ*h80dlac* DNA interaction could be obtained from the data. The fluorimetric titration curve with various λ*h80dlac* DNA concentrations is also shown in Figure 5. The straight line indicates stoichiometric binding of CRP to the DNA without the existence of appreciable free DNA. Due to the large molecular weight and viscosity of λ*h80dlac* DNA, high concentrations of the DNA were difficult to achieve experimentally. At the highest DNA concentration used (6×10^{-9} M), saturation was still not reached, indicating that the binding constant has a magnitude much less than 10^{-9} M.

Fluorescence Depolarization of Dansyl-CRP. The fluorescence depolarization measurements were carried out in order to determine the size and shape of CRP. Dansyl-CRP was selected for this purpose because its high A_0 (emission anisotropy in the absence of rotation) value. The excitation and emission maxima of dansyl-CRP were 340 and 480 nm, respectively. The nanosecond emission kinetics of dansyl-CRP could be fitted to a single excited state lifetime of 21 nsec.

TABLE I: Effect of cAMP Analogs and Other Nucleotides on Fluorescence Properties of AENS–CRP.[a]

Nucleotides Added	Concn (M)	λ_{max} Shift	Fluorescence Intensity at Peak
3′,5′-cAMP	10^{-4}	Blue shift (10 nm)	Increase (30%)
2′,3′-cAMP	10^{-4}	—	—
ε-cAMP	10^{-4}	—	Decrease (12%)
$N^6,O^{2'}$-Dibutyryl-cAMP	10^{-4}	Blue shift (10 nm)	Increase (16%)
3′,5′-cGMP	10^{-4}	—	Decrease (8%)
2′,3′-cGMP	10^{-4}	—	—
cTuMP	10^{-4}	Blue shift (10 nm)	Increase (30%)
AMP	10^{-3}	—	—
ADP	10^{-3}	—	—
ATP	10^{-3}	—	—
GTP	10^{-3}	—	—
UTP	10^{-3}	—	—
CTP	10^{-3}	—	—

[a] — indicates no change.

TABLE II: Effect of Various cAMP Analogs on the Synthesis of RNA with λ*pgal25* DNA as Template.[a]

Cyclic Nucleotides Added	Concn (M)	[³H]GMP Incorporated	
		pmol	Ratio
		145	1.00
cAMP	10^{-6}	178	1.23
	10^{-5}	287	1.98
	10^{-4}	303	2.10
cTuMP	10^{-6}	275	1.90
	10^{-5}	280	1.95
	10^{-4}	303	2.10
$N^6,O^{2'}$-Dibutyryl-cAMP	10^{-6}	164	1.13
	10^{-5}	229	1.58
	10^{-4}	293	2.04
cGMP	10^{-4}	136	0.94
	10^{-3}	153	1.06
ε-cAMP	10^{-4}	153	1.05
	10^{-3}	130	0.89
cGMP + cAMP	10^{-3}, 10^{-5}	170	1.17
ε-cAMP + cAMP	10^{-3}, 10^{-5}	137	0.95

[a] The reaction mixture (0.2 ml) contained 25 mM Tris-HCl (pH 8), 0.125 M KCl, 0.01 M $MgCl_2$, 0.2 mM dithiothreitol, 0.1 mM [³H]GTP (8000 cpm/nmol), 0.15 mM each of ATP, UTP, and CTP, 10 μg of λ*pgal25* DNA, 8 μg of CRP, 3 μg of RNA polymerase, and cyclic nucleotides as indicated. The reaction was initiated with RNA polymerase after preincubation of the reaction mixture without the enzyme for 3 min at 37°. The reaction mixture was further incubated for 10 min at 37°. The trichloroacetic acid precipitable counts were determined in a liquid scintillation counter.

Figure 6 shows the results obtained for the nanosecond fluoresence depolarization measurements of dansyl-CRP. The linear plot of log $A(t)$ *vs.* time indicated that to a good approximation the emission anisotropy of dansyl-CRP obeys the relationships for a rigid sphere (Jablonski, 1961; Perrin, 1934)

$$A(t) = A_0 e^{-t/\phi} \quad (2)$$

where A_0 is the anisotropy at the instant of excitation, and ϕ, the rotational correlation time. The value of ϕ was calculated to be 55 nsec at 22° using a least-squares analysis.

In contrast to what was observed for AENS–CRP, addition of 10^{-4} M cAMP caused a 10% quenching of the fluorescence intensity with no change in the emission maximum of dansyl-CRP. In the presence of 10^{-4} M cAMP the emission anisotropy plot of dansyl-CRP was also linear and a rotational correlation time of 52 nsec was obtained.

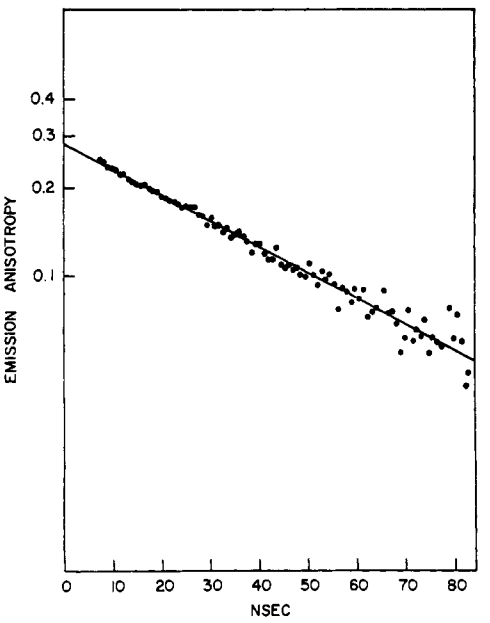

FIGURE 6: Time dependence of the emission anisotropy of dansyl-CRP in buffer C at 22°. The solid line is the least-squares fit of the observed data to a single exponential decay (eq 2) for $A_0 = 0.28$, and $\phi = 55$ nsec.

Discussion

On the basis of amino acid composition, CRP contains 4 mol of half-cystine residues/45,000 g of protein (Anderson *et al.*, 1971), of which only 1.5 residues are accessible for reaction with Nbs_2 in the native protein (at pH 8). It has been suggested (Anderson *et al.*, 1971) that the presence of sulfhydryl groups is essential for activity since *p*-chloromercuribenzoate (10^{-4} M) inhibits cAMP binding activity by about 50%. However, we have found that AENS–CRP, in which over 90% of the free sulfhydryl residues have been modified still retains its full activity in binding cAMP and in promoting transcription of the *gal* operon. This suggests that these sulfhydryl residues do not directly participate in binding cAMP; rather they may assist in maintaining the proper tertiary or quaternary structure of CRP which is required for biological activity. The modification of these sulfhydryl residues by thiol reagents may or may not significantly alter the conformation of CRP, depending on the modifying reagents used.

Hudson and Weber (1973) have shown that 1,5-I-AENS is an environment-sensitive fluorescent probe because the emission maximum shifts continuously to the blue as the polarity of the region around the probe decreases, while the quantum yield increases. Since AENS is covalently attached to the sulfhydryl residues of CRP, the blue shift of emission maximum and increase in fluorescence intensity of the probe accompanying cAMP binding (Figure 3) signal a cAMP-induced conformational change of CRP in such a manner that the microenvironment of these sulfhydryl residues becomes less polar.

Although a number of cAMP analogs apparently bind to CRP (Anderson *et al.*, 1972), only cTuMP (Nissley *et al.*, 1971) and N^6,$O^{2'}$-dibutyryl-cAMP–CRP complex are capable of stimulating *gal* transcription. It is apparent that cAMP is very specific in its ability to combine with CRP in a way that results in enhanced transcription. The specificity observed for the cyclic nucleotide induced conformational change of AENS–CRP (as characterized by the blue shift and increased intensity in fluorescence emission) coincides with the specificity observed for cyclic nucleotide–CRP stimulation of *gal* transcription. Competitive inhibitors of cAMP binding to CRP, such as cGMP and ϵ-cAMP, did not elicit such a conformational change. Thus, the conformational change of CRP induced by cAMP is a prerequisite for the cAMP-dependent gene transcription. This is consistent with the finding that the dissociation constant of the cAMP–AENS–CRP complex determined by fluorimetric titration (5×10^{-6} M) is in good agreement with the concentration of cAMP needed for half-maximal stimulation of *gal* transcription (Nissley *et al.*, 1971).

The nanosecond fluorescence depolarization studies (Figure 6) show that the plot of $A(t)$ *vs.* time for dansyl-CRP is linear, yielding a single rotational correlation time ϕ of 55 nsec. For a rigid sphere, ϕ is related to the molecular weight M by (Perrin, 1934; Jablonski, 1961)

$$\phi = [\eta(\bar{v} + h)M]/RT \quad (3)$$

where η is the viscosity of the solution, $\bar{v}$ is the specific volume, h is the hydration, R is the gas constant, and T is the absolute temperature. Most proteins studied thus far by nanosecond polarization techniques exhibit a single rotational correlation time sufficiently large to correspond to the motion of a rigid sphere. Owing to hydration and departure from spherical symmetry of the protein, the observed rotational correlation time for globular proteins are usually 1.4–2.4 times longer than that calculated for a rigid, unhydrated sphere of the molecular weight of the protein (data summary for eight proteins in Table I; Yguerabide *et al.*, 1970). If dansyl-CRP were a rigid unhydrated sphere, ϕ would be 13 nsec for mol wt 45,000, $\bar{v} = 0.75$ ml/g and $\eta = 0.95$ cP at 22°. The high ratio (4.2) of the observed to the calculated ϕ for AENS–CRP raises the possibility that some oligomers of AENS–CRP larger than dimer (mol wt 45,000) may exist. (Additional physical measurements are necessary to clarify this point.) Nevertheless, an important finding here is that in the presence of 10^{-4} M cAMP, ϕ decreases from 55 to 52 nsec. The magnitude of this change is too small to be associated with a dissociation of CRP into its monomers in the presence of cAMP. The value of ϕ would be 9 nsec for a spherical monomeric CRP (mol wt 22,500) with a hydration of 0.3 ml/g at 22°.

There are two mechanisms by which CRP–cAMP might stimulate the *lac* or *gal* transcription: (a) by interacting with DNA, and/or (b) by interacting with RNA polymerase. A cAMP-dependent binding of CRP to $\lambda h80dlac$ DNA was demonstrated by a membrane filter assay and sucrose density gradient analysis (Nissley *et al.*, 1972; Riggs *et al.*, 1971).

However, no specificity was found for this interaction, since λ*h80* DNA and several other DNAs exhibited similar binding. By use of fluorescent probes, we have been able to distinguish the interaction between CRP and λ*h80dlac* DNA from that between CRP and λ*h80* DNA. A blue shift and a quenching of the emission spectrum of AENS–CRP were observed upon binding to λ*h80dlac* DNA, but not to λ*h80* DNA. This suggests that another conformational change of CRP occurs when CRP interacts specifically with the *lac* operon. The binding of AENS–CRP with λ*h80dlac* DNA is very tight ($K_d \ll 10^{-9}$ M). Unfortunately, the exact dissociation constant and stoichiometry of the binding could not be determined due to the limitation in the sensitivity of the fluorescence titration technique and the large molecular weight of the DNA. It is possible that the technique described by Gilbert and Maxam (1973) may be used to isolate small DNA fragments containing the *lac* operon.

So far no evidence has been presented to indicate that CRP binds to RNA polymerase. Our preliminary results indicate that RNA polymerase causes a blue shift (5 nm) of the emission spectrum of AENS–CRP (F. Y.-H. Wu and C.-W. Wu, unpublished results). However, this effect was not dependent on cAMP and the spectral changes were too small for quantitative analysis. Furthermore, the interaction of CRP with RNA polymerase in the presence of *lac* DNA could not be properly studied due to the difficulty in achieving stoichiometric amounts of DNA with respect to the amount of CRP used in spectroscopic studies. Nevertheless, the observed interaction between CRP and RNA polymerase, though requiring further elucidation, has raised the possibility of a combined mechanism of (a) and (b) described above.

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