Conformational Transitions of Cyclic Adenosine Monophosphate Receptor Protein of *Escherichia coli*. A Temperature-Jump Study†

Cheng-Wen Wu*‡ and Felicia Y.-H. Wu

ABSTRACT: Temperature-jump studies with fluorescence detection were made of the interactions of cAMP and its analogs with the cAMP receptor protein of *Escherichia coli* (CRP) which had been covalently labeled with *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate (1,5-I-AENS). A single relaxation process was observed for a solution containing the labeled CRP (AENS–CRP) in the absence of cyclic nucleotides. The relaxation time of this process is independent of the concentration of AENS–CRP, suggesting an interconversion between two isomeric forms of AENS–CRP. Two relaxation processes were observed for solutions containing AENS–CRP and cAMP. While the faster process reflects the bimolecular binding reaction, the slower process, which is apparently the same as that observed in the absence of cAMP, is associated with the conformational transition of AENS–CRP. The reciprocal relaxation time of the slower process decreases with increasing cAMP concentration, and can be quantitatively analyzed in terms of mechanism in which two different conformational forms of CRP are in equilibrium and the preferential binding of cAMP to one form leads to a shift of the equilibrium to this form. We propose that the form which favors cAMP is the biologically active form required for promoting gene transcription. This hypothesis is consistent with the finding that tubercidin 3′,5′-monophosphate, which is as effective as cAMP in stimulating transcription of the *gal* operon, also shifts the conformational equilibrium toward the active isomer. On the other hand, cGMP, a competitive inhibitor of cAMP, prevents cAMP from affecting the conformational transition. Thus the conformational transitions of CRP may play an important role in regulation of gene transcription.

Important developments have taken place in many areas of biochemical genetics in the past decade. However, molecular mechanisms of gene expression are still open questions in biology. Physicochemical techniques are useful for the elucidation of these molecular mechanisms.

In intact *Escherichia coli* cells cAMP[1] was found to stimulate the synthesis of inducible enzymes (Pastan and Perlman, 1970). This stimulation requires a specific protein, referred to as the cAMP receptor protein (CRP). *In vitro* transcription studies with purified CRP (Anderson *et al.*, 1971; Riggs *et al.*, 1971) have confirmed that in the presence of cAMP, CRP stimulates the transcription of the *lac* (de Crombrugghe *et al.*, 1971) and *gal* (Parks *et al.*, 1971) operons, presumably by binding at the promotor site of the genome and increasing the frequency of initiation.

In order to gain insight into the molecular mechanism of CRP action, we have carried out a fluorescent probe study of CRP (Wu *et al.*, 1974a). An environment-sensitive fluorescent probe, *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate (1,5-I-AENS), was covalently reacted with sulfhydryl residues of CRP. The labeled protein AENS–CRP was fully active in binding cAMP and promoting the *gal* transcription. Addition of cAMP produced a conformational transition of AENS–CRP as characterized by a blue shift and enhancement of its fluorescence emission. More interestingly, the conformational transition observed was induced only by cAMP and its biologically active analogs; cyclic nucleotides which bound to CRP but were biologically inactive did not have such effect. This correlation suggests that the observed conformational transition of AENS–CRP may be related to the biological activity of CRP.

Since the fluorescence properties of AENS–CRP are very sensitive to alterations of its environment, by monitoring fluorescence changes in kinetic experiments both structural and kinetic information can be obtained. Here, we present kinetic studies of the interactions of AENS–CRP with cAMP and other cyclic nucleotides using the fluorescence temperature-jump method. The data obtained are consistent with a mechanism in which a conformational equilibrium exists between two forms of AENS–CRP. cAMP binds preferentially to one of the two isomeric forms and thereby induces the transition from one form to the other. cTuMP, a biologically active analog, shifts the conformational transition in the same direction as cAMP does. On the other hand, cGMP, a competitive inhibitor of cAMP, prevents the alteration of the conformational equilibrium by cAMP. Biological implications of the mechanisms consistent with the kinetic data are discussed.

Experimental Section

Adenosine 3′,5′-monophosphate (cAMP) and guanosine 3′,5′-monophosphate (cGMP) were purchased from Schwarz BioResearch. Tubercidin 3′,5′-monophosphate (cTuMP) was a gift of Dr. G. B. Whitfield, Jr., Upjohn Co. *N*-(Iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate (1,5-I-AENS) was synthesized as described by Hudson and Weber (1973). All

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‡ Research Career Development awardee of the National Institutes of Health.

[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; cTuMP, tubercidin 3′,5′-monophosphate; 1,5-I-AENS, *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate; AENS, *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate group; CRP, cAMP receptor protein; SDS, sodium dodecyl sulfate; *lac*, the lactose operon; *gal*, the galactose operon.

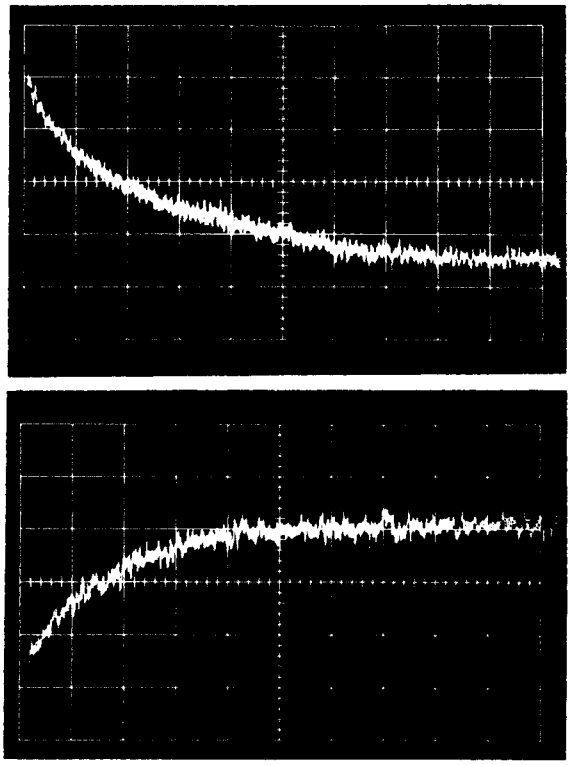

FIGURE 1: Oscilloscope traces of temperature-jump relaxation effects of AENS–CRP in buffer C. The concentration of AENS–CRP was 2.5×10^{-6} M in both traces. The excitation wavelength was 350 nm and the emission was isolated from the excitating light by a Kodak Wratten filter 65(A). The temperature jump was from 17.5 to 25°. The vertical scale is fluorescence intensity in arbitrary units (increase in signal corresponding to decrease in fluorescence intensity). Upper trace: the slow relaxation effect (τ_0) observed in the absence of cyclic nucleotide; the horizontal scale is 20 msec/large division. Lower trace: the fast relaxation effect (τ_2) observed in the presence of 1.8×10^{-5} M cAMP; the horizontal scale is 0.5 msec/large division.

other chemicals were reagent grade or the best commercially available grades.

The cAMP receptor protein (CRP) was purified from *Escherichia coli* B (midlog, General Biochemicals) by a modified procedure of Anderson *et al.* (1971) and Wu *et al.* (1974a). The labeling of CRP with 1,5-I-AENS was carried out as described previously (Wu *et al.*, 1974a).

The equipment used for the temperature-jump relaxation measurements was a combined stopped flow-temperature-jump apparatus constructed in this laboratory based on the description of Faeder (1970) with some modifications. The temperature-jump cell assembly for fluorescence detection was built as described by del Rosario (1970). The reaction volume of this apparatus is about 0.3 ml and utilizes two cornical lenses at right angles to each other. An exciting wavelength of 350 nm from a 200-W Hanovia xenon-mercury arc lamp was isolated with a Bausch and Lomb grating monochromator. The emission wavelength, centered at about 480 nm, was isolated from the exciting wavelength by means of a Kodak Wratten 65(A) filter. The changes in fluorescence were detected with an EMI 9635QB photomultiplier tube. Signal-to-noise ratio of about 1000 was routinely obtained. Temperature jumps of 7.5° were applied to the solution by discharge of a 0.1-μF capacitor which had been charged with 10 kV. The resolution time of the apparatus is approximately 10 μsec.

All solutions used in the temperature-jump experiments were prepared from degassed, deionized, distilled water and

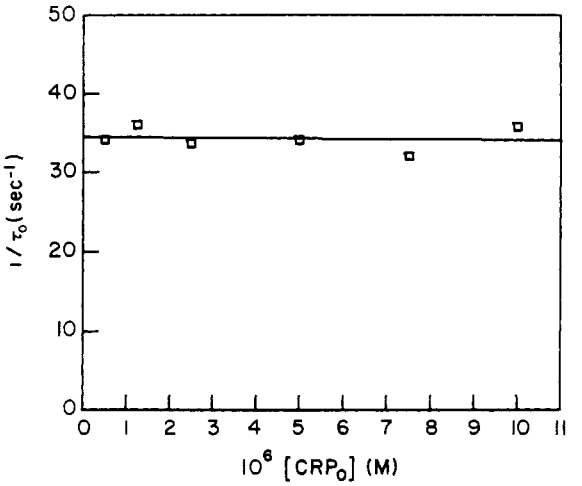

FIGURE 2: A plot of the reciprocal relaxation time, $1/\tau_0$, *vs.* the AENS–CRP concentration. Solutions were buffer C with AENS–CRP, concentrations as indicated.

contained 0.15 M KCl, 0.01 M $MgCl_2$, 0.1 mM dithiothreitol, 0.05 M Tris-HCl (pH 8), and AENS–CRP or cyclic nucleotides as indicated. The temperature-jump was from 17.5 to 25° in all cases.

The kinetic information was recorded on a Tektronic 543 storage oscilloscope and transferred directly on-line to a PDP-11 digital computer by a Biomation Model 802 transient recorder. This system transfers 1024 data points at a maximum rate of 1 point/0.5 μsec with a precision of 8 bits for each point. The relaxation times and amplitudes were calculated by a least-squares analysis that analyzes the data in terms of one or two relaxation processes. The data of successive measurements on the same reaction mixture also were accumulated to enhance the signal-to-noise ratio. A more complete description of a data collection and analysis system similar to the one used here is given by Hilborn (1972). In some cases the oscilloscope traces were photographed and analyzed graphically. The results were in good agreement with the computer analysis. The experimental uncertainty in the relaxation times was estimated to be about 10%.

Results and Treatment of Data

Temperature-Jump Studies with AENS–CRP. A single relaxation process (with the relaxation time τ_0) was seen with the temperature jump for solutions containing AENS–CRP in 0.05 M Tris-HCl (pH 8), 0.15 M KCl, 0.01 M $MgCl_2$, and 0.1 mM dithiothreitol (buffer C). A typical oscilloscope trace of the relaxation process following a temperature jump is given in Figure 1. Changes in fluorescence intensity of AENS–CRP were monitored in the experiment and elimination of the protein abolished the relaxation effect.

Figure 2 shows that the time constant ($\sim$34 sec^{-1}) associated with this relaxation effect is independent of the concentration of AENS–CRP. This suggests the existence of a unimolecular isomerization (or conformational transition) between two different states of CRP

$$(\text{CRP}') \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} (\text{CRP}) \qquad (1)$$

The reciprocal relaxation time for eq 1 is

$$1/\tau_0 = k_0 + k_{-0} \qquad (2)$$

and thus is not dependent on the CRP concentration.

Interaction of cAMP with AENS–CRP. Addition of cAMP to a solution of AENS–CRP in buffer C caused the appearance

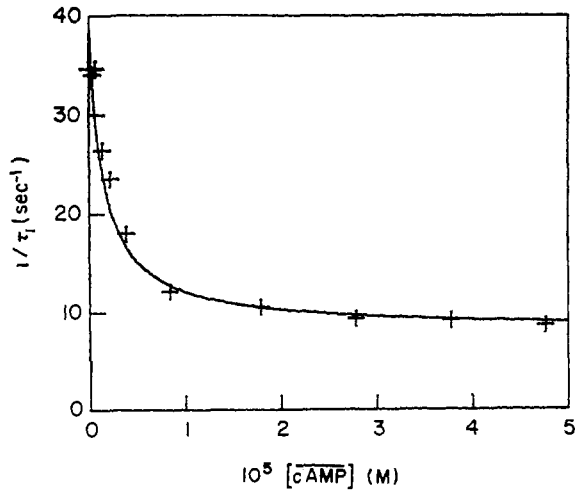

FIGURE 3: The reciprocal relaxation time, $1/\tau_1$, for the interaction of cAMP with AENS–CRP as a function of the equilibrium concentration of free cAMP. The initial concentration of AENS–CRP was 2.5×10^{-6} M. The initial concentration of cAMP varied from 1×10^{-6} to 5×10^{-5} M. The equilibrium concentrations of free cAMP were calculated according to eq 6 using the value of K (5×10^{-6} M) determined by fluorimetric titration (Wu *et al.*, 1974b). The solid line is the computer-fitted curve according to eq 9 and the parameters in Table I.

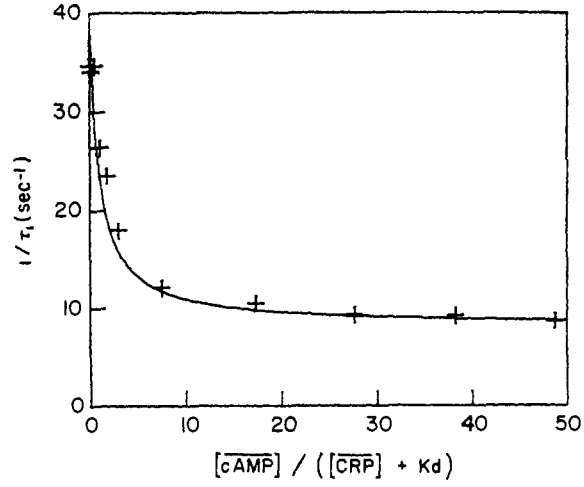

FIGURE 4: A plot of $1/\tau_1$ *vs.* [cAMP]/([cAMP] + K_d) with $K_d = 9.3 \times 10^{-7}$ M. The data points are the same as those plotted in Figure 3. The solid line is the computer-fitted curve according to eq 4 and the parameters in Table I.

of two well-defined relaxation processes, a slower process in the time range 25–125 msec (τ_1) and a faster process in the time range 0.2–2 msec (τ_2). An oscilloscope trace of the faster relaxation process is also shown in Figure 1.

The slower relaxation effect (τ_1) is apparently the same as that observed in the absence of cAMP (τ_0) and is associated with a conformational transition of CRP. The value of $1/\tau_1$ approaches that of $1/\tau_0$ at very low concentrations of cAMP ($<1 \times 10^{-6}$ M). In addition, $1/\tau_1$ is also independent of the total concentration of AENS–CRP added at constant cAMP concentrations which were much higher than the protein concentrations (data not shown).

The concentration dependence of the reciprocal relaxation time, $1/\tau_1$, as a function of the free cAMP concentration is shown in Figure 3. As can be seen in the figure, $1/\tau_1$ decreases rapidly as the concentration of cAMP increases and approaches a constant level at high cAMP concentrations. This rather unusual concentration dependence is characteristic of a mechanism involving an isomerization of the protein, with associated rate constants that are dependent on the degree of saturation of the protein with ligand. The simplest mechanism of this type is a two-step mechanism, as shown in eq 3, where (CRP′) and (CRP) are two isomeric forms of

$$(\text{CRP}') \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} (\text{CRP}) \underset{k_D}{\overset{\text{cAMP},\ k_A}{\rightleftharpoons}} (\text{CRP–cAMP}) \qquad (3)$$

slow fast

AENS–CRP, (CRP–cAMP) is the binary complex of cAMP and (CRP), k_0 and k_{-0} are rate constants associated with the interconversion between (CRP) and (CRP′), and k_A and k_D are rate constants for association and dissociation of the (CRP–cAMP) complex, respectively. If the bimolecular binding step is assumed to equilibrate much faster than the isomerization step, the two relaxation times associated with the two-step mechanism can be written as

$$\frac{1}{\tau_1} = k_0 + \frac{k_{-0}}{1 + \dfrac{[\overline{\text{cAMP}}]}{[\overline{\text{CRP}}] + K_d}} \qquad (4)$$

where $[\overline{\text{CRP}}]$ and $[\overline{\text{cAMP}}]$ are equilibrium concentrations of (CRP) and cAMP, respectively, and K_d ($= k_D/k_A$) is the dissociation constant of the (CRP–cAMP) complex. A PDP-11 computer was programmed to fit the experimental data to eq 4 and 5 using a nonlinear least-squares criterion. It can be seen from eq 4 that as $[\overline{\text{cAMP}}] \to 0$, $1/\tau_1 = k_0 + k_{-0}$ and as $[\overline{\text{cAMP}}] \to \infty$, $1/\tau_1 = k_0$. Thus k_0 and k_{-0} can be estimated by the value of $1/\tau_1$ when the free cAMP concentration is either zero or very high. As shown in Figure 3, $1/\tau_1$ approaches a constant value at high concentrations of cAMP. Furthermore, $[\overline{\text{CRP}}]$ and $[\overline{\text{cAMP}}]$ can be calculated from the overall binding constant (K) obtained by equilibrium measurements (Wu *et al.*, 1974a), and k_0 and k_{-0} estimated as described above, using the following relationships

$$K = \frac{([\overline{\text{CRP}}] + [\overline{\text{CRP}'}])[\overline{\text{cAMP}}]}{[\overline{\text{CRP–cAMP}}]} = \frac{([\text{CRP}]_0 - [\text{cAMP}]_0 + [\overline{\text{cAMP}}])[\overline{\text{cAMP}}]}{[\text{cAMP}]_0 - [\overline{\text{cAMP}}]} \qquad (6)$$

and

$$[\overline{\text{CRP}}] = \frac{[\text{CRP}]_0 - [\text{cAMP}]_0 + [\overline{\text{cAMP}}]}{1 + K_0} \qquad (7)$$

where $K_0 = k_{-0}/k_0$, $[\text{CRP}]_0$ and $[\text{cAMP}]_0$ are the initial concentrations of AENS–CRP and cAMP added, and $[\overline{\text{CRP–cAMP}}]$ is the equilibrium concentration of the (CRP–cAMP) complex. By an iterative procedure, a best-fit value of 9.3×10^{-7} M for K_d was given by the computer. A plot of $1/\tau_1$ *vs.* $[\overline{\text{cAMP}}]/([\overline{\text{CRP}}] + K_d)$ is shown in Figure 4. The computer-fitted curve is given as a solid line.

Figure 5 shows the concentration dependence of $1/\tau_2$ as a function of the sum $([\overline{\text{CRP}}] + [\overline{\text{cAMP}}])$. The linear relationship obtained suggests that the data are consistent with the two-step mechanism (eq 3). The solid straight line represents a least-squares fit of the data according to eq 5. The best-fit kinetic parameters for the two-step mechanism are listed in Table I. It can be seen that the value of K_d obtained from analysis of τ_1 (9.3×10^{-7} M) agrees reasonably with that from analysis of τ_2 (7.3×10^{-7} M).

In the two-step mechanism (eq 3), it is assumed that cAMP binds only to one of the two conformational states, (CRP).

$$1/\tau_2 = k_D + k_A([\overline{\text{CRP}}] + [\overline{\text{cAMP}}]) \qquad (5)$$

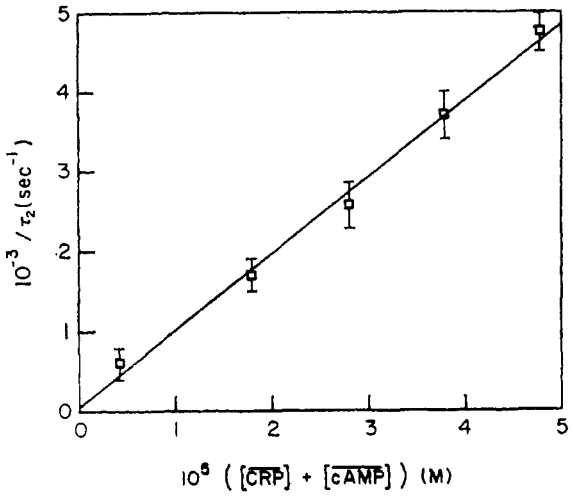

FIGURE 5: The reciprocal relaxation time, $1/\tau_2$, for the interaction of cAMP with AENS–CRP as a function of the sum of equilibrium concentrations of free (CRP) and cAMP. The initial concentration of AENS–CRP was 2.5×10^{-6} M. The initial concentrations of cAMP varied from 5×10^{-6} to 5×10^{-5} M. The calculation of the equilibrium concentrations are described in the text. The straight line was obtained by a least-squares analysis of the data.

One may argue that in reality this might not be the case; cAMP may also bind to the other form, (CRP'), possibly with a different affinity. A general mechanism with this consideration is given in eq 8 (the square mechanism)

$$\begin{array}{ccc} \text{cAMP} + (\text{CRP}') & \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} & (\text{CRP}) + \text{cAMP} \\ {\scriptstyle k_A'}\downarrow\uparrow{\scriptstyle k_D'} & & {\scriptstyle k_A}\downarrow\uparrow{\scriptstyle k_D} \\ (\text{CRP}'\text{–cAMP}) & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & (\text{CRP–cAMP}) \end{array} \tag{8}$$

where (CRP'–cAMP) is the binary complex of (CRP') and cAMP, k_A' and k_D' are rate constants for association and dissociation of this complex, k_1 and k_{-1} are rate constants associated with the interconversion between (CRP–cAMP) and (CRP'–cAMP). Again if the assumptions are made that (a) the vertical steps equilibrate rapidly relative to the horizontal steps, (b) the binding of cAMP to (CRP) is faster than to (CRP'), and (c) the concentration changes of the free cAMP are negligible (this is a good approximation if the cAMP concentration is much higher than that of CRP, a situation which is valid for most of the data obtained), the following analytical expressions are obtained for the three relaxation times associated with the square mechanism (eq 8)

$$\frac{1}{\tau_1} = \frac{k_{-0} + k_{-1}[\overline{\text{cAMP}}]/K_d}{1 + [\overline{\text{cAMP}}]/K_d} + \frac{k_0 + k_1[\overline{\text{cAMP}}]/K_d'}{1 + [\overline{\text{cAMP}}]/K_d'} \tag{9}$$

$$1/\tau_2 = k_D + k_A([\overline{\text{CRP}}] + [\overline{\text{cAMP}}]) \tag{10}$$

$$\frac{1}{\tau_3} = k_D' + k_A'\left\{\frac{[\overline{\text{CRP}'}]}{1 + \dfrac{[\overline{\text{CRP}}]}{K_d + [\overline{\text{cAMP}}]}} + [\overline{\text{cAMP}}]\right\} \tag{11}$$

where K_d' ($= k_D'/k_A'$) is the dissociation constant of the (CRP'–cAMP) complex and $[\overline{\text{CRP}'}]$ is the equilibrium concentration of the isomeric form (CRP').

There are six unknown parameters in eq 9: k_0, k_{-0}, k_1, k_{-1}, K_d, and K_d'. However, it is apparent from the equation that $1/\tau_1 = k_0 + k_{-0}$ as $[\overline{\text{cAMP}}] \rightarrow 0$ and $1/\tau_1 = k_1 + k_{-1}$ as $[\overline{\text{cAMP}}] \rightarrow \infty$. Thus, two of the unknown parameters can be eliminated by estimation of the values $k_0 + k_{-0}$ and $k_1 + k_{-1}$ from the experimental data shown in Figure 2. By varying the other four parameters, the computer gave the best-fit curve for $1/\tau_1$ to eq 9, which is shown as a solid line in Figure 2. The equilibrium concentration of cAMP was calculated from the equilibrium binding constant (5×10^{-6} M) according to an equation analogous to eq 6 for the square mechanism.

TABLE I: Kinetic Parameters.

Rate Constants	Equilibrium Constants
	Two-Step Mechanism
$k_0 = 8.1$ sec^{-1}	$K_0 = k_{-0}/k_0 = 3.8$
$k_{-0} = 30.6$ sec^{-1}	K_d (from τ_1) $= k_D/k_A = 9.3 \times 10^{-7}$ M
$k_A = 9.5 \times 10^7$ M^{-1} sec^{-1}	K_d (from τ_2) $= k_D/k_A = 7.3 \times 10^{-7}$ M
$k_D = 69$ sec^{-1}	$K = K_d(1 + K_0) = 4.5 \times 10^{-6}$ M
	Square Mechanism
$k_0 = 8.0$ sec^{-1}	$K_0 = k_{-0}/k_0 = 3.8$
$k_{-0} = 30.4$ sec^{-1}	$K_d = k_D/k_A = 1.5 \times 10^{-6}$ M
$k_A = 9.5 \times 10^7$ M^{-1} sec^{-1}	$K_d' = k_D'/k_A' = 3 \times 10^{-2}$ M
$k_D = 69$ sec^{-1}	
$k_1 = 8.1$ sec^{-1}	
$k_{-1} = 1.5 \times 10^{-3}$ sec^{-1}	$K = K_d\left(\dfrac{1 + K_0}{1 + \dfrac{K_d'}{K_d}K_0}\right) = 5.6 \times 10^{-6}$ M

The expression for the reciprocal relaxation time associated with the bimolecular reaction of cAMP with (CRP) (eq 10) is identical to that obtained for the two-step mechanism (eq 5). The fitting of the experimental data $1/\tau_2$ to eq 10 is, therefore, essentially the same as demonstrated in Figure 5. The calculated curves for $1/\tau_1$ and $1/\tau_2$ are in good agreement with the experimental data. However, a third relaxation process (τ_3, eq 11), predicted according to the square mechanism to be associated with the binding of cAMP to (CRP'), could not be detected. The best-fit kinetic parameters for the square mechanism are also listed in Table I.

Effects of cGMP and cTuMP. cGMP is a competitive inhibitor of cAMP, whereas cTuMP can replace cAMP in stimulating transcription of the *gal* operon (Nissley *et al.*, 1971). In order to determine whether cGMP or cTuMP affects the conformational transition between (CRP) and (CRP'), kinetic measurements were made to investigate the interactions of these two cyclic nucleotides with AENS–CRP. Two relaxation processes were observed when either cGMP or cTuMP was present in a solution of AENS–CRP. The faster relaxation process (in the time range $\sim 10^{-3}$ sec for cGMP and $\sim 10^{-4}$ sec cTuMP) has small amplitude and is presumably associated with the bimolecular binding of the cyclic nucleotides to AENS–CRP. The slower relaxation process in the time range ~ 100 msec obviously corresponds to the conformational transition of CRP (associated with τ_1), since the relaxation time approaches the same limiting value at low concentrations of cyclic nucleotides. The effects of different concentrations of these cAMP analogs on $1/\tau_1$ are summarized in Table II. Similar to the cAMP effect on $1/\tau_1$, the value of $1/\tau_1$ decreases as the concentration of cTuMP increases. However, variation of cGMP concentration does not change the τ_1 value significantly. Furthermore, the effect of cAMP on τ_1 diminishes when high concentration of cGMP is present.

Discussion

The reciprocal relaxation time associated with the single relaxation process observed in the absence of cyclic nucleotide

is independent of AENS–CRP concentration, suggesting a unimolecular interconversion between two forms of AENS–CRP. Anderson *et al.* (1971) have demonstrated a biologically inactive form of CRP which reactivates spontaneously over time intervals ranging from 20 hr to several days. This is to be contrasted to the relatively rapid conformational transition (in the time range 25–125 msec) of CRP described here. We have also been able to isolate the two forms of CRP which differed in cyclic AMP binding activity, but both displayed the same single band on SDS polyacrylamide gel electrophoresis (Wu *et al.*, 1974b). The characterization of these two forms of the protein is currently in progress. Whether these are related to the two interconverting forms of AENS–CRP observed in temperature-jump experiments remains to be elucidated.

The kinetic data obtained for the binding of cAMP to AENS–CRP are consistent with a simple two-step mechanism (eq 3) in which cAMP is assumed to bind only to one of the two isomeric forms of CRP, and a more general square mechanism (eq 8) in which binding of cAMP to both forms of CRP are considered. The best-fit kinetic parameters for these two mechanisms are summarized in Table I. There are very good agreements between the values of k_0 and k_{-0} calculated for these two mechanisms. The value of K_d obtained for the two-step mechanism (9.3×10^{-7} M) is also roughly consistent with that obtained for the square mechanism (1.5×10^{-6} M). The most striking finding is that in the square mechanism (eq 8) the value of K_d' obtained is four orders of magnitude larger than that of K_d. Physically this means that cAMP binds primarily to (CRP) and its binding to (CRP′) is almost negligible. This may explain why only two relaxation processes were observed experimentally. Furthermore, the rate of conversion of (CRP–cAMP) to (CRP′–cAMP) is very slow relative to the reverse reaction ($k_1 \gg k_{-1}$, Table I). Although thermodynamically a general mechanism, such as the square mechanism, may be of interest to obtain information about all four equilibrium constants, from a kinetic point of view the two-step mechanism is a very good approximation of the general mechanism.

It should be pointed out that the kinetic parameters for the square mechanism in Table I cannot be taken too literally. The four unknown parameters derived by fitting the experimental data to eq 9 (Figure 2) should be regarded as one particular set, among others, that is consistent with the data. Nevertheless, the predicted binding isotherm for cyclic AMP binding to AENS–CRP calculated according to the relationship

$$Y = \frac{1}{1 + \dfrac{1}{(\overline{cAMP})}} \qquad (12)$$

with the parameters in Table I is remarkably similar to the experimentally measured binding isotherm (Wu *et al.*, 1974a). This is shown in Figure 6. The overall binding constants calculated from the kinetic parameters ($K = 5.6 \times 10^{-6}$ M for the square mechanism; $K = 4.5 \times 10^{-6}$ M for the two-step mechanism) are also in excellent agreement with that obtained from fluorimetric titration (5×10^{-6} M). Thus, the analysis that has been carried out shows that a mechanism of the general type of eq 8 is consistent with the data, namely, a mechanism in which two different conformations of CRP exist and cAMP binds preferentially to one of the two forms. This preferential binding to one form results in a shift in the conformational equilibrium of AENS–CRP. This is in accord with our previous observation that addition of cAMP induces a conformational transition of AENS–CRP as characterized

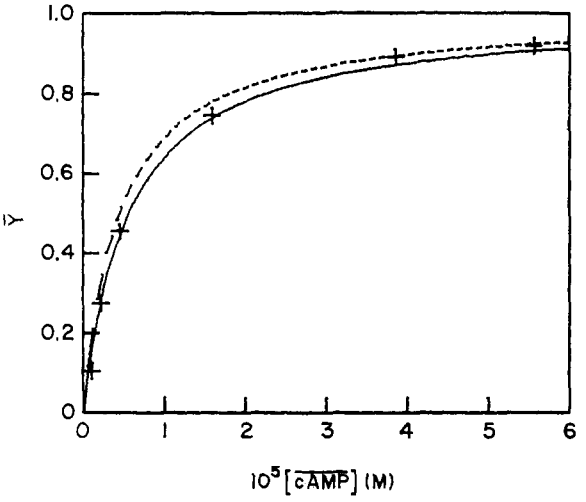

FIGURE 6: The calculated and observed binding isotherms for the cAMP–AENS–CRP interaction in buffer C. The crosses are the observed binding isotherm calculated from the experimental data obtained by fluorimetric titration of AENS–CRP with cAMP published in a previous paper (Wu *et al.*, 1974a). The dashed curve is the theoretical binding isotherm calculated from eq 12 according to the two-step mechanism (eq 3) with the parameters given in Table I. The solid curve is the theoretical binding isotherm calculated from eq 12 according to the square mechanism (eq 8) with the parameters given in Table I.

by a blue shift and an enhancement of fluorescence emission of the probe (Wu *et al.*, 1974a). Furthermore, nanosecond fluorescence depolarization measurements of CRP labeled with dansyl chloride indicate that such conformational transitions do not involve association or dissociation of CRP.

Whether CRP also exists in two forms in the cell is not known. If these forms do exist, they may be involved in the regulation of gene transcription *in vivo*. By kinetic analysis we have demonstrated that there are two forms of CRP in equilibrium. Since cAMP enhances the binding of CRP to *lac* DNA (Anderson *et al.*, 1971) and promotes the CRP-dependent transcription of the *lac* operon (de Combrugghe *et al.*, 1971), the form of CRP which preferentially binds cAMP is probably the biologically active form. In the absence of cAMP the receptor protein exists largely in the inactive form (CRP′). Addition of cAMP shifts the conformational equilibrium so as to enhance the biological activity of CRP by increasing the population of active CRP. It is also possible that cAMP itself may participate directly in the biological activity. In this case, the active form of CRP would be the binary complex (CRP–cAMP) rather than (CRP).

An interesting observation which may be relevant to this hypothesis is the partial stimulation of *gal* transcription by CRP in the absence of cAMP. The extent of stimulation produced by CRP alone is about 25% of that in the presence of 10^{-4} M cAMP. This observation is consistent with the estimation of our kinetic analysis that in the absence of cAMP the ratio of the active form (CRP) to the inactive form (CRP′) is about 1:4 ($K_0 = 3.8$). A similar finding has been reported by Anderson *et al.* (1971), which they attributed to the general protective effect on partial inactivation of RNA polymerase at low concentrations. Under similar experimental conditions, we found that bovine serum albumin (10 times the amount of CRP used) evoked less than 10% stimulation of RNA synthesis.

If the above hypothesis is valid, we would expect that cTuMP, a cAMP analog which is as effective in stimulating the CRP-dependent *gal* transcription (Anderson *et al.*, 1972),

TABLE II: Effect of cAMP Analogs on the Relaxation Times Associated with the Conformational Transitions of AENS–CRP.

Cyclic Nucleotide Added	$1/\tau_1$ (sec^{-1})
None	35
cAMP (1×10^{-6} M)	26
(1×10^{-5} M)	12
cTuMP (1×10^{-6} M)	28
(1×10^{-5} M)	16
cGMP (1×10^{-5} M)	35
(1×10^{-4} M)	37
cGMP + cAMP (1×10^{-4} M)(1×10^{-6} M)	34

would also be able to convert the inactive form of CRP to the active one. In fact, as shown in Table II, cTuMP and cAMP shift the conformational transition of CRP in the same direction, *i.e.*, from (CRP′) to (CRP). On the other hand, cGMP, a competitive inhibitor of cAMP, prevents cAMP from shifting the conformational equilibrium. Although $1/\tau_1$ shows little concentration dependence on cGMP concentration, the effect of cGMP could be due to its binding to (CRP′) rather than (CRP), as demonstrated by the following scheme

$$(\text{CRP}'\text{–cGMP}) \underset{\text{cGMP}}{\rightleftharpoons} (\text{CRP}') \overset{k_0}{\underset{k_{-0}}{\rightleftharpoons}} (\text{CRP}) \overset{\text{cAMP}}{\rightleftharpoons} (\text{CRP–cAMP}) \quad (13)$$

Since k_{-0} is significantly greater than k_0, any perturbation on the equilibrium by cGMP is small, thereby explaining the observed effect. If cAMP pulls the protein equilibrium to the (CRP) form which permits transcription and if cGMP keeps the equilibrium toward the (CRP′) form, then the cyclic nucleotide would be expected to exhibit opposing effects on transcription. This is an important point because recent reports (Goldberg *et al.*, 1973) indicate that cAMP and cGMP may cause opposing effects on a system.

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