Cyclic AMP receptor protein (CRP) regulates the expression of several genes in Escherichia coli. The ability of CRP to bind specific DNA sequences and stimulate transcription is achieved as result of binding of an allosteric ligand: cAMP. Stopped-flow fluorimetry was employed to study the kinetics of the conformational changes in CRP induced by cAMP binding to high and low affinity receptor sites. Results of experiments using CRP labeled at Cys-178 with 1,5-I-AENS indicate change in conformation of the helix-turn-helix, occurring after the formation of CRP-cAMP2 complex, i.e., after saturation of the high affinity sites. The observed conformational change occurs according to sequential model of allostery and is described by rate constants: \( k_c = 9.7 \pm 0.1 \text{ s}^{-1} \) and \( k_{-c} = 0.31 \pm 0.05 \text{ s}^{-1} \), for the forward and backward reaction, respectively. Results of experiments monitored using CRP intrinsic fluorescence suggest that conformational change precedes the formation of CRP-cAMP4 complex and results from displacement of equilibrium between two forms of CRP-cAMP3, caused by binding of cAMP to low affinity sites of one of these forms only. The observed conformational change occurs according to concerted model of allostery and is described by rate constants: \( k_{on} = 28 \pm 1.5 \text{ s}^{-1} \) and \( k_{off} = 75.5 \pm 3 \text{ s}^{-1} \). Results of experiments using single-tryptophan-containing CRP mutants indicate that Trp-85 is mainly responsible for the observed total change in intrinsic fluorescence of wild-type CRP.

CRP\(^1\) regulates the expression of over 100 genes, located in several Escherichia coli operons, most of them being involved in the cell’s response to glucose starvation conditions (1). The protein is a homodimer, composed of 209 amino acid residues per monomer, and each subunit is folded in two domains (2). The larger N-terminal domain contains a binding site for cAMP in anti conformation and is responsible for the dimer stability. The smaller C-terminal domain contains the HTH motif, responsible for DNA recognition and binding. The two domains are connected with a short hinge region (residues 135–138). A recent crystal structure of CRP-DNA complex showed, that each protein subunit binds two cAMP molecules (3). In addition to previously known binding site, located in the N-terminal domain, there is a new site located between the hinge and the turn of HTH, which binds cAMP in the syn conformation.

Several biochemical and biophysical properties of CRP exhibit a bimodal dependence on cAMP concentration (4). This has been interpreted as evidence for existence of three conformational states of the protein, namely: unliganded CRP, CRP-cAMP\(_1\), with one cAMP bound to an anti site, and CRP-cAMP\(_2\) with both anti sites filled. Basing on their most recent crystal structure, Passner and Steitz (3) reinterpreted those results in terms of three conformational states represented by CRP, CRP-cAMP\(_1\), with cAMP bound to two anti sites, and CRP-cAMP\(_4\) with both two anti and two syn sites filled.

In presence of \(-100 \mu\text{M} \text{cAMP}\), CRP becomes “activated” and is able to recognize and bind specific DNA sequences and stimulate transcription (5). Crystallographic (2) and equilibrium dialysis studies (6) indicate that the “active” form of the protein is represented by CRP-cAMP\(_2\), complex, with two anti sites filled. However, in the presence of millimolar concentrations of cAMP, where CRP-cAMP\(_4\) complex predominates, there is a loss in affinity and sequence specificity in DNA binding and consequently loss in transcription stimulation (7). This strongly suggests that the saturation of syn sites is causing protein “deactivation.”

Previous biochemical and biophysical studies were focused on the identification of structural changes accompanying binding of cAMP to CRP, as well as on the determination what specific residues are involved in effecting observed transitions (reviewed in Ref. 1). However, none of those studies enabled identification of the mechanism, underlying the observed allosteric changes, in terms of KNF or MWC models. This paper presents the results of kinetic investigations of cAMP-induced conformational changes in CRP. Presented results provide evidence that allosteric “activation” of CRP at micromolar cAMP concentrations, occurs according to sequential (KNF) model, while conformational change observed at millimolar concentrations of cAMP, occurs according to concerted (MWC) model.

### EXPERIMENTAL PROCEDURES

**Materials**—1,5-I-AENS, phenylmethylsulfonyl fluoride, EDTA, and Tris were purchased from Sigma. DTt, cAMP, and cGMP were either from Sigma or Fluka. The Fractogel EMD SO\(_4\) 650 (M) was from Merck; Q Sepharose Fast Flow and Sephadex G-25 were from Amersham Pharmacia Biotech. The nutrients for bacterial growth were either from Difco Laboratories or Life Sciences. All other chemicals were analytical grade products from POCh-Gliwice. All measurements were performed in buffers prepared in water purified by a Millipore system.

**Plasmids and Cells**—CRP wild-type (wt) was isolated from E. coli strain SA100 containing the plasmid pHA7, which encodes the crp gene (8). The plasmid pHA7 was a generous gift from Dr. S. Garges. Trypophan (Trp) at position 13 or 85 of CRP was replaced by phenylalanine with the use of pHA7 plasmid. The mutagenesis was performed using overlap extension method with Pwo DNA polymerase. Plasmids encod-
ing mutant crp genes were introduced into E. coli strain M1824crp, kindly provided by Dr. S. Busby. Bacteria were grown on Terrific broth at 37 °C overnight, in a Biostat B fermentor from Braun, Germany, and then harvested by centrifugation.

Protein Purification—CRP wt and mutants were purified at 4 °C, as described previously (9), with some important modifications. The cells were disrupted only by sonication using six 30-s pulse bursts, and the cell debris were removed by centrifugation. The crude extract was loaded onto a column filled with Fractogel EMD S0, equilibrated with buffer: 20 mM sodium phosphate, pH 6.8, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, and the column was washed over-night. Proteins were eluted from the column with a linear gradient of NaCl (0–1.0 M) in same buffer, and under such conditions CRP was eluted at about 0.7 M NaCl. Fractions containing CRP were pooled, dialyzed against buffer: 50 mM Tris-HCl, pH 7.9 (at 20 °C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol, and passed through a column filled with Q Sepharose, equilibrated with same buffer. The protein was collected in the void volume, dialyzed, and stored in aliquots at −20 °C. After this two-step procedure, the protein was highly pure (>97%), as judged by SDS-polyacrylamide gel electrophoresis and Comassie Brilliant Blue staining.

All measurements were performed in buffer A (50 mM Tris-HCl, pH 7.9 (at 20 °C), 100 mM KCl, and 1 mM EDTA). Before measurements the protein aliquots stored at −20 °C were thawed on ice, and dialyzed extensively against buffer A to remove DTT and glycerol. Before measurements all samples were filtered through a microporous filter (0.45 μm) to remove all undissolved impurities.

Concentration Determination—The concentration of protein, cyclic nucleotides, and fluorescence probe was determined by absorption spectroscopy using the following extinction coefficients: 40,800 M⁻¹ cm⁻¹ at 278 nm for CRP wt dimer (6), 14,650 M⁻¹ cm⁻¹ at 259 nm and 12,950 M⁻¹ cm⁻¹ at 254 nm for cAMP and eGMP (10), respectively, and 6000 M⁻¹ cm⁻¹ at 340 nm for AENS group (11). Extinction coefficients of CRP mutants were determined using the method described elsewhere (12) and were 29,700 M⁻¹ cm⁻¹ and 33,100 M⁻¹ cm⁻¹ at 278 nm for W85F and W131F dimers, respectively.

Fluorescent Labeling—CRP wt was covalently labeled with 1,5,1-
AENS using the procedure described elsewhere (13), with some minor modifications. Protein and label were mixed at molar ratio 1:20 and incubated at 4 °C in buffer A, overnight at dark. Labeled CRP was purified by Sephadex G-25 column and dialyzed extensively against buffer A. The stoichiometry of labeling was determined by absorption spectroscopy and was in the range of 2.2–2.6 mol of label/mol of CRP dimer.

Fluorescence Stopped-flow Experiments—Kinetic measurements were performed at 20 °C (± 0.2 °C) using a stopped-flow spectrophluorimeter SX-17 MV from Applied Photophysics, United Kingdom. The dead time of mixing was determined to be less than 2 ms. Changes in the protein conformation induced by cAMP binding were monitored using two methods: 1) fluorescence intensity of AENS-CRP, observed through a cut-off filter at wavelengths using two methods: 1) fluorescence intensity of AENS-CRP, observed through a cut-off filter at wavelengths

Mechanism of Allosteric Changes of CRP

Mechanism of Allosteric Changes of CRP

At millimolar cAMP concentrations, CRP undergoes a conformational change according to sequential (KNF) model of allostery (see “Discussion”). This can be represented by the scheme shown by Reactions 1 and 2 and Equation 3.

$$K_1 \text{ CRP} + 2\text{cAMP} \rightarrow \text{CRP-CAMP} + \text{cAMP} \rightarrow \text{CRP-CAMP}_2$$

**REACTION 1**

$$k_1$$

$$K_{CRP-CAMP} \rightarrow \text{CRP*-cAMP}_2$$

**REACTION 2**

$$K_C = k_2 / k_1$$

**EQ. 3**

$$K_1 \text{ and } K_2 \text{ are intrinsic dissociation constants for binding of the first and the second cAMP molecule to low affinity (anti) sites, CRP and CRP* represent protein before and after the conformational change, } K_{CRP-CAMP} \text{ the equilibrium constant between these two forms, while } k_1 \text{ and } k_2 \text{ are rate constants that describe the conformational change step.}$$

The observed rates (hobs) derived in kinetic experiments performed at micromolar cAMP concentrations can be fit to the following equation to yield the kinetic and thermodynamic parameters $k_1$, $k_2$, and $K_{CRP-CAMP}$.

$$h_{obs} = k_2 + k_1 [\text{cAMP}] + K_{CRP-CAMP} \cdot [\text{cAMP}]^2$$

**EQ. 4**

For the simplicity of the analysis, the two high affinity sites were considered identical and independent and characterized by an average dissociation constant $K_{CRP-CAMP}$. The change in the fluorescence intensity, resulting from the conformational change of CRP occurring at micromolar cAMP concentrations, can be fit to the following equation to yield thermodynamic parameters $K_{CRP-CAMP}$ and $K_C$.

$$\Delta F_{obs} = \Delta F_{max} \frac{K_{CRP-CAMP} \cdot [\text{cAMP}]}{1 + K_{CRP-CAMP} \cdot [\text{cAMP}]} + K_C [\text{cAMP}]$$

**EQ. 5**

$$\Delta F_{obs} \text{ and } \Delta F_{max} \text{ are the observed and the maximum change in fluorescence intensity going in from CRP-cAMP}_2 \text{ to CRP*-cAMP}_2 \text{ respectively.}$$

At millimolar cAMP concentrations, CRP undergoes a conformational change according to concerted (MWC) model of allostery (see “Discussion”), which can be represented by the scheme shown by Reactions 3 and 4 and Equation 6.

$$\text{P} \rightarrow \text{P}^+$$

**REACTION 3**

$$K_3 \text{ P} + 2\text{cAMP} \rightarrow \text{P}^+ - \text{cAMP} + \text{cAMP} \rightarrow \text{P}^+ - \text{cAMP}_2$$

**REACTION 4**

$$P^+ \text{ and } P \text{ are the binding and non-binding form of protein, } K_3 \text{ is the equilibrium constant between these two states, } k_{on} \text{ and } k_{off} \text{ are rate constants that describe the isomerization step, while } K_4 \text{ and } K_4 \text{ are intrinsic dissociation constants for binding of the first and the second cAMP molecule to low affinity (syn) sites.}$$

The observed rates (hobs) derived in kinetic experiments performed at millimolar cAMP concentrations can be fit to the following equation to yield the kinetic and thermodynamic parameters $k_{on}$, $k_{off}$, and $K_{syn}$.

$$k_{obs} = k_{on} + k_{off} + K_{syn} + 2K_{syn} \cdot [\text{cAMP}] + [\text{cAMP}]^2$$

**EQ. 7**

For simplicity of analysis, the two low affinity sites were considered identical and independent and characterized by an average dissociation constant $K_{syn}$. The change in the fluorescence intensity, resulting from the conformational change of CRP occurring at millimolar cAMP concentrations,
can be fit to the following equation to yield thermodynamic parameters $K_{\text{syn}}$ and $K_0$.

$$
\Delta F_{\text{obs}} = \Delta F_{\text{max}} \frac{2K_{\text{syn}}[\text{cAMP}]}{K_{\text{syn}} + K_0 + 2K_{\text{syn}}[\text{cAMP}]} + [\text{cAMP}]^2
$$

(Eq. 8)

$\Delta F_{\text{obs}}$ and $\Delta F_{\text{max}}$ are the observed and the maximum change in the fluorescence intensity in going from P to P$^\ast$ form of protein, respectively.

**RESULTS**

**AENS-CRP Fluorescence**—After excitation at 340 nm, the AENS-CRP emits a characteristic fluorescence with a maximum set at about 490 nm. Addition to the solution of 100 $\mu$M [cAMP] causes about 20% increase in the fluorescence intensity of AENS-CRP and concomitant blue shift in the emission maximum by about 10 nm. A further increase in [cAMP] to millimolar levels does not change the fluorescence characteristics of the labeled protein.

When AENS-CRP is stopped-flow mixed with cAMP of various concentrations, a characteristic increase in the fluorescence intensity of the label is observed (Fig. 1). The obtained kinetic traces were fit to a single exponential or a sum of such terms (Equation 2). In all cases the data were satisfactorily described by a single exponential, while double exponential fitting did not improve the goodness of the fit.

The rates obtained from fitting of AENS-CRP fluorescence detected kinetic traces to single exponential were plotted against the final cAMP concentration and the resulting graph is presented in Fig. 2, while the corresponding amplitudes are presented in Fig. 3. As the total cAMP concentration increases, the observed rate ($k_{\text{obs}}$) also increases. For ligand concentrations below 40 $\mu$M, an approximate linear dependence of $k_{\text{obs}}$ on [cAMP] is observed. Above 40 $\mu$M [cAMP], the $k_{\text{obs}}$ deviates from linearity and approaches a constant value at very high (millimolar) concentrations of cAMP. Such behavior indicates a sequential two-step mechanism, where the process of conformational change is consecutive versus the ligand-binding step. Previous studies indicate that the association of cAMP to CRP is a very fast process (15), suggesting that the observed change in the fluorescence intensity of AENS-CRP results from conformational change of the double liganded protein, i.e.: CRP-cAMP$^2$ (Equation 3 and Reactions 1 and 2).

From the dependence of $k_{\text{obs}}$ on cAMP concentration, it is possible to evaluate the rate constants that describe the conformational change step and the average dissociation constant for cAMP binding to high affinity sites of CRP (Equation 4). The following values of the fitted parameters were obtained: $k_c = 9.7 \pm 0.1$ s$^{-1}$, $k_c' = 0.31 \pm 0.05$ s$^{-1}$, and $K_{\text{diss}} = 27.5 \pm 1$ $\mu$M (Table I). The above values enable to calculate the equilibrium constant between CRP-cAMP$^2$ before and after the conformity...
The dependence of the amplitudes, associated with the observed rates, on the final [cAMP] is shown in Fig. 3. The presented binding curve saturates fully at about 100 μM [cAMP], with an apparent half-saturation point set at about 5.5 μM. This corresponds well with another binding curve (Fig. 3), which represents cAMP-induced changes in the fluorescence intensity of AENS-CRP, obtained from equilibrium studies. Both sets of data were fit to Equation 5, and the best-fit parameters are summarized in Table I. Presented results are in good agreement with values obtained from rate-based analysis. However, the analysis of the amplitude-based data gives a slightly lower value of the average dissociation constant $K_{\text{diss}} = 9.6 \pm 2.5$ μM and a higher value of the equilibrium constant $K_C = 0.2 \pm 0.09$, comparing to rate-based data analysis.

**Trp Fluorescence Measurements**—When CRP is stopped-flow mixed with cAMP at concentrations below 300 μM, only very small change in the fluorescence intensity of Trp residues can be detected. However, when CRP is mixed with cAMP of higher concentrations, a characteristic increase in the fluorescence intensity of Trp residues is observed (Fig. 4). The obtained kinetic traces were fit to a single exponential or a sum of such terms (Equation 2). In all cases studied, the data were satisfactorily described by a single exponential, while double exponential fitting did not improve the goodness of the fit.

The rates obtained from fitting of Trp fluorescence detected kinetic traces were plotted against the final [cAMP] and the resulting plot is presented in Fig. 5. The corresponding amplitudes are shown in Fig. 6. As the total cAMP concentration increases, the observed rate ($k_{\text{obs}}$) decreases, reaching a constant value at very high ligand concentrations. Such behavior is indicative of displacement of an equilibrium between two conformational states of the protein, caused by the binding of ligand to one of these forms only (Equation 6 and Reactions 3 and 4).

Because the association of cAMP to CRP was shown to be a very fast process (15), it can be assumed that $P$ and $P'$ denote two interconvertible forms of CRP-cAMP complex (see “Discussion”). Moreover, as the observed changes occur at millimolar [cAMP], they must be associated with binding of cAMP to low affinity sites of CRP, but not with cAMP binding to high affinity sites.

In order to evaluate the rate constants of the conformational change associated with $P$ to $P'$ transition, as well as the average dissociation constant for cAMP binding to low affinity sites of CRP, the rate dependence from Fig. 5 was fit to Equation 7. The following values of the fitted parameters were obtained: $k_{\text{on}} = 28 \pm 1.5$ s$^{-1}$, for the forward reaction, $k_{\text{off}} = 75.5 \pm 3$ s$^{-1}$ for the backward reaction, and $K_{\text{syn}} = 2.0 \pm 0.2$ mM for the average dissociation constant of the syn sites. From these values one can calculate the equilibrium constant between the two forms of CRP to be: $K_C = 2.7$. All presented results are summarized in Table II.

**Fig. 4.** Kinetics of cAMP binding to CRP measured by the change in the fluorescence intensity of Trp residues. Measurements were performed at 20 °C, in buffer A, pH 7.9, with cAMP concentration of 2 μM and various cAMP final concentration: ○, 0 mM; □, 1 mM; △, 3.5 mM; ▽, 8 mM. The solid lines are the single exponential fits.

**Fig. 5.** Dependence of the observed rate ($k_{\text{obs}}$) of cAMP-induced changes in Trp fluorescence intensity of CRP wt (○), CRP wt incubated with 200 μM cAMP (●), and W13F mutant (□) on the final cAMP concentration. The solid line is the best fit of data to Equation 7, with the parameters of the fit summarized in Table II.

| Protein  | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_C$ | $K_{\text{diss}}$ |
|----------|----------------|----------------|-------|------------------|
| CRP wt   | 28 ± 1.5 s$^{-1}$ | 75.5 ± 3 s$^{-1}$ | 9.6 ± 2.5 μM | 0.2 ± 0.09 |

$^a$ Obtained from rate-based analysis of kinetic data.

$^b$ Obtained from equilibrium data.

$^c$ Obtained from amplitude-based analysis of kinetic data.
case CRP was incubated at 200 μM [cAMP] for 1 h and subsequently stopped-flow mixed with cAMP of various concentrations. Additionally, in that case, the characteristic increase in the fluorescence intensity of Trp residues could be satisfactorily fit to a single exponential. The dependence of \( k_{\text{obs}} \) on final cAMP concentration is virtually identical to that obtained for CRP in initial absence of the ligand (Fig. 5). When Equation 7 was used for fitting these data, the following values of the estimated parameters were obtained: \( k_{\text{on}} = 28.5 \pm 2.0 \text{ s}^{-1} \), \( k_{\text{off}} = 85 \pm 7.5 \text{ s}^{-1} \), and \( K_{\text{syn}} = 1.8 \pm 0.3 \text{ mM} \). From the values above, one can estimate the equilibrium constant between two interconvertible forms of CRP-cAMP, which is \( K_q = 3.0 \). This is exactly the same as obtained during kinetic experiments where unliganded CRP wt was used (Table II).

Analogous fluorescence stopped-flow experiments using Trp fluorescence detection were performed using two single-tryptophan-containing CRP mutants: W85F and W13F. In case of CRP W85F, only very small decrease in intrinsic fluorescence intensity was observed at micromolar [cAMP]. However, a strong increase in Trp fluorescence at micromolar [cAMP] was observed in case of CRP W13F, and the obtained kinetic traces could be well described by a single-exponential. The dependence of \( k_{\text{obs}} \) on cAMP concentration obtained for W13F mutant is shown in Fig. 5, while the corresponding amplitude dependence is presented in Fig. 6. Both the rate and amplitude dependences are similar to results obtained in case of CRP wt. The values of parameters: \( k_{\text{on}}, k_{\text{off}}, K_0 \), and \( K_{\text{syn}} \) derived from fitting those data to Equations 7 and 8 are also quite similar between CRP wt and W13F mutant (Table II).

![Graph](image)

**Fig. 6.** Dependence of the amplitudes associated with cAMP-induced changes in Trp fluorescence intensity of CRP wt (□) and W13F mutant (●), on the final cAMP concentration. The solid line denotes the best fit of data to Equation 8, with the parameters of the fit summarized in Table II.

### TABLE II

**Mechanism of Allosteric Changes of CRP**

| Protein  | \( k_{\text{on}} \) | \( k_{\text{off}} \) | \( K_0 \) | \( K_{\text{syn}} \) |
|----------|---------------------|---------------------|---------|---------------------|
|          | \text{ s}^{-1}   | \text{ s}^{-1}   | \text{ mM} | \text{ mM}         |
| CRP wt   | 28.5 ± 2.0         | 85 ± 7.5           | 2.0 ± 0.2| 4.4 ± 1.1          |
| CRP W13F | 18.2 ± 1.5         | 80.5 ± 4.4         | 4.4 (3.9, 5.1) | 6.4 ± 2.0 |

a Obtained from rate-based analysis.
b Obtained from amplitude-based analysis.
c Obtained from rate-based analysis, initial 200 μM [cAMP].

Each CRP subunit contains three cysteine residues (8); however, only Cys-178, which is located in the turn of the HTH motif, is solvent-accessible (4, 18) and can be used for fluorescent labeling (13, 19). Because of their interesting location, Cys-178 can be used as a relevant source of information about conformational changes occurring within the DNA binding domain.

The fluorescence intensity of AENS-CRP increases only in presence of micromolar concentrations of cAMP, while millimolar ligand concentrations do not promote further changes in AENS-CRP fluorescence properties. Therefore, the environment of Cys-178 residues responds only to cAMP binding to anti sites, while occupancy of syn sites does not cause any apparent changes in the conformation of the HTH motif. Binding of cGMP to AENS-CRP does not influence fluorescence properties of the label, over a wide range of cGMP concentrations (data not shown), indicating that cGMP binding to anti and syn sites does not lead to changes in the microenvironment of Cys-178 residues. Since the HTH motif and the anti sites are located in different domains and are spatially separated, the increase of AENS-CRP fluorescence due to cAMP binding to high affinity sites is a clear demonstration of allosteric changes, which reflect interdomain cross-talking. Apparently, cGMP fails to induce such proper communication between the domains.

Upon binding of cAMP to anti sites, the fluorescence intensity of AENS-CRP increases with concomitant blue shift in the emission maximum by about 10 nm. Differential fluorescence spectra reveals that such blue shift results from increase in the fluorescence intensity of a fraction of fluorophores, whose emission maximum is set at about 465 nm. The observed changes can be explained in terms of decreased internal mobility and/or increased hydrophobicity of the local surrounding of Cys-178 residues. Conformational changes occurring within the HTH motif were reported also by Baichoo and Heyduk (20). Using a "protein footprinting" technique, these authors observed a substantial decrease in the solvent exposure of α-helices D and E with concomitant increase in solvent accessibility of α-helix F. Therefore, changes in AENS-CRP fluorescence intensity reported in the present study may reflect “freezing” of the HTH motif in the proper (active) conformation, which enables sequence-specific DNA recognition and binding.

AENS-CRP fluorescence was used to study the kinetics of the conformational change of the HTH motif, due to cAMP binding to high affinity sites. The process was studied under pseudo-first-order conditions and is well described by a simple exponential. The rate of the observed process increases with increasing ligand concentration, reaching an asymptotic value at
Moreover, the values of thermodynamic constants served experimentally in the amplitude dependence of AENS-should exhibit marks of positive cooperativity. This is because and both Trp-13 and Trp-85 are part of the N-terminal domain.

The basic assumption that underlies such interpretation of the rate dependence is that the association step is much faster, as compared to the conformational change step. Temperature-jump studies (15) seem to confirm the above assumption, since the bimolecular rate constant for cAMP association to high affinity sites was estimated to be $k_a = 9.7 \times 10^7$ M$^{-1}$ s$^{-1}$. Similarly, the reported values of the rate constants for association of various ligands to a number of different proteins usually fall in the range between $10^7$ and $10^9$ M$^{-1}$ s$^{-1}$ (21, 22), showing that the association is generally a very fast process. On the other hand, results of Wu and Wu (15) enable to estimate the rate of cAMP dissociation from CRP in the range $k_d = 69$ s$^{-1}$, which is in agreement with F$^{39}$NMR data of 3-fluorotyrosine containing CRP, suggesting $k_d < 75$ s$^{-1}$ (24). Since the dissociation constant for high affinity sites is $\sim 30$ mM (Refs. 4 and 6; this work), the bimolecular association rate constant can be estimated in the range of $k_a \sim 2 \times 10^6$ M$^{-1}$ s$^{-1}$. This is high enough for the association to be at least 100 times faster than the conformational change, for cAMP concentrations above 1 mM.

If the association is truly a fast process, then it is logical to assume that statistically the conformational change will start from the double-ligated protein, i.e. CRP-cAMP$_2$, where both anti sites are filled. The dependence of the $k_{a,b}$ on the final cAMP concentration can be then used to evaluate the rate constants, which govern the conformational change step, as well as the dissociation constant for the high affinity sites. For simplicity of analysis, it was assumed that the two anti sites are identical and independent. Analysis of data according to Equation 4 gives the following values for the rate constants: $k_a = 9.7 \pm 0.1$ s$^{-1}$ and $k_c = 0.31 \pm 0.05$ s$^{-1}$, which enable to estimate the equilibrium constant between protein before and after the conformational change, $K_C = 0.032$. Low value of $K_C$ indicates that virtually all double-ligated CRP will be switched into the active conformation.

Low value of $K_C$ also suggests that the binding isotherm should exhibit marks of positive cooperativity. This is because the conformational change would help in stabilization of the double-ligated CRP. Such positive cooperativity is indeed observed experimentally in the amplitude dependence of AENS-CRP fluorescence intensity on cAMP concentration (Fig. 3). Moreover, the values of thermodynamic constants $K_{syn}$ and $K_{con}$, obtained from rate-based and amplitude-based analysis, as well as equilibrium measurements are very similar, confirming the validity of the model employed.

There are two tryptophan residues in each CRP subunit (8), and both Trp-13 and Trp-85 are part of the N-terminal domain. When cAMP is present in concentrations below 300 $\mu$M, only very small change in Trp fluorescence intensity can be observed, which is too small to be assessed precisely. However, when cAMP is present in millimolar concentrations, there is a considerable increase in Trp fluorescence intensity of CRP wt. This suggests that Trp fluorescence intensity is sensitive to binding of cAMP to low affinity sites, and their occupancy is causing a conformational change occurring within the N-terminal domain.

In order to determine the kinetic mechanism of such changes occurring as result of cAMP binding to syn receptor sites, stopped-flow experiments were performed and monitored using Trp fluorescence. The process was studied under pseudo first-order conditions and is well described by a single exponential. The rate of the observed changes in Trp fluorescence intensity is decreasing with increasing [cAMP], reaching a constant value at ligand concentrations exceeding 10 mM (Fig. 5). Such characteristic behavior is usually interpreted as evidence of displacement of an equilibrium between two conformational states of the protein, caused by the binding of ligand to one form only (21, 22). Therefore, the observed process of conformational change of CRP occurs according to concerted (MWC) model of allostery (25).

The basic assumption that underlies such interpretation of the rate dependence is that the isomerization step is slow compared to the fast ligand-binding step, which follows the conformational change. The affinity of the low affinity sites for cAMP is about 50 times lower than the affinity of the high affinity sites (Ref. 4; this work). On the other hand, the F$^{39}$NMR data (24) suggest that the dissociation rate from low affinity sites is $k_d \approx 350$ s$^{-1}$. This allows us to estimate the bimolecular rate constant for cAMP association to syn sites, in the range of $k_a \sim 2 \times 10^6$ M$^{-1}$ s$^{-1}$. In such case, cAMP binding would be effectively faster than the isomerization step for ligand concentrations above 1 mM.

It was mentioned earlier in this section that the process of cAMP binding to high affinity sites of CRP is a very fast process. If so, then statistically any changes in Trp fluorescence intensity should start from the double-ligated CRP-cAMP$_2$, where both anti sites are filled. This assumption is confirmed by the results of kinetic studies, performed using CRP in presence of 200 $\mu$M [cAMP] as starting conditions. Regardless, whether stopped-flow experiments are initiated from CRP in absence of cAMP or CRP equilibrated at 200 $\mu$M [cAMP], virtually identical results are obtained. This proves that CRP becomes double-ligated within the dead time of stopped-flow mixing. Since the double-ligated form of CRP is achieved so fast, it is also logical to conclude that the equilibrium between two forms of the protein, described in Equation 6 and Section 3, relates to CRP-cAMP$_2$ complex. Moreover, such equilibrium seems to be independent of the conformational change occurring in the HTH motif. In experiments which initiate from CRP in presence of 200 $\mu$M [cAMP], exclusively all protein will be found in the active conformation (CRP*-cAMP$_2$), while in experiments starting from CRP in absence of cAMP, only small portion of protein will reach the active state within the dead-time of mixing. However, identical results are obtained in both cases, demonstrating that equilibrium between P and P* forms is independent of the amount of protein in the CRP*-cAMP$_2$ state.

The dependence of $k_{a,b}$ on the final cAMP concentration (Fig. 5) can be used to evaluate the rate constants, which govern the isomerization step and the dissociation constant for the low affinity sites (Equation 8). Such analysis gives the values of the rate constants: $k_{a,b} = 28 \pm 1.5$ s$^{-1}$ and $k_{a,b} = 75.5 \pm 3$ s$^{-1}$, while the average dissociation constant is $K_{syn} = 2 \pm 0.2$ mM. The dissociation constant is similar to that reported by Heyduk and Lee (4) based on equilibrium Trp fluorescence studies. The ratio between the rate constants of the isomerization step gives an estimate of the equilibrium constant between the two forms of double-ligated protein, which is $K_a = 2.7$. Although $K_a$ is not very high, it can be anticipated that binding of cAMP to syn sites will exhibit marks of positive cooperativity. Such positive cooperativity in binding is indeed observed experimentally, in the amplitude dependence of the Trp fluorescence signal on ligand concentration (Fig. 6). Very similar values of $K_{syn}$ and $K_0$ are obtained from rate-based and amplitude-based analysis, supporting validity of the model employed.

The two forms of CRP-cAMP$_2$ complex, termed P and P*,
differ in the activity of the low affinity sites. As a first approximation, the isomer denoted as P (Eqn. 7) has an affinity close to zero, while the isomer denoted as P’ can bind cAMP with an average dissociation constant \( K_{syn} = 2 \pm 0.2 \) mM. It can be concluded that, in P, the syn sites are not properly formed and that, in order to convert them into active sites in P’, conformation of the N-terminal domain must be changed. Results obtained using single-tryptophan-containing CRP mutants W85F and W13F strongly suggest that it is Trp-85 that is mostly responsible for the observed total change in Trp fluorescence of CRP wt (Figs. 5 and 6). The crystal structure of Passner and Steitz (3) also emphasizes the importance of N-terminal domain in the formation of the syn receptor pocket, as several important contacts between syn-cAMP and CRP, come from residues of the larger domain or adjacent hinge region. Previous studies show that conformational changes occurring at millimolar cAMP concentrations involve also changes in CRP secondary structure (26), increased protection against proteolytic attack on \( \alpha \)-helix C (4, 27), and large contraction of the protein dimensions (19).

Since the syn sites have a relatively high dissociation constant, one may doubt if they have any significant biological role in vivo. After all, millimolar concentrations of cAMP are rarely, if ever, found in E. coli. It must be noted, however, that P and P’ forms of CRP-cAMP\(_2\) complex are present also in micromolar concentrations of the ligand, and cAMP binding to syn sites only stabilizes the state P’ of the protein, via formation of the CRP’-cAMP\(_4\) complex. Earlier studies (4, 5) and recent investigations (7) show that, in millimolar cAMP concentrations, nonspecific DNA competes more efficiently with specific DNA binding to CRP, arguing that the P’ may represent a weak DNA-binding form of CRP. This observation finds support in the crystallographic work of Passner and Steitz (3), who postulate that cAMP molecule bound in the syn conformation interferes with proper interaction between DNA and residues of the recognition helix of HTH motif. These authors noted also a significant change in the conformation of Lys-52, between CRP-cAMP\(_2\) and CRP-cAMP\(_4\)-DNA complexes. Since Lys-52 was shown to be important in activation of transcription at class II promoters (28), the P’ form of the protein might play some role in CRP’s ability to differentially regulate promoters. Further experiments must be done in order to assess the role of syn sites and the conformational state P’ of CRP, in the regulation of gene expression.

Acknowledgments—We are grateful for Prof. S. Garges for supplying us with the plasmid pHAl for overproduction of CRP and Prof. S. Busby for supplying us with E. coli strain M182Δcrp. We thank Dr. Barbara Kepys and Aneta Kaczmarczyk for technical assistance in preparation of CRP W13F mutant.

REFERENCES

1. Kolb, A., Busby, S., Buc, H., Garges, S., and Adhya, S. (1993) *Annu. Rev. Biochem.* **62**, 749–795
2. Weber, I. T., and Steitz, T. A. (1987) *J. Mol. Biol.* **196**, 311–326
3. Passner, J. M., and Steitz, T. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2843–2847
4. Heyduk, T., and Lee, J. C. (1989) *Biochemistry* **28**, 6914–6924
5. Taniguchi, T., ONeil, M., and de Crombrugghe, B. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 5090–5094
6. Takahashi, M., Blazy, B., and Baudras, A. (1980) *Biochemistry* **19**, 5124–5130
7. Mukhopadhyay, J., Sur, R., and Parrack, P. (1989) *FEBS Lett.* **243**, 215–218
8. Aiba, H., Fujimoto, S., and Otsaki, N. (1982) *Nucleic Acids Res.* **10**, 1345–1361
9. Malecki, J., and Wasylewski, Z. (1997) *Eur. J. Biochem.* **243**, 660–669
10. Merck & Co., Inc. (1976) *The Merck Index*, 9th Ed., p. 353, Merck & Co., Inc., Rahway, NJ
11. Hudson, E. N., and Weber, G. (1973) *Biochemistry* **12**, 4154–4161
12. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182**, 319–326
13. Wu, F. Y.-H., Nath, K., and Wu, C.-W. (1974) *Biochemistry* **13**, 2567–2572
14. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York
15. Wu, C.-W., and Wu, F. Y.-H. (1974) *Biochemistry* **13**, 2573–2578
16. Garges, S., and Adhya, S. (1988) *J. Bacteriol.* **170**, 1417–1422
17. Kim, J., Adhya, S., and Garges, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9700–9704
18. Eilen, E., and Krakow, J. S. (1977) *J. Mol. Biol.* **114**, 47–60
19. Heyduk, T., Heyduk, T., and Lee, J. C. (1992) *J. Biol. Chem.* **267**, 3200–3204
20. Baichoo, N., and Heyduk, T. (1997) *Biochemistry* **36**, 10830–10836
21. Cantor, C. R., and Schimmel, P. R. (1980) *Biophysical Chemistry Part III: The Behavior of Biological Macromolecules*, W. H. Freeman and Co., San Francisco
22. Fersht, A. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman and Co., New York
23. Kosland, D. E., Nemethy, G., and Filmer, D. (1960) *Biochemistry* **5**, 365–385
24. Hinds, M. G., King, R. W., and Keeseay, J. (1991) *FEBS Lett.* **283**, 127–130
25. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118
26. DeGrazia, H., Harman, J. G., Tan, G., and Wartell, R. M. (1990) *Biochemistry* **29**, 3557–3562
27. Cheng, X., Kovac, L., and Lee, J. C. (1995) *Biochemistry* **34**, 10816–10826
28. Williams, R. M., Rhodius, V. A., Bell, A. I., Kolb, A., and Busby, S. J. W. (1996) *Nucleic Acids Res.* **24**, 1112–1118
Kinetic Studies of cAMP-induced Allosteric Changes in Cyclic AMP Receptor Protein from Escherichia coli

J.IJdrzej Malecki, Agnieszka Polit and Zygmunt Wasylewski

J. Biol. Chem. 2000, 275:8480-8486.
doi: 10.1074/jbc.275.12.8480

Access the most updated version of this article at http://www.jbc.org/content/275/12/8480

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 24 references, 5 of which can be accessed free at http://www.jbc.org/content/275/12/8480.full.html#ref-list-1