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Kinetic and Structural Studies of the Allosteric Conformational Changes Induced by Binding of cAMP to the cAMP Receptor Protein from *Escherichia coli*†

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ABSTRACT: The cAMP receptor protein, allostericaly activated by cAMP, regulates the expression of more than 100 genes in *Escherichia coli*. CRP is a homodimer of two-domain subunits. It has been suggested that binding of cAMP to CRP leads to a long-distance signal transduction from the N-terminal cAMP binding domain to the C-terminal domain of the protein responsible for interaction with specific sequences of DNA. In this study, the stopped-flow and time-resolved fluorescence lifetime measurements were used to observe the kinetics of the distance changes between the N-terminal and C-terminal domain of CRP induced by binding of cAMP to high-affinity binding sites. In these measurements, we used the constructed CRP heterodimer, which possesses a single Trp85 residue localized at the N-terminal domain of one CRP subunit, and fluorescently labeled by 1,5-I-AEDANS Cys178 localized at the C-terminal domain of the same subunit or at the opposite one. The Förster resonance energy transfer method has been used to study the distance changes, induced by binding of cAMP, between Trp85 (fluorescence donor) and Cys178-AEDANS (fluorescence acceptor) in the CRP structure. The obtained results show that the allosteric transitions of CRP at micromolar cAMP concentrations follow the sequential binding model, in which binding of cAMP to high-affinity sites causes a 4 Å movement of the C-terminal domain toward N-terminal domains of the protein, with kinetics faster than 2 ms, and CRP adopts the "closed" conformation. This fast process is followed by the slower reorientation of both CRP subunits.

The cyclic AMP receptor protein (CRP),[1] in the presence of the allosteric effector cAMP, activates transcription of more than 100 genes in *Escherichia coli* (*1*, *2*). Transcription activation by CRP can serve as a model system for understanding the molecular structural mechanism of transcription activation, since at the simplest CRP-dependent promoters only three macromolecules such as DNA sequence, RNA polymerase, and CRP interact (*3*). The high-resolution three-dimensional crystal structure of the CRP−cAMP complex shows that the homodimeric protein is composed of two identical 209-amino acid subunits, each subunit is folded into two distinct domains, and the liganded protein shows structural asymmetry (*4*, *5*). In the homodimeric CRP−cAMP complex, one subunit adopts an open conformation and the second adopts a closed conformation, in contrast to the CRP−cAMP−DNA complex in which the symmetric closed conformation of CRP is observed (*4*). Since the crystal structure of apo-CRP has not yet been elucidated, one can only speculate about the causes of this homodimeric protein asymmetry. In CRP, the larger N-terminal domain (residues 1−134) is responsible for dimerization of the protein and for interaction with the allosteric effector cAMP (*2*). The smaller C-terminal domain (residues 139−209) is responsible for interaction with the promoter DNA sequence through a helix−turn−helix (HTH) motif. The two functional domains are connected by a hinge region (residues 135−138) as shown in Figure 1. The crystal structure of CRP has revealed that each protein subunit binds two cAMP molecules with different affinities (*4*). The high-affinity sites, where cAMP binds in the anti conformation, are buried within the N-terminal domain, whereas lower-affinity binding sites, where cAMP binds in the syn conformation, are located at the interface formed by the C-terminal domain of the CRP subunit. Therefore, one can expect that CRP exists in solution in at least three conformational states: free CRP, CRP with two cAMP molecules bound to high-affinity sites at the N-terminal domain [CRP−$(cAMP)_2$], and CRP with four cAMP molecules bound to both low- and high-affinity sites at N-terminal and C-terminal domains [CRP−$(cAMP)_4$]. It has been well documented by a variety of biophysical and biochemical studies in solution that CRP, upon cAMP binding, undergoes allosteric con-

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[1] Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; CRP, cAMP receptor protein; wt, wild type; CRPW13F, CRP without Trp13 residues in both subunits; CRPW85-CRP, CRP without tryptophan residues in one subunit and without Trp13 in the second subunit; CRPW85-CRPC178, CRP without tryptophan residues and Cys178 labeled with 1,5-I-AEDANS in one subunit and without Trp13 in the second subunit; CRP(W85,C178)-CRP, CRP without tryptophan residues in one subunit and without Trp13 and Cys178 labeled with 1,5-I-AEDANS in the second subunit; 1,5-I-AEDANS, *N*-iodoacetylaminoethyl-1-naphthylamine-5-sulfonate; AEDANS-CRP, CRP covalently labeled at Cys178 with 1,5-I-AEDANS; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl *β*-D-thiogalactoside; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate; FRET, fluorescence (Förster) resonance energy transfer; CD, circular dichroism.

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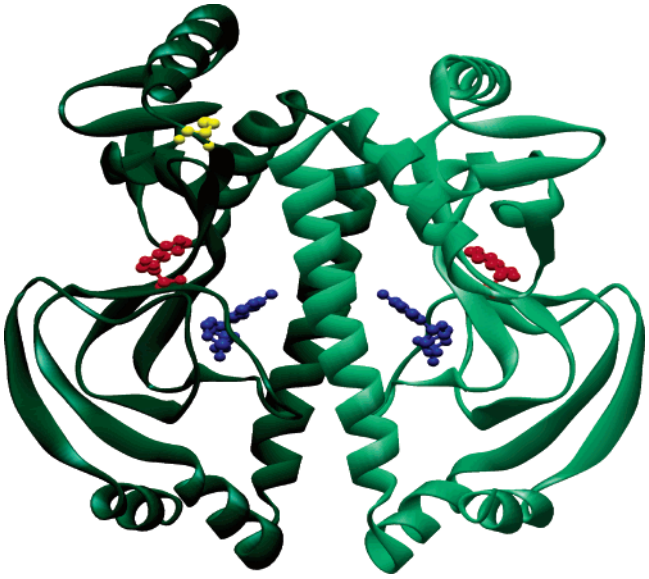

FIGURE 1: Structure of the CRP dimer. Included in this figure are Cys178 (colored yellow), Trp85 (colored red), and cyclic AMP binding to anti sites (colored blue). The figure was generated with WEBLAB VIEWERPRO (version 3.7) using atomic coordinates for the cAMP−CRP complex (*5*). The coordinates were obtained from the Protein Data Bank entry 1G6N.

formational changes that enable the protein to recognize specific DNA promoter sequences (*2*). This process can be accomplished through a long-range conformational signal transduction from the N-terminal domain to the HTH structural motif at the C-terminal domain of the protein. In fact, our fluorescence quenching studies of CRP−DNA interactions also indicate the occurrence of long-distance conformational signal transduction within the protein from the C-terminal DNA-binding domain to the N-terminal domain of CRP (*6*). Our stopped-flow kinetics studies (*7*, *8*) have shown that in solution cAMP-induced allosteric transitions in CRP can be described by two independent models of allostery. Binding of cAMP in an anti conformation in the CRP−$(cAMP)_2$ complex can be described by the sequential binding model, contrary to binding of cAMP in a syn conformation to low-affinity sites, where the concerted model of allostery is required for description of the kinetic data. On the other hand, the isothermal titration calorimetry (ITC) studies have shown that binding of two cAMP molecules at N-terminal domains exhibits negative cooperativity, and the binding to the first site is exothermic in contrast to that at the second site in the CRP−$(cAMP)_2$ complex, the occupancy of which is endothermic; the binding reaction is entropically driven (*9*). This observation has led the authors to the conclusion that the increase in entropy is a reflection of an increased dynamics of the CRP upon binding of cAMP to these strong binding sites of the protein. Indeed, our anisotropy decay of Trp13, located in the N-terminal domain of CRP (*10*), and fluorescence quenching measurements (*6*) clearly indicate an increase in the local protein dynamics upon binding of cAMP. Recently, using the Förster energy transfer (FRET) method, we have shown (*10*) that the binding of cAMP in the CRP−$(cAMP)_2$ complex caused a substantial increase in the FRET efficiency between Trp85, localized at the N-terminal domain of CRP, and Cys178 fluorescently labeled with AEDANS localized in the HTH structural motif of the C-terminal domain of CRP. These changes in FRET efficiency indicate a decrease in the distance between the two domains of the protein upon cAMP binding. The observations, via intrinsic or extrinsic fluorophore conformational changes in homodimeric CRP in solution upon binding of cAMP, do not allow us to draw any conclusions about conformational changes, which can take place in the particular CRP subunits.

We decided to study the structural and kinetic changes in the apo-CRP induced by binding of cAMP, using the heterodimeric protein, with the FRET method as well as by stopped-flow techniques. Since each subunit of CRP contains two tryptophan residues at positions 13 and 85 localized in N-terminal domains of the protein as well as one reactive cysteine residue, Cys178, localized in the HTH motif in the C-terminal domain, we have used site-directed mutagenesis as well as fusion CRP−His_6 and CRP−GST proteins to construct a heterodimer of CRP. The CRP heterodimer (Figure 1) contains a single Cys178 residue, fluorescently labeled with 1,5-I-AEDANS, localized at only one of the C-terminal domains of the protein as well as a single Trp85 residue localized at only one of the N-terminal domains of a given subunit of the protein. The fluorescently labeled Cys178 and single Trp85 residue of the heterodimer protein have been used to study the allosteric changes in CRP structure induced by binding of cAMP.

MATERIALS AND METHODS

Materials. 1,5-I-AEDANS was purchased from Molecular Probes. EDTA and Tris were from MP Biomedical. Phenylmethanesulfonyl fluoride, reduced glutathione, and cAMP were from Fluka. IPTG was from Sigma. Thrombin and glutathione−Sepharose 4 Fast Flow were from Amersham Biosciences. Ni−NTA His-Bind Superflow was from Novagen. *Taq* polymerase, dNTP, and restriction endonucleases were from Fermentas. The nutrients for bacterial growth were from either Difco Laboratories or GibcoBRL. All other chemicals were analytical grade products from POCh-Gliwice. All measurements were performed in buffers prepared in water purified with a Millipore system.

Plasmids and Cells. Wild-type (wt) CRP was isolated from *E. coli* strain SA500 containing the pHA7 plasmid, which encodes the *crp* gene. The pHA7 plasmid was a generous gift from S. Garges. The pET-15b plasmid was purchased from Novagen, and the pGEX-4T-2 plasmid was purchased from Amersham Biosciences. Tryptophan (Trp) at position 13 of CRP was replaced with phenylalanine, and tryptophan at position 85 was replaced with leucine. The mutagenesis was performed using the overlap extension method with *Taq* DNA polymerase. Plasmids encoding mutant *crpW13F* and *crpW13FW85L* genes were introduced into *E. coli* strain BL21 purchased from Novagen. Bacteria were grown on LB medium at 37 °C, in a Biostat B fermentor from B. Braun. The expression was induced in the host bacteria by adding IPTG to the final concentration 0.4 mM when the OD reached 0.6; the incubation was continued for 3 h, and then the cells were harvested by centrifugation.

Protein Purification. CRP mutants were purified at 4 °C. The cells were disrupted by only sonication using six 45 s pulse bursts, and the cell debris was removed by centrifugation. The crude extract of GST-tagged CRP was loaded onto a column filled with glutathione−Sepharose 4 Fast Flow

equilibrated with buffer [140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium phosphate, 1.8 mM monopotassium phosphate (pH 7.3_, and 1 mM PMSF], and the column was washed overnight. GST-tagged proteins were eluted from the column with a buffer that contained 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione. Fractions containing CRP were pooled and dialyzed against a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM KCl, and 1 mM EDTA (buffer A).

The crude extract of His-tagged CRP was loaded onto a column filled with Ni−NTA His-Bind Superflow equilibrated with buffer [50 mM monosodium phosphate (pH 8.0), 300 mM sodium chloride, and 10 mM imidazole]. His-tagged proteins were eluted from the column with a linear gradient of imidazole (from 20 to 250 mM) in the same buffer, and under such conditions, CRP was eluted at ∼200 mM imidazole. Fractions containing CRP were pooled and dialyzed against buffer A. After purification, the proteins exhibited only one band on SDS−polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

To obtain heterodimers, CRPW85−CRP His-tagged CRP and GST-tagged CRP were mixed at molar ratio of 1:5 in buffer A with 6 M guanidine hydrochloride and incubated for 1−2 h at room temperature. After that, they were intensively dialyzed against buffer A at 4 °C for 24 h. To separate heterodimers of CRP from homodimers, the mixture after the dialysis and centrifugation was loaded at first onto a column filled with Ni−NTA His-Bind Superflow, and than onto a column filled with glutathione−Sepharose 4 Fast Flow. The fractions containing heterodimers of CRP were pooled and dialyzed against a buffer containing 140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium phosphate, and 1.8 mM monopotassium phosphate (pH 7.3). After that, the GST tag and the His tag were removed by using thrombin (1 unit of protease/0.1 mg of fusion proteins, incubation for 16 h at room temperature).

All measurements were performed in 50 mM Tris-HCl (pH 8.0) containing 100 mM KCl and 1 mM EDTA (buffer A). Before the measurements, all samples were filtered through a microporous filter (0.45 μm) to remove all undissolved impurities.

Fluorescence Labeling of CRP. Details of the preparation of I-AEDANS-labeled CRP were described previously (*11*). Fluorescence labeling of CRP was done before heterodimers were prepared. The stoichiometry of labeling was determined by absorption spectroscopy and was in the range of 0.9−1.1 mol of label/mol of CRP monomer. It has been shown previously that only Cys178 can be chemically modified under conditions preserving its native structure (*11*, *12*). Fractions exhibiting a high absorbance at both 280 and 340 nm were joined and dialyzed extensively against buffer A.

Concentration Determination. The concentration of the protein, cyclic nucleotide, and fluorescence label was determined by absorption spectroscopy using the following extinction coefficients: 14 650 M^{-1} cm^{-1} at 259 nm for cAMP (*13*), 33 100 M^{-1} cm^{-1} at 278 nm for CRPW13F dimers (*14*), and 6000 M^{-1} cm^{-1} at 340 nm for I-AEDANS. The concentrations of heterodimers were determined by using the formula described previously (*15*).

Fluorescence Stopped-Flow Experiments. Kinetic measurements were performed at 20 ± 0.2 °C using a SX-17 MV stopped-flow spectrofluorimeter from Applied Photophysics (Leatherhead, U.K.) in two-syringe mode. 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 tryptophan (Trp) residues, observed through a cutoff filter at wavelengths of >320 nm, after excitation at 295 nm, and (2) energy transfer between Trp residues and the 1,5-I-AEDANS moiety covalently attached to Cys178 CRP. Trp residues were excited at 295 nm, and the total fluorescence emission was observed at 350 nm through an interference filter and a cutoff filter at >320 nm.

The reaction mixtures contained final concentrations of 1 μM AEDANS-CRP and 1.5 μM CRP (for tryptophan experiments). In a typical series of experiments, the protein at fixed concentration was stopped-flow mixed with cAMP at various concentrations (mixing ratio of 1:1). Ten to fifteen kinetic traces were collected and averaged for each concentration point. Such averaged kinetic traces were fit to a single exponential or to a sum of such terms:

$$F(t) = A \exp(-kt) + C \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t, A and k are the amplitude and observed rate constant, respectively, and C is the fluorescence at infinite time. All kinetic traces were analyzed using software supplied by Applied Photophysics.

Data Analysis. Details of analysis of stopped-flow results were described previously (*7*).

FRET Measurements. Fluorescence decays were measured using a homemade time-correlated single-photon counting system based on Ortec electronics. It consisted of a Philips 2020Q photomultiplier with a response time of 1.5 ns, a 1 GHz preamplifier, a model 935 quad constant fraction discriminator, and a model 457 time-to-amplitude converter (TAC). A nanosecond nF 900 flash lamp from Edinburgh Instruments was used as a light source. Both excitation and emission light were polarized by Glan-Thompson prism polarizers.

Energy transfer was observed between the tryptophan residues and the 1,5-I-AEDANS moiety covalently attached to Cys178. The tryptophan was excited at 297 nm. Fluorescence decays were observed at 350 nm using a >320 nm cutoff filter and an interference filter centered at 350 nm. Fluorescence decays were recorded at a resolution of 23 ps per channel, resulting in a total time window of 100 ns. The final concentrations of CRP were 1 μM.

Intensity decay data were analyzed using the following multiexponential decay law:

$$I_t = \sum_i \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \quad (2)$$

where α_i and τ_i are the pre-exponential factor and decay time of component i, respectively. The fractional fluorescence intensity of each component is defined as $f_i = \alpha_i\tau_i/\sum\alpha_i\tau_i$. The data were analyzed with the software from Edinburgh Instruments. Best-fit parameters were obtained by minimization of the reduced χ^2 value.

The average efficiency of energy transfer $\langle E \rangle$ was calculated from the average donor lifetime in the presence ($\langle\tau_{DA}\rangle$) and the absence of acceptor ($\langle\tau_D\rangle$):

$$\langle E \rangle = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle} \quad (3)$$

The average lifetime was obtained from the equation

$$\langle \tau \rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \quad (4)$$

As $\langle \tau_{DA} \rangle$ and $\langle \tau_D \rangle$ are obtained without needing to know the absolute protein concentrations, uncertainties associated with protein concentration determination are eliminated in the time-resolved fluorescence measurements.

The average distance between the donor−acceptor pair ($\langle R \rangle$) was calculated from the equation

$$\langle R \rangle = R_0[(E^{-1} - 1)^{1/6}] \text{ Å} \quad (5)$$

where R_0 is the Förster critical distance (the distance at which 50% energy transfer occurs). R_0 is given by

$$R_0 = 9.78 \times 10^3[\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \text{ Å} \quad (6)$$

where n is the refractive index of the medium, Q_D is the quantum yield of the donor, $J(\lambda)$ is a spectral overlap integral of the donor fluorescence and acceptor absorption, and κ^2 is the orientation factor and accounts for the relative orientation of the donor emission and acceptor absorption transition dipole. Generally, κ^2 is assumed to be equal to $^2/_3$, which is the value for donors and acceptors that was randomized by rotational diffusion before energy transfer.

The fluorescence quantum yield of the donor in the absence of the acceptor (Q_D) was calculated from the equation

$$Q_D = Q_{RF} \frac{S_D A_{RF}}{S_{RF} A_D} \quad (7)$$

where S_D and S_{RF} are the areas under the emission spectra of the donor and a reference compound, respectively, and A_{RF} and A_D are the absorbances of the reference compound and donor, respectively, at the excitation wavelength. Q_{RF} is the quantum yield of the reference compound L-tryptophan and was taken to be 0.14 in water at 25 °C (*17*).

Circular Dichroism Measurements. Circular dichroism (CD) spectra were measured with JASCO 710 spectropolarimeter at 20 °C in a 0.1 cm cuvette. The spectra were run from 190 to 250 nm. Each spectrum was recorded at least three times and averaged. The final concentrations of CRP were 15 μM (0.71 mg/mL). The circular dichroism measurements have been used to show that denaturation of heterodimeric CRP by guanidine hydrochloride is fully reversible.

RESULTS

Binding of cAMP Monitored by the Fluorescence of a Tryptophan Residue. The rate of binding of cAMP to CRPW85-CRP was measured by the fluorescence intensity of the tryptophan residue using a stopped-flow method. When CRP wt is stopped-flow mixed with cAMP, one can observe the significant changes in the Trp intensity at only high ligand concentrations (*7*). Similar measurements were made with CRPW85-CRP.

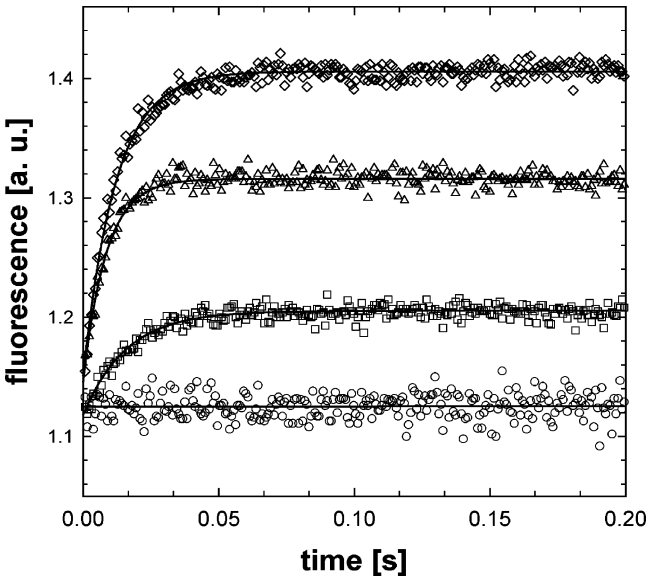

FIGURE 2: Kinetics of binding of cAMP to CRPW85-CRP measured by the changes in the fluorescence intensity of Trp residues. Measurements were performed at 20 °C in buffer A at pH 8.0 with a CRP concentration of 1.5 μM and various cAMP final concentrations: 0 (○), 1.0 (□), 2.4 (△), and 3.25 mM (◇). The fluorescence is in arbitrary units (au). The solid lines are the best single-exponential fits.

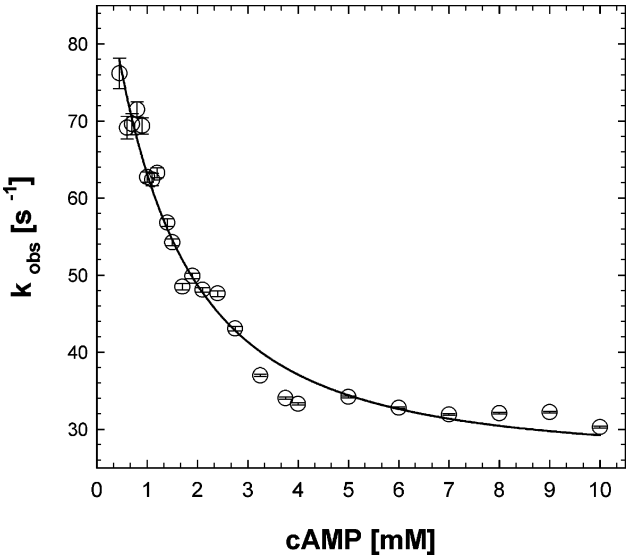

FIGURE 3: Dependence of observed rate constants (k_{obs}) for cAMP-induced changes in the Trp fluorescence intensity of CRPW85-CRP on the final cAMP concentration. The solid line is the best fit of the data, with the parameters of the fit summarized in Table 1.

Figure 2 presents a characteristic increase in the fluorescence intensity of the Trp residue. For all concentrations of cAMP, the kinetic traces could be fitted well using a single-exponential curve. Double-exponential fitting did not improve the goodness of the fit, as can be judged from the normalized variance and residual distribution. The rate constants k_{obs} calculated from the kinetic traces were plotted against the concentration of the ligand (cAMP), and the plot is presented in Figure 3. When the total cAMP concentration increased, the observed rate k_{obs} decreased, reaching a plateau at a very high ligand concentration. A description of the

Table 1: Kinetics and Thermodynamic Parameters Describing Binding of cAMP to Low-Affinity Sites of CRPW85-CRP

protein	k_{on} (s^{-1})	k_{off} (s^{-1})	K_0	K_{syn} (mM)
CRP wt[a]	28.0 ± 1.5	75.5 ± 3.0	2.7	2.0 ± 0.2
CRPW85-CRP	26.5 ± 2.5	78.0 ± 7.4	2.9	2.1 ± 0.5

[a] Obtained from ref *7*. k_{on} and k_{off} are constants describing the CRP ↔ CRP* isomerization step, and K_{syn} is a geometric average of dissociation constants for binding of cAMP to low-affinity binding sites of CRP.

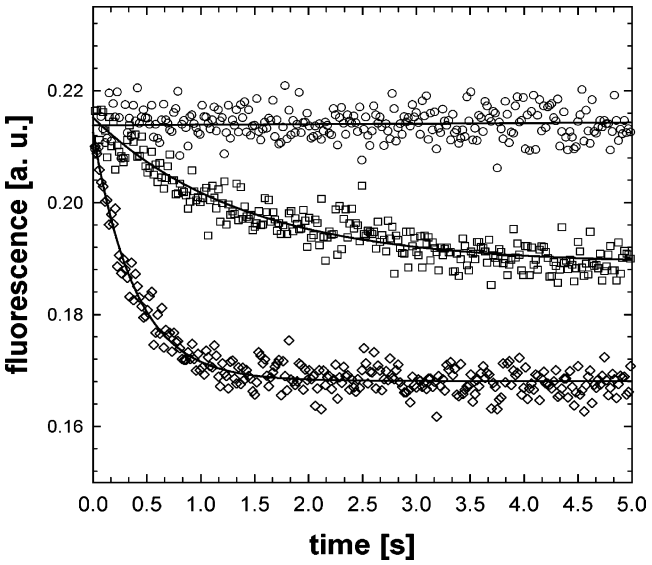

FIGURE 4: Kinetics of binding of cAMP to CRPW85-CRPC178 measured by the changes in the fluorescence intensity of the donor in energy transfer between the Trp residue (donor) and 1,5-I-AEDANS (acceptor). Measurements were performed at 20 °C in buffer A at pH 8.0 with a CRP concentration of 1.0 μM and various cAMP final concentrations: 0 (○), 0.0075 (□), and 0.06 mM (◇). The fluorescence is in arbitrary units (au). The solid lines are the best single-exponential fits.

interactions of CRPW85-CRP with cAMP was obtained using a model that was proposed in our previous study, where the binding of cAMP to wild-type CRP was analyzed (*7*). The kinetic and thermodynamic parameters obtained by fitting the data as described previously (*7*) are presented in Table 1.

Binding of cAMP Monitored by Energy Transfer between the Trp Residue (donor) and 1,5-I-AEDANS (acceptor) Covalently Attached to Cys178. The kinetics of the binding of cAMP to CRPW85-CRPC178 and CRP(W85,C178)-CRP mutants were measured as changes in the fluorescence intensity of the donor in energy transfer between the Trp residue (donor) and 1,5-I-AEDANS (acceptor). In the case of mutant CRP(W85,C178)-CRP, the addition of the ligand did not cause any significant changes in energy transfer (data not presented). Therefore, all further considerations refer to CRPW85-CRPC178. In all cases, the data were satisfactorily described by a single exponential (eq 1), while double-exponential fitting did not improve the goodness of the fit. Figure 4 shows a typical kinetic plot of the binding reaction between cAMP and CRPW85-CRPC178. For the CRPW85-CRPC178 mutant, the observed rate constants (k_{obs}) increased with an increase in ligand concentrations and reached a constant value at very high (millimolar) concentrations of cAMP, as shown in Figure 5. These results remain in agreement with our previous kinetic studies using wild-type

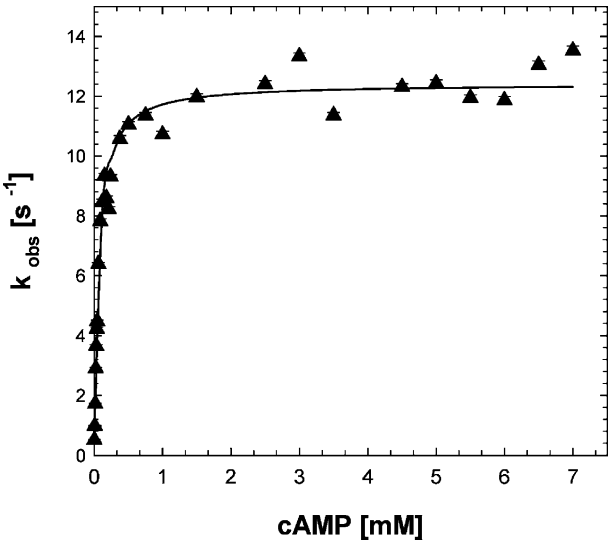

FIGURE 5: Dependence of observed rate constants (k_{obs}) for cAMP-induced changes in the Trp (donor) fluorescence intensity of CRPW85-CRPC178 on the final cAMP concentration. The solid line is the best fit of the data, with the parameters of the fit summarized in Table 2.

Table 2: Kinetics and Thermodynamic Parameters Describing Binding of cAMP to High-Affinity Sites of CRPW85-CRPC178

protein	k_c (s^{-1})	k_{-c} (s^{-1})	K_C	K_{anti} (μM)
CRP wt[a]	9.7 ± 0.1	0.31 ± 0.05	0.032	27.5 ± 1.0
CRPW85-CRPC178	12.2 ± 0.3	0.80 ± 0.30	0.070	29.2 ± 2.6

[a] Obtained from ref *7*. k_{-c} and k_c are rate constants describing the CRP−$(cAMP)_2$ ↔ CRP*−$(cAMP)_2$ conformational change step. K_C is the equilibrium constant between two forms of CRP, and K_{anti} is a geometric average of dissociation constants for binding of cAMP to high-affinity binding sites.

CRP (*7*). The parameters of fitting the data performed as described previously (*7*) are presented in Table 2.

FRET Measurements. Lifetime measurements for mutants CRPW85-CRPC178 and CRP(W85,C178)-CRP pointed to a decreased lifetime of the tryptophan fluorescence when energy transfer occurs. Figure 6 shows the time-dependent donor decays for the proteins bearing donor alone and for those with donor and acceptor. Lifetime measurements were repeated several times for each protein. The fluorescence decays were analyzed as a multiexponential decay, and in each case, the double-exponential decay was characterized by lower values of reduced χ^2. In some cases, the triple-exponential decays were recorded. The average lifetimes of Trp85 in the absence and presence of acceptor and the efficiency of energy transfer are presented in Table 3. The transfer efficiency varied between the apoprotein and the protein after binding of cAMP at a micromolar (200 μM) concentration. In the absence of the ligand, CRPW85-CRPC178 exhibited an efficiency of transfer of $14.6 \pm 2.1\%$. The addition of cAMP had an effect on energy transfer. The value of its efficiency in the case of CRPW85-CRPC178 with two cAMP molecules bound to anti sites was higher ($23.8 \pm 4.4\%$) than that for apo-CRP. The result obtained for the mutant CRP(W85,C178)-CRP in the presence of 200 μM cAMP was equal to $77.6 \pm 3.8\%$.

The energy transfer efficiency values were used to calculate the average distance between the donor and the acceptor. The fluorescence quantum yield of the Trp (donor)

Table 3: Summary of Energy Transfer Measurements

protein	$\langle\tau_D\rangle$ (ns)	$\langle\tau_{DA}\rangle$ (ns)	$\langle E\rangle$ (%)	$\langle R\rangle$ (Å)
CRPW85-CRPC178	2.45 ± 0.40	2.09 ± 0.22	14.6 ± 2.1	29.5 ± 0.9
CRPW85-CRPC178−$(cAMP)_2$[a]	2.61 ± 0.18	1.99 ± 0.29	23.8 ± 4.4	26.7 ± 1.9
CRP(W85,C178)-CRP	2.45 ± 0.40	1.26 ± 0.45	60.9 ± 5.0	22.0 ± 2.5
CRP(W85,C178)-CRP−$(cAMP)_2$[a]	2.61 ± 0.18	0.58 ± 0.28	77.6 ± 3.8	17.9 ± 2.1

[a] The concentration of cAMP was 200 μM.

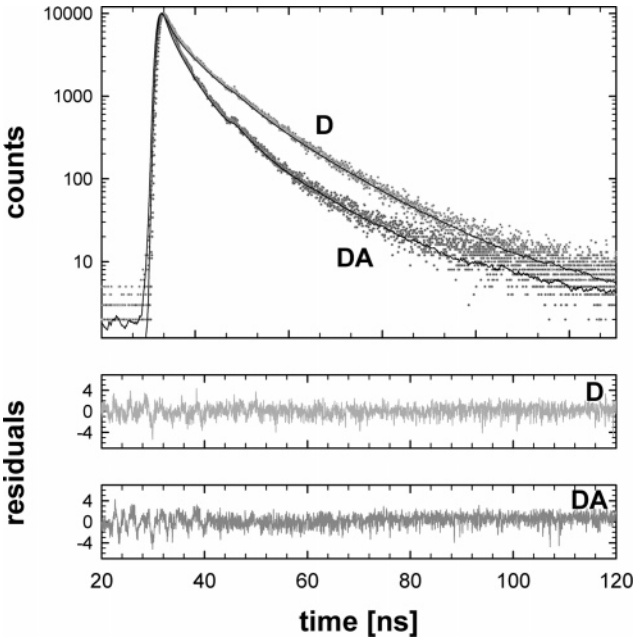

FIGURE 6: Trp85 fluorescence intensity decays for mutant CRPW85-CRPC178 without and with 1,5-I-AEDANS covalently attached to Cys178. The gray dotted curve shows the intensity decay of donor alone (D), and the dark gray dotted curve shows the intensity decay of the donor in the presence of acceptor (DA). The black solid lines and weighted residuals are for the best exponential fits. Experiments were performed in buffer A at 20 °C.

in the absence of 1,5-I-AEDANS (acceptor) was estimated using eq 7. The fluorescence quantum yield of Trp was 0.082 for apo-CRP. The Förster distance R_0 was calculated using eq 6, and the orientation factor, κ^2, was assumed to be equal to $^2/_3$ (*10*). The distance between Trp85 and Cys178 in CRP mutants was calculated using eq 5, and the Förster distance was calculated to be 22.8 Å. The estimated distances are listed in Table 3. There were significant differences between the calculated distance in apo-CRPW85-CRPC178, apo-CRP(W85,C178)-CRP, and CRP complexed with cAMP.

Circular Dichroism Measurements. The CD spectra of CRP wt and heterodimer CRPW85-CRP show no significant differences between these proteins (data not presented), which indicates that there are no changes in the secondary structure of the heterodimer compared to the homodimer of CRP.

DISCUSSION

It has been proposed that the molecular mechanism of signal transduction, induced upon binding of the allosteric inducer cAMP to CRP, involves a change in the relative orientation of the protein subunits and a change in the orientation of the C-terminal domain relative to the N-terminal domain of the protein (*2*, *5*). These changes allow the CRP to switch from a low affinity for DNA and a nonspecific DNA binding state to the state characterized by high affinity and DNA sequence specificity (*2*). Recently, we have also proposed (*6*) that the binding of CRP via C-terminal domains of the protein to the different DNA promoter sequences can induce various conformational changes in the N-terminal domains, which in turn allow the protein to interact with additional participants of transcriptional machinery such as RNA polymerase and other regulatory proteins. It has been assumed that in solution CRP can exist in several conformational states, and the distribution among these states is affected by binding of the inducer or by binding of the protein to specific DNA sequences (*6*, *9*, *10*). We believe that to understand this allosteric mechanism of CRP function it is essential to elucidate the kinetic mechanism of this long-distance signal transduction as well as to associate with this process the changes in the distance between the C-terminal and N-terminal domains of the protein upon inducer binding.

Bearing these findings in mind, in this study, we have used fast kinetic as well as resonance energy transfer (FRET) methods to demonstrate the symmetry in the signal transduction between the N-terminal and C-terminal domains of CRP upon binding of the allosteric cAMP inducer. We have used the constructed heterodimer of CRP, in which a single Trp85 residue was located at only one subunit of the protein and the reactive Cys178 residue was positioned at the same subunit or at another. This CRP heterodimer mutants, denoted as CRPW85-CRPC178 or CRP(W85,C178)-CRP, gave us a unique possibility to observe how the binding of cAMP can influence conformational changes in each protein subunit. The Trp85 residue, positioned at the N-terminal domain, and Cys178 (fluorescently labeled by 1,5-I-AEDANS probe), positioned in the HTH motif in the C-terminal domain of CRP, have been used as reporter groups, which can give relevant information regarding conformational changes which occur upon binding of the inducer to the protein. The mutants of the heterodimer of CRP, tagged with His_6 and GST, were separated for homogeneity using affinity chromatography as described in Materials and Methods. The observed reversibility of the protein denaturation is in agreement with our previous studies, which show that wild-type CRP (*19*) as well as its S128A mutant (*20*) undergoes a fully reversible denaturation by guanidine hydrochloride, as can be judged by CD spectra as well as by fluorescence measurements. The heterodimer CRP mutants, after removal of the His_6 and GST tags, possess the same secondary structure as the wild-type protein, as judged by CD spectroscopy in the wavelength range of 190−250 nm as well as by the cAMP binding constant, K_{anti} (Table 2). The substitution of Trp residues in the heterodimer of CRP as well as labeling by AEDANS of Cys178 of the protein did not induce any observable structural changes in free CRP, as monitored by circular dichroism (data not shown). Although in this work the free

energy of subunit interaction of CRP mutants has not been studied, one can expect that these substitutions do not lead to free energy changes in CRP heterodimer association. This suggestion can be supported by the observation that the S128A and S128P substitutions do not lead to substantial changes in the free energy of CRP subunit interactions (*20*, *21*). Ser128 of CRP, in contrast to Trp85 and -13 as well as Cys178 (*4*), is involved in the protein subunit interactions, and this mutation does not significantly perturb the structural integrity of free CRP. These heterodimeric mutants of CRP provided a means of studying the symmetry of the protein and its complexes with the cAMP effector in solution using the FRET method.

Our previous kinetic studies performed on homodimeric wild-type CRP have demonstrated that the formation of the CRP−$(cAMP)_4$ complex can be described by the equilibrium between the two forms of the CRP−$(cAMP)_2$ complex, where only one form is able to bind cAMP at low-affinity sites (*7*). This process was observed by conformational changes in CRP, which occur in surroundings of Trp85 residues present in both subunits of the protein. At present, the kinetics of binding of cAMP to the heterodimer CRP mutant, which possesses a single Trp85 residue located at only one of the protein subunits, were measured under pseudo-first-order conditions, and experimental data could be fitted to single-exponential curves. The observed rate constants for the CRPW85-CRP heterodimer decreased with an increase in cAMP concentration, as observed in the case of wild-type CRP. In this process, two conformational states of CRP exist, and only one of these states is able to bind the ligand. The calculated parameters are presented in Table 1, and these values are in very good agreement with those found for CRP wt. This agreement strongly suggests that the kinetic mechanism of binding of cAMP to both syn sites of CRP is very similar.

We have used the fluorescence energy transfer (FRET) method to study the kinetics of the distance changes between the N-terminal and C-terminal domain of CRP due to cAMP binding to high-affinity anti sites of the CRP. In these studies, we used single-tryptophan mutants of CRP, in which Trp85 was used as the fluorescence donor and the single Cys178 residue fluorescently labeled with 1,5-I-AEDANS was used as an acceptor. Because Trp85 does not change its fluorescence intensity in the presence of a micromolar concentration of cAMP (*10*), this residue can be used as a convenient fluorescence donor in these measurements. The kinetic measurements were performed when both the donor and the acceptor were localized at the same subunit of heterodimeric CRP or the donor and the acceptor were positioned on opposite subunits. These experimental setups allowed us to follow the kinetics of cAMP binding by observing changes in the fluorescence intensity of Trp85 as a result of the FRET phenomenon. When cAMP binds to anti sites, the fluorescence intensity of Trp85 decreases due to resonance energy transfer only in the case where the fluorescence acceptor Cys178-AEDANS was localized in the opposite subunit of CRP. When the donor and acceptor were on the same subunit of CRP, no significant decrease in the fluorescence of the Trp85 donor has been detected upon cAMP binding (data not shown). The apparent lack of a distance change between the N-terminal and C-terminal domains in the same subunit of CRP induced by cAMP binding can be due to the fact that this domain movement was faster then the dead time of the our stopped-flow device. Indeed, our equilibrium results of the FRET experiments presented in Table 3 support this suggestion.

This suggestion is in agreement with steady-state fluorescence intensity measurements of Trp85, serving as a FRET donor in the CRP(W85,Cys178-AEDANS)-CRP mutant, which indicate the decrease of Trp85 fluorescence intensity upon addition of cAMP (data not shown). This decrease does not result from the inner filter effect, which can be neglected under the conditions used in the fluorescence intensity measurements.

The FRET phenomenon was used to study the kinetics of the distance changes between two subunits of CRP, due to binding of cAMP to high-affinity sites, and the process was performed under pseudo-first-order conditions. Under these conditions, the kinetic process is described well by a simple single-exponential model, and in all cases, this model was superior, as judged from the normalized variance and residual distribution. The rate of the observed processes increased with the increasing ligand concentration, reaching the asymptotic values at cAMP concentrations above 1 mM. Because the association of cAMP with CRP can be considered a fast process, which is much faster than the stopped-flow device dead time of ~2 ms (*7*, *8*), one can assume that the changes (observed by FRET) in the distance between the N-terminal domain of one subunit and the C-terminal domain of the second subunit of CRP will start from the doubly ligated CRP−$(cAMP)_2$ complex. The observed process of binding of cAMP to the anti sites can be described as a unimodal conformational change in two subunits of CRP orientation that occurs according to sequential binding model. For this process, the observed rate constant (k_{obs}) should increase with an increase in cAMP concentration. This explanation remains in agreement with our previous kinetic studies of interactions of cAMP with wild-type CRP (*7*) as well as with its T127I and S128A mutants (*8*), which have shown that the binding of the ligand to high-affinity anti sites results in conformational changes in the HTH motifs localized in the C-terminal domains of the protein. The values of kinetic constants k_c and k_{-c} as well as dissociation constant K_{anti} and isomerization constant K_c are presented in Table 2. In Table 2, the kinetic and thermodynamic parameters, describing binding of cAMP to wild-type CRP, determined by the measurements of fluorescence intensity changes in the surroundings of Cys178 residues in the HTH motif, are also presented for comparison. The kinetic and thermodynamic parameters determined in this study, describing the distance changes between the C-terminal domains of one subunit and the N-terminal domains of the second subunit of CRP upon binding of cAMP to high-affinity sites, are very close to the values found in case of the conformational changes in the HTH motif of CRP wt. This agreement supports the mechanism proposed previously (*7*), which describes the cooperative sequential model of binding of cAMP to CRP. An analysis of the data gave a value of 0.07 for the equilibrium constant, K_c, between the two conformational forms of the CRP−$(cAMP)_2$ complex. This low value is comparable to the K_c value of 0.032, which has been estimated for wild-type CRP, and one can expect that almost all doubly liganded CRP would exist in solution in the conformational active state. The dissociation constant, K_{anti},

which is a geometric average of constants K_1 and K_2 for two cAMP molecules sequentially bound to heterodimeric CRP is very close to the value found for CRP wt, and this agreement indicates that the heterodimeric form of the protein exhibits the same affinity for the cAMP inductor as wild-type CRP. The results presented in this report provide further evidence of a kinetic mechanism of long-distance communication between N-terminal and C-terminal domains within one subunit as well as between two subunits of CRP, induced by binding of cAMP to high-affinity binding sites of CRP. In our previous work, we have shown by the FRET method that the average distance between Trp85 and fluorescently labeled Cys178 in wild-type CRP decreased from 26.6 to 18.7 Å upon binding of two cAMP inductor molecules in the CRP−$(cAMP)_2$ complex. To confirm this observation and to elucidate more precisely the cAMP-induced allosteric transitions, which are involved in a change in the orientation of the CRP subunit and a change in the distance between two domains in each of the protein subunit, we used the FRET method. The calculated distance between Trp85 localized at the N-terminal domain and Cys178 at the C-terminal domain of CRP of the same subunit in apo-CRP is equal to 22.0 Å and decreased to 17.9 Å upon binding two cAMP molecules to make a CRP−$(cAMP)_2$ complex. The distance between the sulfur atom of Cys178 and the C9−C10 bond of the indole ring of Trp85 of the CRP subunit, derived from the crystal structure of the CRP−$(cAMP)_2$ complex (PDB entry 1G6N), is equal to 18.9 and 21.9 Å for the subunit present in the "closed" and "open" conformation, respectively. The results presented here clarify the problem of CRP−$(cAMP)_2$ structural asymmetry resulting from conformational differences between the protein subunits, observed in the crystal structure (*4*, *5*). Our results clearly show that in solution both subunits in apo-CRP possess an open conformation and, in the CRP−$(cAMP)_2$ complex, both subunits adopt a closed conformation. This observation experimentally supports the dynamic simulation studies (*18*), which have predicted that in solution, both subunits in the CRP−$(cAMP)_2$ complex possess a closed conformation. The distance, determined by the FRET method, between Trp85 localized at one N-terminal domain in apo-CRP and Cys178 localized in the C-terminal domain of the second subunit of the protein is equal to 29.5 Å. Binding of cAMP to the high-affinity binding sites in the CRP−$(cAMP)_2$ complex causes the relative reorientation of the subunits and change this distance to 26.7 Å.

To summarize, the results obtained in this study show that in solution both subunits of apo-CRP exhibit a symmetrical structure of the open conformation, in which both C-terminal domains of the protein are the same distance from the N-terminal domains. The mechanism of allosteric transitions of CRP at a micromolar cAMP concentration occurs according to a sequential binding model, in which binding of the inducer to the high-affinity sites causes an ∼4 Å movement of the C-terminal domains toward the N-terminal domains of the protein, with kinetics faster then 2 ms, and the protein adopts a symmetrical closed conformation. This fast process associated with adoption of the closed conformation is followed by a slower reorientation of both CRP subunits. At millimolar concentrations of cAMP, when the next two molecules of the effector are bound to low-affinity sites making the CRP−$(cAMP)_4$ complex, the mechanism of this process can be described by displacement of the equilibrium between two forms of the CRP−$(cAMP)_2$ complex.

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