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# Allosteric Control of cAMP Receptor Binding Dynamics

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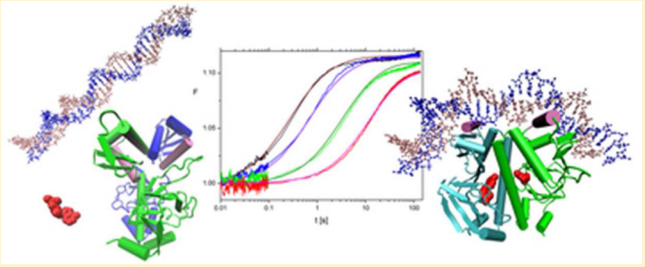
**ABSTRACT:** The intrinsic fluorescence of the cyclic AMP receptor is a sensitive indicator of the reaction with DNA, but signals are perturbed by a photoreaction. A ratio procedure is shown to be useful for correction. The reaction of the protein with DNA indicated by corrected transients extends over a broad time range not only at low salt concentrations but also at physiological salt concentrations. The initial binding step can be recorded preferentially at low salt pH 7 and is shown to be very similar for specific and nonspecific DNA. The rate constant for initial binding at 13.5 mM salt pH 7 is $2 \times 10^8$ $M^{-1}$ $s^{-1}$. Slow reaction steps up to times of several hundred seconds are observed both at low and high salt; the magnitude and sign of fluorescence amplitudes are strongly dependent on salt and pH. At 100 mM salt pH 8, the slow reaction step observed for the binding of the cyclic AMP receptor protein to promoter DNA is strongly shifted to longer times upon reduction of the cAMP concentration. The observed cAMP dependence is described quantitatively by a model implying that binding of the receptor to promoter DNA requires two cAMP molecules per protein dimer and is not consistent with a model assuming that a single cAMP is sufficient for activation. The rate constant for binding of the protein·dimer·$(cAMP)_2$ complex to the promoter is $1.3 \times 10^8$ $M^{-1}$ $s^{-1}$, close to the limit of diffusion control. Equilibration of specific complexes takes ~100 s at physiological concentrations of the reaction components.



The cyclic AMP receptor protein (CAP) controls the activity of more than 150 promoters.[1−3] The control is based on cAMP as a messenger for intracellular signal transduction. High-resolution CAP structures have been determined in different states of ligand binding.[4−9] Binding of cAMP to the protein induces release of the DNA binding domain from an embedded state.[8,9] The protein is a dimer both in crystals and in solution under usual conditions. In the complex with the promoter, the subunits of the protein form specific contacts with the palindromic DNA and induce bending of the double helix by an angle[5,7,10−14] of ~90°. Because CAP is a paradigm for the regulation of gene activity, structures have been studied extensively.

The information about the dynamics of the CAP system is not as advanced yet, but some results[15−17] obtained by various approaches have been presented. In a recent study based on stopped-flow electro-optics,[17] a rather complex sequence of reactions was detected for the binding of CAP to promoter DNA at a reduced salt concentration of 13.5 mM. For technical reasons, stopped-flow electro-optics could not be applied at physiological salt concentrations. In this investigation, the intrinsic fluorescence of CAP is measured to study the reaction with DNA at reduced salt concentrations used previously for the electro-optical measurements and also at physiological salt concentrations. The advantage of the approach based on the intrinsic fluorescence is the fact that there are no artificial reporter groups that may perturb the reaction response. However, the CAP fluorescence is affected by a photoreaction; correction is required even when the photoeffect is reduced to a minimum. Using a ratio correction procedure, DNA binding could be characterized under various conditions, and also at salt concentrations close to physiological ones. The fluorescence data measured at low salt concentrations can be compared with the electro-optical results, and thus, fluorescence changes can be assigned to structural changes during the reaction. Measurements in the physiological range of conditions clearly demonstrate the details of the allosteric mechanism used for regulation of gene activity by the cyclic AMP receptor.

## MATERIALS AND METHODS

The kinetics was recorded with a home-built stopped-flow instrument. A Hamamatsu 200 W mercury−xenon lamp together with a Bausch&Lomb high-intensity grating monochromator was used for excitation at 296.7 nm. By comparison with a commercial instrument, it was shown that the signal-to-noise ratio of the home-built instrument is favorable. This is partly due to the potential for individual adjustment of experimental parameters and the availability of high light intensities. The photoelectric signal was digitized with a Spectrum PCI.212 with 12 bit amplitude resolution and a 128 M-sample storage (Spectrum, Grosshansdorf, Germany). Most kinetic traces were sampled at 500 kHz up to 2.13 min. Extensive condensation of data, in particular at long times, was applied for optimal signal-to-noise ratios of each individual transient. This is the reason for the increase in the signal-to-noise ratio at t >0.1 s (cf. Figures 1−5). The signal-to-noise

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ratio was also increased by averaging of up to 20 transients. The averaged reaction transients were corrected for photoeffects by a procedure described in Results and with limitations specified in Discussion. Origin (OriginLab Corp.) was used for the numerical operations. The final transients were fit to kinetic models by DYNAFIT.[18,19]

The cAMP receptor protein was prepared according to the procedure by Ghosaini et al.[20] and personal communications kindly provided by A. Kolb. CAP concentrations are always given in units of protein dimer and were determined using an extinction coefficient of 40800 $M^{-1}$ $cm^{-1}$ at 278 nm.[21,22]

The single strands for the consensus DNA fragment with 40 bp were synthesized on the basis of the sequence given by Ebright et al.,[23] but the single "dangling" G residues at each end were omitted. The single strands were annealed in 10 mM NaCl, 20 mM sodium cacodylate (pH 7), and 0.1 mM EDTA . The single strands for a nonspecific DNA double helix with 40 bp (ns-40bp, sequence GCGTCCAGTT CGTTGAGCTT CTCCAGCAGC GTTAATGTCG) were synthesized and annealed by the same procedure. The sequence of ns-40bp was selected for its minimal content of segments corresponding to pseudosites.[17]

The following buffers were used: 5 mM NaCl, 10 mM sodium cacodylate pH 7, and 0.1 mM EDTA/DTT (buffer A); 10 mM NaCl, 5 mM Tris pH 8.0, and 0.1 mM EDTA/DTT (buffer B); 90 mM NaCl, 10 mM sodium cacodylate pH 7, and 0.1 mM EDTA/DTT (buffer C); 90 mM NaCl, 20 mM Tris pH 8.0, and 0.1 mM EDTA/DTT (buffer D).

Moleculer graphics was designed with VMD;[17] the structures of Protein Data Bank entries 1J59[7] and 3HIF[9] together with a DNA structure generated with NAMOT[24] were used.

## RESULTS

**Ratio Procedure for the Correction of Photoeffects.** When solutions of CAP are exposed to UV light for excitation of its fluorescence, the observed fluorescence is continuously decreased because of some photoreaction. When the light intensity is reduced to minimize the photoreaction, the signal-to-noise ratio is decreased, which is a serious problem in particular for kinetic measurements at high time resolutions. Thus, a procedure for the correction of photoeffects was tested. Fluorescence-detected transients measured at high light intensities are presented in Figure 1 for two different conditions. (1) CAP and consensus DNA are mixed in the stopped flow in buffer A and react with each other to form the protein·DNA complex. The signal represents both the binding and the photoreaction. (2) The solutions of CAP and consensus DNA are mixed and allowed to equilibrate before the mixture is exposed to the stopped flow. In this case, the observed fluorescence change is due to the photoreaction, either directly or indirectly. The fluorescence intensity of the transients given in Figure 1 at time zero is normalized to 1. Under the assumption that the change in the fluorescence intensity due to the photoreaction is the same in both cases, the transient representing binding and photoreaction can be corrected by division of this transient by the transient representing the photoreaction. The first part of the resulting transient up to t ≈0.1 s is hardly affected by the correction, whereas large changes are generated at longer times. Because this correction procedure involves assumptions (cf. Discussion), which need not be justified, the result was controlled by separate measurements using reduced light intensity. In Figure 1b, the corrected transient from Figure 1a is compared with a

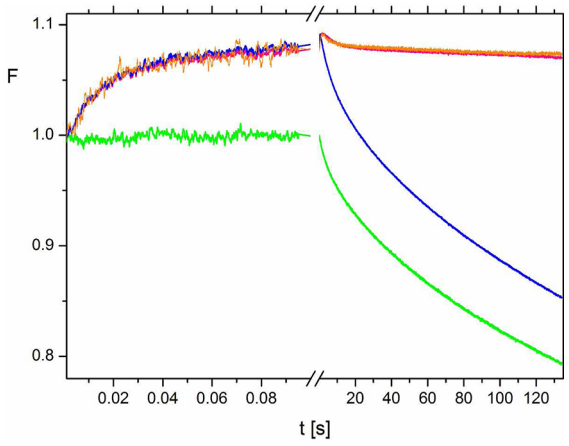


**Figure 1.** Stopped-flow transients detected by fluorescence (normalized to 1 at time zero) for the reaction of 0.1 μM CAP with 0.1 μM consensus DNA in buffer A with 20 μM cAMP at 2 °C at a high light intensity [binding and photoreaction (blue)]. Transient observed under the same conditions with the reactants equilibrated before the measurement in the stopped flow [photoreaction without binding (green)]. Transient obtained by ratio correction, i.e., the transient measured for the binding and photoreaction divided by the transient measured for the photoreaction without binding (magenta). Transient measured for the same reaction but at a light intensity reduced by a factor of 10, ratio corrected (orange).

transient recorded at a much lower light intensity but without changing all the other conditions. This transient has been subjected to the equivalent correction procedure, again using transients recorded with and without the binding reaction. A simple measure of the different light intensities is the reduction of the fluorescence in the absence of the binding reaction: at the higher light intensity, the fluorescence is reduced by 20.7% after exposure for 135 s, whereas the reduction is only 5.1% at the lower light intensity after the same exposure time. Given the large difference in the correction amplitude, the transients are remarkably similar. A major difference is the signal-to-noise ratio, which is of course favorable at high light intensities.

Another control showed that the change in the fluorescence intensity due to the photoreaction under stopped-flow conditions was very similar for CAP alone and CAP in binding equilibrium with DNA (at the same light intensity and in the same buffer). As a precaution against unexpected changes in the photoeffect (e.g., due to a change in the light emission of the arc lamp), the data for the photoreaction were recorded after each set of reaction transients, but variations under given reaction conditions were negligible.

The ratio correction was applied to all stopped-flow fluorescence transients shown in this work. The reference curves measured without the binding reaction may contribute to the noise of the corrected transients. This noise contribution was avoided by fitting the reference curves with sums of exponentials. These fits represent the reference curves with high accuracy and were used for ratio corrections. Obviously, the ratio correction procedure may not be sufficiently accurate under all conditions or for different reaction systems, and thus, controls are essential. It turned out that already a change in pH from 7 to 8 affected the photoreaction: at pH 8, lower light intensities had to be used, which is reflected by the higher noise of the transients recorded at pH 8. However, the variation of the cAMP concentration used in Binding Reaction at High Salt did not induce detectable changes in the photoreaction.


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**Binding Reaction at Low Salt.** The transients observed for the reaction of CAP with consensus DNA at low salt are quite different for pH 7 and 8 (cf. Figure 2). In buffer A at pH

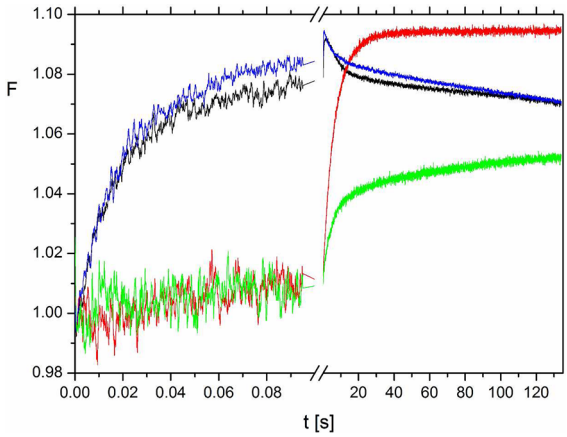


**Figure 2.** Stopped-flow transients detected by fluorescence, ratio corrected, for the following reactions of 0.1 $\mu$M CAP with 0.1 $\mu$M DNA fragments in buffers containing 20 $\mu$M cAMP at 2 °C: (1) consensus DNA in buffer A at pH 7 (black), (2) consensus DNA in buffer B at pH 8 (magenta), (3) ns-40bp in buffer A at pH 7 (blue), and (4) ns-40bp in buffer B at pH 8 (green).

7, there is a clear increase in the fluorescence already at short times of up to 100 ms, whereas the corresponding transient in buffer B at pH 8 exhibits almost no change in this time range. In contrast, a large increase in fluorescence in buffer B at pH 8 is observed at times beyond 100 ms, whereas a fluorescence decrease is found in buffer A in the corresponding time range. For an assignment of these effects, it is useful to compare the reaction of specific DNA with that of nonspecific DNA. As shown in Figure 2, the difference in the transients observed in buffer A at pH 7 is relatively small. Thus, it is likely that the effects recorded for the consensus DNA also mainly reflect binding to nonspecific sites, resulting from the fact that nonspecific sites are abundant. Conversion of nonspecific to specific complexes is expected in the case of consensus DNA, but it is hardly possible to assign any of the observed components in the fluorescence transients to such conversion, unless additional information, e.g., by stopped-flow electro-optics,[17] is used.

The transient observed for nonspecific DNA in buffer B at pH 8 shows an amplitude smaller than that of the corresponding transient for consensus DNA. An estimation of the binding constant for nonspecific DNA in buffer B based on the data of Giraud-Panis et al.[25] shows that there is only partial binding under the given experimental conditions. Nevertheless, the transient clearly reveals a slow reaction effect similar to that found for consensus DNA and, thus, suggests that this slow effect reflects some change in the conformation of the protein occurring with both specific and nonspecific DNA.

The dependence of the transients on the reactant concentration observed in buffer A (cf. Figure 3) can be used to fit rate constants. The parts of the transients at >0.1 s were not used in the fits, because these parts could not be fit with a simple model. The reason for this complication is not established; apparently, a more complex kinetic model is required (cf. Discussion). Fitting of the data recorded for ≤100 ms to the following model

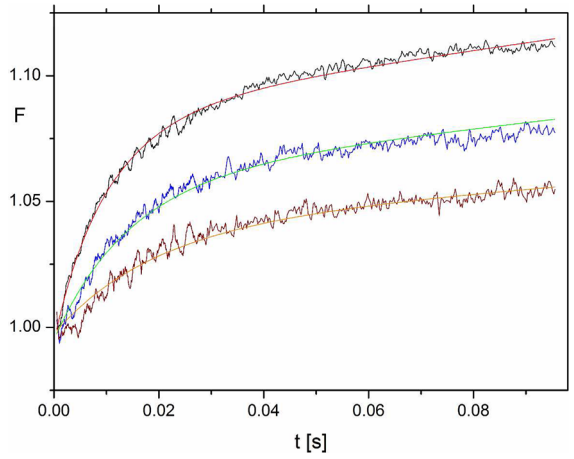


**Figure 3.** Stopped-flow transients detected by fluorescence, ratio corrected, for the reaction of CAP with consensus DNA in buffer A with 20 $\mu$M cAMP at 2 °C in a 1:1 mixing ratio at the following reactant concentrations after mixing: 200 (black), 100 (blue), and 50 nM (red). The least-squares global fits by the model described in the text are colored magenta, green, and orange lines for 200, 100, and 50 nM, respectively.

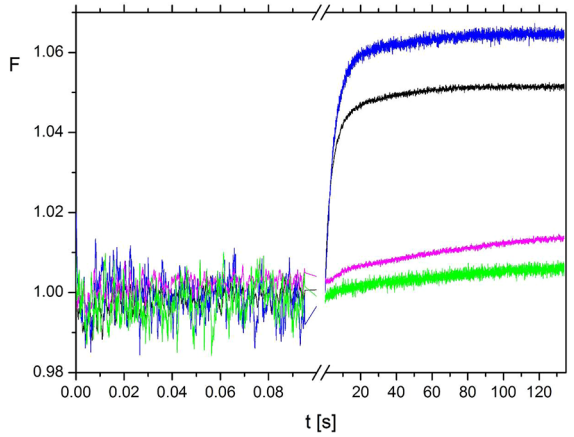


**Figure 4.** Stopped-flow transients detected by fluorescence, ratio corrected, at 2 °C of 100 nM CAP with 100 nM DNA fragment with 20 $\mu$M cAMP: consensus DNA in buffer C at pH 7 (black) and buffer D at pH 8 (blue) and ns-40bp in buffer C at pH 7 (magenta) and buffer D at pH 8 (green).

$$P + D \underset{k_1^-}{\overset{k_1^+}{\rightleftarrows}} C \overset{k_2^+}{\rightarrow} E$$

provided the following parameters: $k_1^+ = 2 \times 10^8$ $M^{-1}$ $s^{-1}$, $k_1^- = 32$ $s^{-1}$, and $k_2^+ = 1.3$ $s^{-1}$. Similar results were obtained from a corresponding set of transients recorded for ns-40bp in buffer A (not shown): $k_1^+ = 2 \times 10^8$ $M^{-1}$ $s^{-1}$, $k_1^- = 28$ $s^{-1}$ ,and $k_2^+ = 0.02$ $s^{-1}$. The values for $k_1^+$ are close to the level expected for a diffusion-controlled reaction. The accuracy of the $k_1^+$ and $k_1^-$ values is estimated to be ±20%; the corresponding estimate for $k_2^+$ is ±30%.

**Binding Reaction at High Salt.** When 0.1 $\mu$M CAP is mixed with 0.1 $\mu$M consensus DNA at salt concentrations of ~100 mM (buffers C and D), fluorescence-detected transients do not show any significant effect in the time range up to 100 ms but do show a clear increase in fluorescence in the time range t ≥1 s (Figure 4). This effect is very similar at pH 7 and 8. A slow effect in the same time range is observed for the


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nonspecific fragment ns-40bp at pH 7, but the amplitude is much smaller. A further decrease in this amplitude is found for the nonspecific fragment at pH 8. These results indicate that the binding of CAP to nonspecific DNA at high salt and pH 8 is negligible under the conditions of these experiments.

The slow effect found at high salt for consensus DNA has been analyzed in more detail at pH 8 by recording its dependence on cAMP concentration. As shown in Figure 5 the

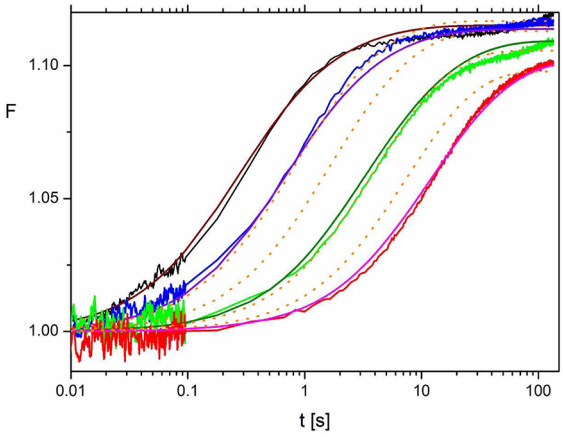


**Figure 5.** Stopped-flow transients detected by fluorescence, ratio corrected, for the reaction of 100 nM CAP with 100 nM consensus DNA in buffer C at 20 °C at 20 (black), 10 (blue), 4 (green), and 2 $\mu$M cAMP (red). The least-squares global fits according to the model with a CAP·$(cAMP)_2$ complex binding to the DNA are colored brown, purple, green, and magenta for 20, 10, 4, and 2 $\mu$M, respectively. The four yellow dotted traces represent the least-squares global fits according to the model with a CAP·cAMP complex binding to the DNA.

main part of the slow process is strongly shifted to longer times, when the cAMP concentration is reduced stepwise from 20 to 2 $\mu$M. This effect reflects coupling of binding of cAMP to CAP with the binding of CAP to the DNA. A simple model of the reaction

$$\mathrm{CAP} + \mathrm{cAMP} \underset{k_a^-}{\overset{k_a^+}{\rightleftarrows}} \mathrm{CAP{\cdot}cAMP}$$

$$\mathrm{CAP{\cdot}cAMP} + \mathrm{cAMP} \underset{k_a^-}{\overset{k_a^+}{\rightleftarrows}} \mathrm{CAP{\cdot}(cAMP)_2}$$

$$\mathrm{CAP{\cdot}(cAMP)_2} + \mathrm{DNA} \underset{k_d^-}{\overset{k_d^+}{\rightleftarrows}} \mathrm{CAP{\cdot}(cAMP)_2{\cdot}DNA}$$

considers the fact that there are two sites for binding of cAMP on CAP and assumes that both of these sites should be occupied for sufficiently strong binding to DNA. This model can be used to fit the experimental data at a satisfactory accuracy (cf. Figure 5). The parameters of this fit are as follows: $k_a^+ = 1.2 \times 10^8$ $M^{-1}$ $s^{-1}$, $k_a^- = 2.9 \times 10^3$ $s^{-1}$, $k_d^+ = 1.3 \times 10^8$ $M^{-1}$ $s^{-1}$, and $k_d^- = 1.5 \times 10^{-3}$ $s^{-1}$. The accuracy of the rate constants is estimated to be ±20%.

For comparison, a model with a single step of cAMP binding followed by DNA complexation according to CAP·cAMP + DNA ↔ CAP·cAMP·DNA was used for fitting, but in this case, the result is not satisfactory: the shift of the transients with a decreasing cAMP concentration on the time scale is much smaller than that found in the experiments (cf. Figure 5).

The three-step model presented above assumes equivalent macroscopic rate constants for the two cAMP binding steps. The data can also be described using a set of different macroscopic equilibrium constants of $6.4 \times 10^4$ and $3.1 \times 10^4$ $M^{-1}$ derived from fluorescence measurements of Lin and Lee[26] for binding of cAMP to the protein in the absence of DNA. These binding constants were confirmed by birefringence data[14] for the cAMP-induced transition between straight and bent forms of promoter DNA in binding equilibrium with CAP. For technical reasons, it is not possible to fit rate constants using these binding constants as boundary conditions (not yet provided by DYNAFIT).

## DISCUSSION

The cyclic AMP receptor is of special interest because of its central role in gene regulation and its particular mode of action involving specific DNA binding in combination with strong bending of the double helix. The determination of structures at an atomic level of detail has been pushed much further than the characterization of their dynamics. Various approaches have been used to study the dynamics of the cyclic AMP receptor and its binding to DNA.[15−17] In a recent investigation, structures during the DNA binding reaction have been identified by stopped-flow electro-optics[17] at a salt concentration of 13.5 mM. In the investigation presented here, the intrinsic fluorescence of the receptor has been used to study the reaction both under the conditions used for the electro-optical study and at higher ionic strengths over the range of physiological conditions. The intrinsic fluorescence provides the advantage for gaining information about the system in its natural state, without potential perturbations introduced by reporter groups. In this case, however, a photoreaction affects the fluorescence signals and, thus, a correction procedure has to be used.

**Ratio Correction of Transients Affected by a Photoreaction.** The procedure used for the correction of transients is simple and appears to be straightforward, but the results may not be correct in all cases. Errors may appear because the photoreaction may be different in the individual states during the reaction. The change in fluorescence observed in the final state of the reaction is used for correction of the fluorescence during the whole course of the reaction. This approximation is justified, when the photoreaction is not changed during the binding reaction. The first steps of CAP binding to DNA are relatively fast under most conditions, and thus, the extent of the photoreaction is minor during the initial period of the binding reaction. In any case, the result must be controlled because details cannot be predicted. The controls executed in this study justify the conclusion that the main features of the reaction are represented properly in the corrected transients, whereas details may require further investigation.

**Binding Reactions at Low Salt.** Sufficiently large changes in fluorescence intensity during the initial 100 ms of the binding reaction were observed only in low-salt buffer A at pH 7 (cf. Figure 3). The similarity of the transients for specific and nonspecific DNA under these conditions clearly demonstrates that this part of the reaction reflects binding to nonspecific sites. The rate constant $k_1^+$ of $2 \times 10^8$ $M^{-1}$ $s^{-1}$ determined for the first association step reflects a reaction close to the diffusion-controlled limit. A comparison of the binding constant $k_1^+/k_1^- = 7-8 \times 10^6$ $M^{-1}$ with a linear extrapolation of the equilibrium data presented by Giraud-Panis et al.[25] in their Figure 4 seems to indicate a difference of a factor of ∼100.


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However, these authors report in their Figure 1 a change in the slope of log($K$) versus log[Na] at low salt. When this effect is considered, extrapolation of their data provides a binding constant similar to that derived from the kinetic experiments. Because of extrapolation, the comparison can be only approximate; furthermore, there are relatively large differences in the equilibrium parameters presented by different authors; finally, the $k_1^+/k_1^-$ value need not be equivalent with the equilibrium constant, because there are more reaction steps. The experimental transients demonstrate the existence of further reaction steps, but a satisfactory quantitative interpretation of these data failed, because the optical parameters of the transients measured at different reactant concentrations at >100 ms were not consistent with a simple model. There are different possibilities for a qualitative interpretation. Binding of CAP to nonspecific DNA is known to be cooperative. A first step of binding is expected to produce complexes at "isolated sites" of the DNA "lattice". Thus, the reaction subsequent to the initial step may be occupation of adjacent binding sites, leading to complexes stabilized by cooperative interactions. Because the size of a CAP binding site includes ~15 nucleotide residues,[27,28] the cooperative growth reaction is limited for the 40 bp DNA fragments used in this study. Another type of reaction expected to occur is redistribution of CAP molecules between different DNA strands.[15] The special dependence on the DNA concentration resulting from this type of reaction has not been included in the models used for data fitting until now. Consideration of exchange reactions between strands may resolve problems with fitting of transients at low salt for times of >100 ms. In addition to the processes discussed above, reaction steps that reflect conformation changes like the release of the specific DNA binding site from the embedded state of the receptor protein must be expected.[8,9]

In the case of the consensus DNA, the final state of the reaction is the specific complex, and thus, again rearrangement reactions are expected to occur. Kinetic models including all the different types of rearrangements have not yet been tested.

**Binding Reaction at High Salt.** Most of the complications resulting from binding to nonspecific sites at low salt are avoided at high salt and pH 8, because the level of nonspecific binding is reduced to a minimum. Although complexes with nonspecific sites are not expected to appear in a significant population, they may contribute as short-lived intermediates. The standard approach used in the determination of binding kinetics using transients measured at different CAP and DNA concentrations was tested, but the experimental transients did not provide sufficiently clear results. Variation of the cAMP concentration provided more useful information. The distinct shift of the reaction transients observed upon variation of the cAMP concentration (cf. Figure 5) proved to be very useful for a detailed characterization of the binding reaction. It is well-known that binding of cAMP to CAP enhances the DNA binding affinity. However, the views of the mechanism of this regulation presented in the literature are very different. Many authors concluded until recently[22,29−31] that binding of a single AMP to the CAP dimer is sufficient for activation. As shown in Figure 5, the kinetic data are not consistent with a model assuming that binding of a single cAMP to the CAP dimer is enough for promoter binding under physiological conditions. The model implying two cAMP molecules for sufficient activation of DNA binding is in exact agreement with the strong shift of the reaction transients induced by changes in the cAMP concentration. Recently, an electro-optical analysis of the equilibrium transition between bent and straight DNA resulting from changes in the cAMP concentration[14] also led to the conclusion that two cAMP molecules are required for activation. Now there are results obtained by two completely independent techniques supporting the same conclusion. In comparison with previous investigations, it should be noted that the kinetic approach is relatively simple and provides the possibility of comparing the experimental result with predictions of the alternative models by simple visual inspection (cf. Figure 5), illustrating the high sensitivity of kinetics. The models can be distinguished because the different concentration dependencies are clearly separated out on the time scale. Another essential argument in favor of this interpretation is the fact that the fitted parameters come in the expected range of magnitude without external constraints: for example, the equilibrium constant for specific DNA binding is on the order of magnitude given by literature data,[23] and the rate constant for specific binding is very close to those found for corresponding protein−DNA complexes[32,33] (cf. below).

The problem of the allosteric control mechanism is coupled with the more general question of the action principle of the cAMP receptor: the palindromic promoter DNA and the protein dimer appear to be selected for optimal use of the specific recognition module by combination of two units leading to doubling of the free energy. Along this line, activation of both subunits by the same mechanism may be expected. The results presented here support this view. According to the results of Lin and Lee,[22] however, binding of cAMP to the second protein unit even leads to a reduction in the affinity for the promoter. Because of the general implications, investigations by independent approaches should be useful,

The rate constants both for binding of cAMP to the protein and for binding of the cAMP·CAP complex to the promoter DNA are relatively high. Thus, the rather long time required for formation of the specific complex under physiological conditions is mainly due to the low concentration of the active reactants. Under the conditions used for regulation in living cells,[34,35] the cAMP concentration is ~1 $\mu$M, leading to a fraction of the receptor with both cAMP sites occupied of ~$10^{-3}$. The rate constant for binding of the receptor·$(cAMP)_2$ complex to the promoter obtained from these experiments $1.3 \times 10^8$ $M^{-1}$ $s^{-1}$ is well within the range of values reported for corresponding cases of DNA recognition.[32,33] This rate is close to the limit of diffusion control. Fried and Crothers[15] reported different rate constants of $6.7 \times 10^6$ and $5 \times 10^7$ $M^{-1}$ $s^{-1}$ for the reaction of CAP with a wild-type promoter fragment in a low-salt buffer using a gel electrophoresis technique with and without quenching, respectively. They attributed the difference to "removal of non-specifically bound CAP during the quenching step". Tworzydlo et al.[16] used reactants with fluorescence labels at an ionic strength of ~130 mM and found rate constants in the range of $1.1-3.4 \times 10^6$ $M^{-1}$ $s^{-1}$. It is likely that the difference from the value presented here is due to the labels, which seem to report a different mode of the reaction.

**General Survey of Assignments.** A comparison of the transients observed for the different fragments indicates that nonspecific binding is the first reaction at reduced salt concentrations, also in the case of the promoter DNA fragment. This is due to the large excess of nonspecific sites on DNA, even for a relatively short promoter DNA. At high salt, the fast process reflecting nonspecific binding is not observed anymore.

Thus, the conclusion that the remaining slow process reflects specific binding is suggested. However, the slow processes observed at reduced salt concentrations cannot be assigned automatically to formation of specific complexes, because slow processes appear for nonspecific DNA, as well. In summary, part of the processes found in the fluorescence transients can be assigned reasonably well, but there are obvious difficulties in the assignment of some processes.

General conclusions obtained by comparison of data for specific and nonspecific DNA are equivalent for fluorescence and electro-optical data. The main difference is the structural information resulting from electro-optical transients. Because this information is mainly on the level of the global structure, specific complexes are not detected from individual contacts between bases and amino acid side chains directly but are distinguished by the global implications of these contacts. In the case of CAP−promoter complexes, the DNA double helix is strongly bent, indicated by a clear reduction of electro-optical decay time constants. The experiments at 13.5 mM salt show the appearance of bent DNA over a broad time range from ∼1 s up to hours, depending on the lifetime of nonspecific complexes. The fluorescence experiments under corresponding conditions reveal fluorescence changes in the same time range. The direction of this change is pH-dependent: at pH 7, the fluorescence decreases, whereas at pH 8, an increase is observed.

The electro-optical data demonstrate that the broad time range for formation of the specific complex with bent DNA at low salt is due to a slow transfer of the protein from nonspecific sites to the specific site.[17] This delay effect is reduced when the lifetime of nonspecific complexes is decreased. By this argument, it may be expected that specific complexes are formed at a high rate, when the salt concentration is high. However, the reaction observed at high salt is not as fast as might have been expected, because binding to specific sites requires the active form of CAP, which is induced by binding of cAMP. This is reflected in the kinetics of CAP binding to the promoter by a very strong dependence of the rate on cAMP concentration. The experimental data clearly demonstrate that two cAMP molecules must be bound to the CAP dimer for full activation of promoter binding. X-ray structures[8,9] show that the DNA binding domain of CAP is embedded in the absence of cAMP and is exposed upon cAMP binding. For sufficiently strong promoter binding, both subunits must be converted to the active form. In the physiological range of cAMP concentrations, the equilibrium concentration of CAP with two cAMP molecules is rather low, leading to a relatively slow complex formation.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

(1) Zubay, G., Schwartz, D., and Beckwith, J. (1970) Mechanism of Activation of Catabolite-Sensitive Genes: A Positive Control System. *Proc. Natl. Acad. Sci. U.S.A. 66*, 104−110.

(2) Zheng, D. L., Constantinidou, C., Hobman, J. L., and Minchin, S. D. (2004) Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. *Nucleic Acids Res. 32*, 5874−5893.

(3) de Crombrugghe, B., Busby, S., and Buc, H. (1984) Cyclic AMP Receptor Protein: Role in Transcription Activation. *Science 224*, 831−838.

(4) McKay, D. B., and Steitz, T. A. (1981) Structure of catabolite gene activator protein at 2.9 Å resolution suggests binding to left-handed B-DNA. *Nature 290*, 744−749.

(5) Schultz, S. C., Shields, G. C., and Steitz, T. A. (1991) Crystal Structure of a CAP-DNA Complex: The DNA Is Bent by 90°. *Science 253*, 1001−1007.

(6) Passner, J. M., and Steitz, T. A. (1997) The structure of a CAP-DNA complex having two cAMP molecules bound to each monomer. *Proc. Natl. Acad. Sci. U.S.A. 94*, 2843−2847.

(7) Parkinson, G., Wilson, C., Gunasekera, A., Ebright, Y. W., Ebright, R. E., and Berman, H. M. (1996) Structure of the CAP-DNA complex at 2.5 angstrom resolution: A complete picture of the protein-DNA interface. *J. Mol. Biol. 260*, 395−408.

(8) Popovych, N., Tzeng, S. R., Tonelli, M., Ebright, R. H., and Kalodimos, C. G. (2009) Structural basis for cAMP-mediated allosteric control of the catabolite activator protein. *Proc. Natl. Acad. Sci. U.S.A. 106*, 6927−6932.

(9) Sharma, H., Yu, S. N., Kong, J. L., Wang, J. M., and Steitz, T. A. (2009) Structure of apo-CAP reveals that large conformational changes are necessary for DNA binding. *Proc. Natl. Acad. Sci. U.S.A. 106*, 16604−16609.

(10) Kapanidis, A. N., Ebright, Y. W., Ludescher, R. D., Chan, S., and Ebright, R. H. (2001) Mean DNA bend angle and distribution of DNA bend angles in the CAP-DNA complex in solution. *J. Mol. Biol. 312*, 453−468.

(11) Lin, S. H., and Lee, J. C. (2003) Determinants of DNA bending in the DNA-cyclic AMP receptor protein complexes in *Escherichia coli*. *Biochemistry 42*, 4809−4818.

(12) Heyduk, T., and Lee, J. C. (1992) Solution studies on the structure of bent DNA in the cAMP receptor protein-lac DNA complex. *Biochemistry 31*, 5165−5171.

(13) Kahn, J. D., and Crothers, D. M. (1998) Measurement of the DNA bend angle induced by the catabolite activator protein using Monte Carlo simulation of cyclization kinetics. *J. Mol. Biol. 276*, 287−309.

(14) Porschke, D. (2010) Allosteric Control of Promoter DNA Bending by Cyclic AMP Receptor and Cyclic AMP. *Biochemistry 49*, 5553−5559.

(15) Fried, M. G., and Crothers, D. M. (1984) Kinetics and Mechanism in the Reaction of Gene Regulatory Proteins with DNA. *J. Mol. Biol. 172*, 263−282.

(16) Tworzydlo, M., Polit, A., Mikolajczak, J., and Wasylewski, Z. (2005) Fluorescence quenching and kinetic studies of conformational changes induced by DNA and cAMP binding to cAMP receptor protein from *Escherichia coli*. *FEBS J. 272*, 1103−1116.

(17) Porschke, D. (2012) Structures during binding of cAMP receptor to promoter DNA: Promoter search slowed by non-specific sites. *Eur. Biophys. J. 41*, 415−424.

(18) Kuzmic, P. (1996) Program DYNAFIT for the analysis of enzyme kinetic data: Application to HIV proteinase. *Anal. Biochem. 237*, 260−273.

(19) Kuzmic, P. (2009) DynaFit: A Software Package for Enzymology. *Methods Enzymol. 467*, 247−280.

(20) Ghosaini, L. R., Brown, A. M., and Sturtevant, J. M. (1988) Scanning Calorimetric Study of the Thermal Unfolding of Catabolite Activator Protein from *Escherichia coli* in the Absence and Presence of Cyclic Mononucleotides. *Biochemistry 27*, 5257−5261.

(21) Takahashi, M., Blazy, B., and Baudras, A. (1980) An Equilibrium Study of the Cooperative Binding of Adenosine Cyclic 3′,5′-

Monophosphate and Guanosine Cyclic 3′,5′-Monophosphate to the Adenosine Cyclic 3′,5′-Monophosphate Receptor Protein from *Escherichia coli*. *Biochemistry 19*, 5124−5130.
(22) Lin, S. H., and Lee, J. C. (2002) Linkage of multiequilibria in DNA recognition by the D53H *Escherichia coli*, cAMP receptor protein. *Biochemistry 41*, 14935−14943.
(23) Ebright, R. H., Ebright, Y. W., and Gunasekera, A. (1989) Consensus DNA site for the *Escherichia coli* catabolite gene activator protein (CAP): CAP exhibits a 450-fold higher affinity for the consensus DNA site than for the *E. coli* lac DNA site. *Nucleic Acids Res. 17*, 10295−10305.
(24) Tung, C. S., and Carter, E. S. (1994) Nucleic-Acid Modeling Tool (Namot): An Interactive Graphic Tool for Modeling Nucleic Acid Structures. *Comput. Appl. Biosci. 10*, 427−433.
(25) Giraud-Panis, M. J., Toulme, F., Blazy, B., Maurizot, J. C., and Culard, F. (1994) Fluorescence study on the nonspecific-binding of cyclic-AMP receptor protein to DNA: Effect of pH. *Biochimie 76*, 133−139.
(26) Lin, S. H., and Lee, J. C. (2002) Communications between the high-affinity cyclic nucleotide binding sites in *E. coli* cyclic AMP receptor protein: Effect of single site mutations. *Biochemistry 41*, 11857−11867.
(27) Saxe, S. A., and Revzin, A. (1979) Cooperative Binding to DNA of Catabolite Activator Protein of *Escherichia coli*. *Biochemistry 18*, 255−263.
(28) Takahashi, M., Blazy, B., and Baudras, A. (1979) Non-specific Interactions of CRP from *E. coli* with native and denatured DNAs: Control of binding by cAMP and cGMP and by cation concentration. *Nucleic Acids Res. 7*, 1699−1712.
(29) Takahashi, M., Blazy, B., Baudras, A., and Hillen, W. (1989) Ligand-Modulated Binding of a Gene Regulatory Protein to DNA: Quantitative Analysis of Cyclic-AMP Induced Binding of CRP from *Escherichia coli* to Non-Specific and Specific DNA Targets. *J. Mol. Biol. 207*, 783−796.
(30) Heyduk, T., and Lee, J. C. (1989) *Escherichia coli* cAMP Receptor Protein: Evidence for 3 Protein Conformational States with Different Promoter Binding Affinities. *Biochemistry 28*, 6914−6924.
(31) Tutar, Y. (2008) Chemical linkage at allosteric activation of *E. coli* cAMP receptor protein. *Protein J. 27*, 21−29.
(32) Kleinschmidt, C., Tovar, K., Hillen, W., and Porschke, D. (1988) Dynamics of Repressor Operator Recognition: The Tn10-Encoded Tetracycline Resistance Control. *Biochemistry 27*, 1094−1104.
(33) Halford, S. E., and Marko, J. F. (2004) How do site-specific DNA-binding proteins find their targets? *Nucleic Acids Res. 32*, 3040−3052.
(34) Lis, J. T., and Schleif, R. (1973) Different Cyclic-AMP Requirements for Induction of Arabinose and Lactose Operons of *Escherichia coli*. *J. Mol. Biol. 79*, 149−162.
(35) Epstein, W., Rothmandenes, L. B., and Hesse, J. (1975) Adenosine 3′-5′-Cyclic Monophosphate as Mediator of Catabolite Repression in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 72*, 2300−2304.