Eur Biophys J (2012) 41:415–424
DOI 10.1007/s00249-012-0791-y

ORIGINAL PAPER

EBSA
Biophysics in Europe

Structures during binding of cAMP receptor to promoter DNA: promoter search slowed by non-specific sites

Dietmar Porschke

Received: 11 October 2011 / Revised: 31 December 2011 / Accepted: 16 January 2012 / Published online: 24 February 2012

Abstract The kinetics of cAMP receptor (CAP) binding to promoter DNA has been studied by stopped-flow electric-dichroism at a reduced salt concentration, where the coupling of non-specific and specific binding can be observed directly. Amplitudes, rise and decay times of dichroism transients provide detailed information about the reaction and the structure of intermediates over more than six orders of magnitude on the time scale. CAP binding during the first milliseconds after mixing is indicated by an increase of both rise- and decay-time constants. A particularly large increase of rise times reflects initial formation of non-symmetric complexes by protein binding to non-specific sites at DNA ends. The increase of the hydrodynamic dimensions continues up to ~ 1 s, before a decrease of time constants reflects transition to compact states with bent DNA up to the time range of $\sim 10^3$ s. The slow approach to CAP-induced DNA bending is due to non-specific complexes, which are formed initially and are converted slowly to the specific complex. At the salt concentration of 13.5 mM, conversion to specific complexes with bent DNA is completed after ~ 40 s at pH 8 compared to $>10^3$ s at pH 7, resulting from a higher affinity of CAP to non-specific sites at pH 7 than 8 by a factor of ~ 100. Thus, under the given conditions non-specific sites delay rather than facilitate formation of the specific complex with bent DNA. Experimental data obtained for a non-specific DNA clearly indicate the impact of pseudo-sites. The different electro-optical parameters have been combined in global fits.

D. Porschke (✉)
AG Biomolecular Dynamics, Max Planck Institut für biophysikalische Chemie, Am Fassberg 11, 37077 Göttingen, Germany
e-mail: dpoersc@gwdg.de

Keywords Kinetics · DNA binding · Bending · Stopped flow · Electric dichroism · Pseudo-sites

Introduction

The cyclic AMP receptor (CAP) together with its complex with promoter DNA is the paradigm for gene regulation via DNA bending (Kolb et al. 1993). The structure of this complex has been analyzed and described in detail (Schultz et al. 1991; Passner and Steitz 1997; Parkinson et al. 1996). Investigations on the kinetics of CAP promoter interactions have also been reported (Fried and Crothers 1984; Tworzydlo et al. 2005), but key steps of the kinetics, in particular the time course of CAP induced promoter bending, have not been characterized yet. This is partly due to technical difficulties associated with the identification of macromolecular structures during fast reactions. The potentials of currently available approaches are compared in the discussion. In the present investigation a special technique is applied, which is based on stopped flow mixing with electro-optical detection. Electro-optical transients provide very sensitive information on global structures in solution. For the case of ethidium intercalation into double helices a resolution with respect to DNA length of 1 intercalated residue, corresponding to ~ 3.4 Å length resolution, at a time resolution up to 0.2 ms has been demonstrated (Porschke 1998). A major advantage of the technique is the fact that processes may be detected even in the absence of any spectroscopy change—simply due to changes of hydrodynamic dimensions and/or due to changes of the electric parameters (polarizability, dipole moment). Because electro-optical signals can be calculated from crystal or simulated structures, a detailed analysis is possible.

The entire metabolism is dependent on gene regulation and, thus, the reactions involved in this regulation are expected to be particularly fast. The results on lac repressor binding to its DNA target (Riggs et al. 1970; Winter et al. 1981; Barkley 1981), with a rate much higher than diffusion controlled, support this view. The results obtained in the present investigation demonstrate that there are barriers on the reaction path of CAP to the specific promoter site: CAP-induced promoter bending is observed after a relatively long reaction time. Contributions to the special reaction mechanism of CAP are characterized and the results are compared with those obtained for other cases of protein-DNA recognition.

Materials and methods

Samples

CAP and the DNA fragments were prepared as described previously (Ghosaini et al. 1988; Porschke 2010, 1991). The restriction fragment "lac121" has 121 base pairs with the primary CAP binding site of the lac promoter at its center. A restriction fragment with 84 base pairs, blunt-ended and without the specific site, is used for comparison (from vector component pVH51 by HaeIII restriction). The reaction of the receptor with the DNA fragments was measured in the following buffers: "T" with 10 mM Na-ClO_4, 5 mM Tris, pH 8.0, 0.1 mM EDTA/DTT; "C" with 10 mM Na-cacodylate, pH 7, 5 mM NaCl, 0.1 mM EDTA/DTT. The concentration of cAMP was 20 μM in all measurements.

Stopped-flow electro-optics

The general construction of the stopped-flow electro-optics instrument has been described (Porschke 1998). For the present project a new measuring cell has been prepared (Fig. 1). This cell has an optical path of 10 mm, which is longer than that of the cell used previously (2 mm). The increased path length provides a higher sensitivity of optical detection, but involves the drawback of a decreased time resolution. A dead time of 2 ms was measured for the new cell at 2°C. The electro-optical transients were recorded in the standard setup for measurements of the electric dichroism with a polarizer in front of the electro-optical cell, generating polarized light parallel to the field vector. The wavelength was 248.2 nm (mercury line).

The transients were fitted by a deconvolution procedure described previously (Porschke and Jung 1985). The reference curve representing the response curve of the detector was obtained by measuring birefringence transients. For this purpose an aqueous 1 M urea solution was

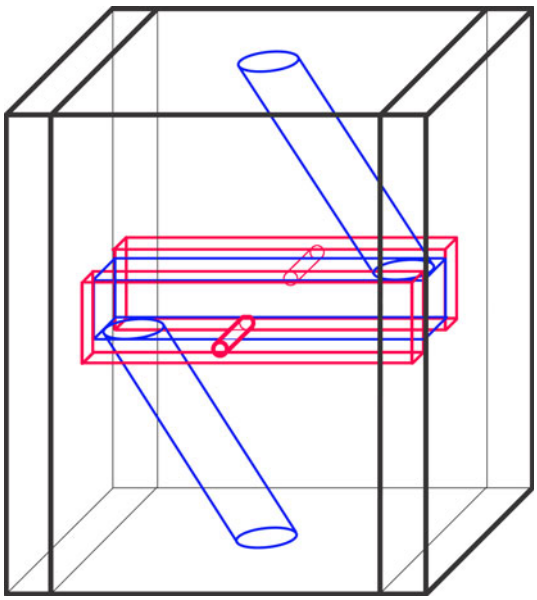

Fig. 1 Construction of the stopped-flow electric field-jump cell: The flow channel is shown in *blue* with the circular entrance/exit channels and the rectangular observation chamber at the center. The optical path length is 10 mm. The Pt electrode plates with the connector pins are shown in *red*. The rectangular cuboid with 20-, 14- and 10-mm lengths of the outer edges is melted together from quartz pieces. Most of these pieces are made from black quartz, with the exception of the windows for the observation chamber, which are prepared from quartz with high UV transmission

filled into the stopped flow cell, and polarizers were introduced into the light beam before and after the cell in the standard birefringence detection mode. Birefringence transients were generated by application of electric field pulses and recorded by the same detection system used for the measurements of dichroism transients.

As discussed previously (Porschke 2010), an excess of CAP over the lac121 fragment by a factor of ~3 is required for complete formation of the specific complex. Because the electro-optical data record the state of the DNA, the excess known to be required for complete complex formation was used in all experiments.

Results

The binding reaction of CAP to DNA double helices was initiated by mixing of the components in the stopped flow instrument, and the progress of the reaction was recorded by transients of the electric dichroism, induced by application of electric field pulses at given time intervals after mixing. Dichroism transients were measured over a broad range of delay time intervals, starting directly after stop of the flow and ending at times of approximately 1 h. As demonstrated by the examples shown in Fig. 2, there is a considerable change in these transients during the reaction.

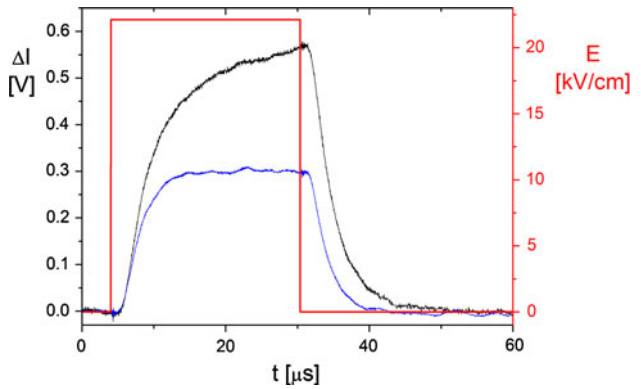

Fig. 2 Dichroism transients (change of light intensity ΔI as a function of time t) at 0.1 (*black line*) and 83 s (*blue line*) after stop of the flow for the reaction of 0.575 μM CAP with 0.2 μM lac121 in buffer C at 2°C. The electric field pulse E is shown in *red*. The rise time of the detector was limited for an optimal signal to noise ratio, which is reflected in the rise and decay transients by a sigmoidal delay phase due to convolution. The time constants were evaluated by a deconvolution routine using the response curve of the detector measured separately

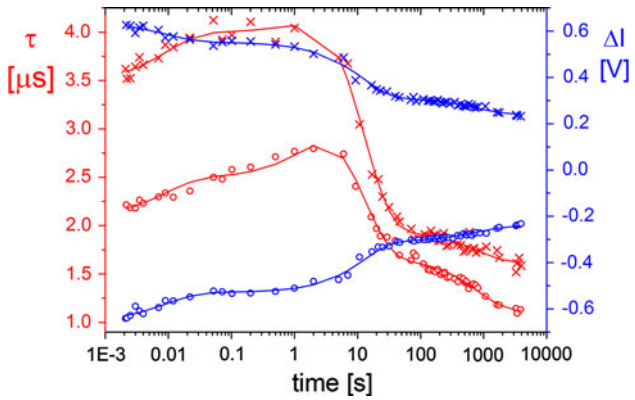

Fig. 3 Reaction of 0.575 μM CAP with 0.2 μM lac121 in buffer C at 2°C reflected by dichroism time constants τ (*left scale*) and amplitudes ΔI (*right scale*). The symbols and the scale in *red* denote time constants; the symbols and the scale in *blue* denote amplitudes. The *cross* symbols refer to rise curves; the *open circle* symbols refer to decay curves. The *continuous lines* show the representation of the data by a global fit based on a mechanism with four reaction steps ($k_1^+ = 2 \times 10^8$ M^{-1} s^{-1}, $k_1^- = 0.4$ s^{-1}, $k_2^+ = 0.4$ s^{-1}, $k_2^- = 0.1$ s^{-1}, $k_3^+ = 0.08$ s^{-1}, $k_3^- = 0.02$ s^{-1}, $k_4^+ = 0.001$ s^{-1}, $k_4^- = 0.0003$ s^{-1})

The information provided by these transients was extracted by fitting of amplitudes and time constants to both rise and decay curves.

The decay curves were measured after pulse termination and, thus, reflect free rotational diffusion of the particles. The DNAs used in the present investigation can be regarded as rigid rods in a close approximation, and their dichroism decay can be described by single exponentials. The effective decay time constant of the free promoter DNA fragment under the conditions of the experiments presented in Figs. 3, 4, 5 is 1.85 μs. The decay time constant measured 2 ms after mixing of this DNA with protein is already 2.2 μs (cf. Fig. 3). This value represents an average and indicates that at this time already part of the DNA molecules formed a complex with the protein. The increase of the decay time constant resulting from protein binding continues over a broad time range up to ~1 s. At reaction times $t > 1$ s, the decay time constants start to decrease, indicating formation of more compact structures. Again this process is extended over a broad time range—from ~1 s up to ~1 h. After reaction times of ~1 h the decay time constant arrives at $\tau \sim 1$ μs, which is consistent with the value observed under stationary conditions.

During most of the reaction time, a heterogeneous mixture of reaction states is expected to exist, which should be reflected by a spectrum of different exponential time constants. Most of these time constants are close to each other. The fraction of reaction states with divergent time constants is usually quite low and, thus, these states cannot be detected separately. Because the signal-to-noise ratio of the transients obtained from single shot experiments is not sufficiently high for separation of exponentials with closely

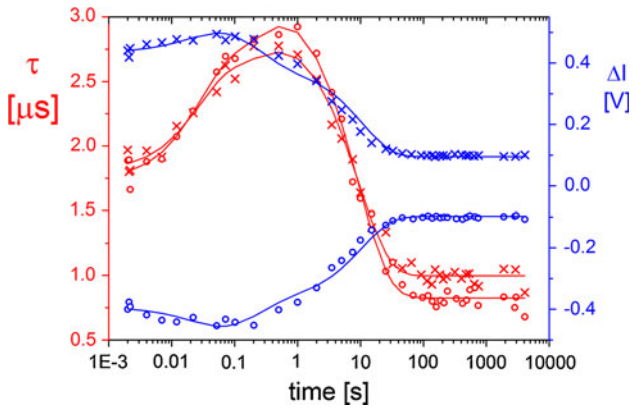

Fig. 4 Reaction of 0.517 μM CAP with 0.1712 μM lac121 in buffer T at 2°C reflected by dichroism time constants τ (*left scale*) and amplitudes ΔI (*right scale*). The symbols and the scale in *red* denote time constants; the symbols and the scale in *blue* denote amplitudes. The *cross* symbols refer to rise curves; the *open circle* symbols refer to decay curves. The *continuous lines* show the representation of the data by a global fit based on a mechanism with three reaction steps ($k_1^+ = 10^7$ $M^{-1}s^{-1}$, $k_1^- = 1$ s^{-1}, $k_2^+ = 30$ s^{-1}, $k_2^- = 0.1$ s^{-1}, $k_3^+ = 0.09$ s^{-1}, $k_3^- = 0.01$ s^{-1})

spaced time constants, the transients were fitted by single exponentials throughout the course of the reaction. Thus, single exponentials are used as an approximation and represent an average.

The dichroism rise observed for the free DNA under the action of the electric field at the given experimental conditions can also be described by single exponentials in a reasonable approximation. The electric field induces a torque, which accelerates rotation towards the direction of the field vector. Thus, the rise-time constants decrease with

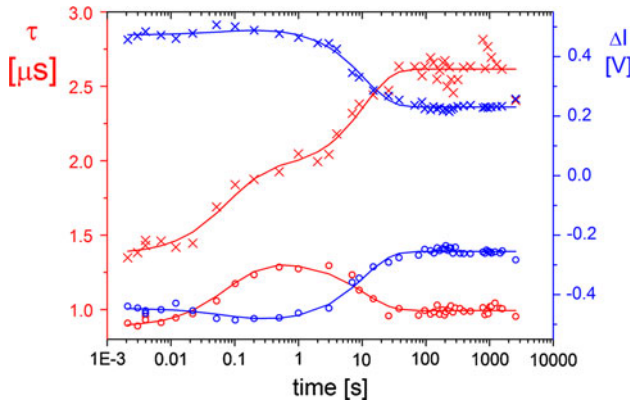

Fig. 5 Reaction of 0.4875 μM CAP with 0.2995 μM 84-bp fragment in buffer T at 2°C reflected by dichroism time constants τ (*left scale*) and amplitudes ΔI (*right scale*). The symbols and the scale in *red* denote time constants; the symbols and the scale in *blue* denote amplitudes. The *cross* symbols refer to rise curves; the *open circle* symbols refer to decay curves. The *continuous lines* show the representation of the data by a global fit based on a mechanism with two reaction steps ($k_1^+ = 3 \times 10^7\ M^{-1}\ s^{-1}, k_1^- = 0.5\ s^{-1}, k_2^+ = 0.1\ s^{-1}, k_2^- = 0.02\ s^{-1}$)

increasing electric field strength. This decrease is dependent on the ion concentration. Under the conditions of the experiments represented in Fig. 3, the rise-time constant for the free DNA is approximately 0.9 μs. Compared to this value, the rise time of ~3.6 μs observed 2 ms after mixing with the protein is unexpectedly large.

A comparison with the decay-time constant measured for the same state of reaction demonstrates that the large increase of the rise time cannot be explained by the change of the diffusion coefficient resulting from protein binding alone. There is an additional effect resulting from a change in the orientation mechanism. Free DNA rods are symmetric, also with respect to their charge distribution, and thus their field induced orientation is driven by an induced dipole mechanism. When a protein is bound to such a DNA rod, most binding states are non-symmetric, and thus the charge distribution is also non-symmetric. The loss of symmetry is equivalent with generation of a "quasi-permanent" dipole moment (Porschke 2007). The change of the dipole character causes a change in the orientation mechanism (Fredericq and Houssier 1973), leading to a change in the form of the rise curves and a slowdown of the rise. If the rise curve for the DNA with protein attached at one end could be measured separately at a high signal-to-noise ratio in the limit of low electric field strengths, two exponentials would be required for an accurate description. Under the present experimental conditions with a broad distribution of many different reaction states and at the given limitation of the signal-to-noise ratio, these processes cannot be resolved at a sufficient accuracy. Because of these limitations the overall rise curves are described approximately by single exponentials. Thus, the contribution to the rise curves from particles with a permanent dipole is simply reflected by a slowdown in the approach to the stationary dichroism, corresponding to an increased exponential time constant.

The dichroism amplitudes also provide useful information about the progress of the reaction. Because rise and decay curves are fitted separately, both the rise and decay amplitudes are presented. In principle the absolute values of these amplitudes should be identical for a given transient, but there are some variations due to a limited accuracy of the fitted parameters. As shown in Fig. 3, the amplitudes decrease continuously during the reaction. This decrease may be explained to some extent by a decrease in the polarizability; another contribution may come from a decrease in the optical anisotropy. A quantitative determination of these contributions is possible in principle, but would require extensive measurements. The amplitudes in the given form are useful as a simple indicator for the progress of the reaction. The changes observed over the broad time range from 2 ms to 1 h support the conclusion derived from the time constants on the existence of an unexpectedly complex sequence of reactions.

The data shown in Fig. 3 were obtained in buffer C with an ionic strength of 13.5 mM at pH 7. For comparison another set of data was collected in buffer T at pH 8 with the same ionic strength (Fig. 4). The general course of the reaction is very similar, but the final bent state is formed more quickly at pH 8 than at pH 7. Another comparison is enabled by Fig. 5, where data are shown for the binding of CAP to a non-specific DNA fragment in buffer T. The decay time constants indicate a reaction similar to that observed for the specific DNA (Figs. 3 and 4), including a reduction of rotation time constants at reaction times >1 s. However, this reduction is much smaller than observed for the specific DNA. Apparently bending of the double helix upon binding of CAP to non-specific DNA is limited. A detailed examination of all data provides more information. A clear difference between the specific and the non-specific DNA is observed with respect to the time dependence of the rise times. The basis of this effect is discussed below (Sect. "Pseudo-sites").

Global fitting

The electro-optical measurements provide different parameters describing the reaction from various distinct points of view, but nevertheless all these parameters reflect the same reaction. Thus, it is possible to combine these parameters in a global fit, which should provide one set of rate constants describing the time course of the different parameters together with individual amplitudes reflecting the changes of the parameters. The global fits shown in Figs. 3, 4, 5 were prepared by Dynafit (Kuzmic 1996). Simple sequential reaction models with a first bi-molecular

step followed by intra-molecular steps were used. Rate constants are given with subscripts denoting the number of the reaction step and superscripts ± for forward/backward directions, respectively (legends to Figs. 3, 4 and 5). Although there are deviations of the fitted line from data points in some time ranges, the fits are quite satisfactory in general. The quantitative representation of the data requires many parameters—not only rate constants but also amplitudes. Because of mutual coupling, the individual parameters cannot be determined yet at the desired accuracy. This is mainly due to the relatively large number of coupled reaction steps. Moreover, it is very difficult to estimate the accuracy of fitted rate constants. However, the different modes of the reaction can be clearly identified and can be assigned to the structure changes indicated by the hydrodynamic, optical and electrical parameters. The rate constants for the individual reaction steps should be useful as first approximation. Future refinement of the numerical values is possible by including information from other experiments. For example, known values of overall equilibrium constants should be used in the fits as boundary conditions, but currently this option is not yet implemented in Dynafit. Furthermore, experimental data at different concentrations should be included. Data obtained by a spectroscopic procedure with continuous recording will be useful for this purpose. However, special procedures are required for the analysis of CAP-DNA reactions because of technical difficulties such as photoreactions during fluorescence measurements.

The description of the data measured for the reaction of CAP with promoter DNA in buffer C, pH 7, by a simple sequential reaction scheme requires four reaction steps; the rate constants for the last step are $k_4^+ = 0.001\ s^{-1}$ and $k_4^- = 0.003\ s^{-1}$. In the case of buffer T, pH 8, the number of steps for the reaction of CAP with promoter DNA is reduced to three, and the rate constants for the last step are $k_3^+ = 0.09\ s^{-1}$ and $k_3^- = 0.01\ s^{-1}$. The transfer of non-specific complexes to the specific one is described in the global fits by simple standard reaction steps, although this transfer may involve a broad distribution of steps.

Models of reaction states

The procedures of quantitative molecular electro-optics have been discussed (Porschke and Antosiewicz 2007), and results have been presented for the complex formed from CAP and lac121 (Porschke 2010). These results [Fig. 6 in Porschke (2010)] can be used to evaluate the bending angle for the final reaction state observed under the present experimental conditions. The decay time constant τ observed after long reaction times ($t \rightarrow \infty$) indicates the following bending angles: in buffer C (pH 7) the decay time 1 ± 0.1 μs shows a bending angle of ∼119° ± 8°; in buffer T (pH 8) the decay time $\tau = 0.8 \pm 0.1$ μs demonstrates a bending angle of ∼137° ± 10°. The bending angle of 92° was derived previously (Porschke 2010) by electro-optical measurements under equilibrium conditions at ∼100 mM salt, pH 8. Similar values were obtained for corresponding conditions by other methods (Kahn and Crothers 1998; Kapanidis et al. 2001; Lin and Lee 2003). The increase of the bending angle with decreasing ionic strength is consistent with independent electro-optical measurements at equilibrium (in preparation).

The procedures of quantitative molecular electro-optics can also be used to analyze the structures of intermediates during the reaction. The increase of the time constants during the first part of the reaction is attributed to non-specific binding, which is expected to result in non-symmetric complexes preferentially because of statistics. As an example, electro-optical parameters were calculated for a linear DNA of 121 bp with a CAP dimer molecule bound at the end of this DNA. The dichroism decay time at 2°C for this complex is 2.82 μs, which is already close to the experimental values in the reaction time range between 0.1 and 1 s. Addition of a second CAP dimer molecule adjacent to the first one leads to an increase of the decay time constant to 3.02 μs. These data support the conclusion that non-specific complexes without significant bending of the DNA are formed in the first reaction period up to ∼1 s. The absence of strong bending effects in non-specific CAP-DNA complexes observed during the initial binding reaction is consistent with models presented by Weber and Steitz (1984) and by Pendergrast et al. (1994).

The decay time constants observed for the reaction of CAP with the 84-bp fragment show a maximum with τ ∼1.3 μs at a reaction time ∼0.5 s, and a final decrease to ∼1.0 μs at reaction times >100 s. A simulation of a complex with a CAP dimer bound to straight 84-bp DNA at pseudo-site α (see below) provided a decay time constant of 1.18 μs. Addition of a second CAP molecule close to the first one provided a decay time constant of 1.28 us. Introduction of DNA bending by about 50° at pseudo-site α (cf. below) in the complex with one CAP dimer reduced the decay time constant to about 0.9 μs. A comparison of these model simulations with the experimental data (Fig. 5) indicates that there is some bending of the double helix when CAP is bound to a pseudo-site in the 84-bp fragment at equilibrium ($t \rightarrow \infty$).

Non-symmetric complexes are reflected by relatively slow rise processes. In the case of lac121 the specific binding site is at the center, and, thus, effects due to non-symmetric complexes disappear in the final stage of the reaction. A quite different result is obtained in the case of the 84-bp fragment, where the rise times are particularly large at the end of the reaction. This effect has been

observed in buffer C at pH 7 and in buffer T at two different ionic strengths (data at 26.2 mM salt, pH 8, not shown). The magnitude of the effect raises the question of whether it can be explained by statistics alone.

Pseudo-sites

The electro-optical data provide evidence for stable non-symmetric complexes formed by CAP with the 84-bp fragment. In search of a basis for this result, the sequence of the 84-bp fragment was compared with the sequence of the consensus CAP-binding site (Ebright et al. 1989). The analysis was partly conducted by programs of the "Wisconsin Package" (GCG Version 11.1, Accelrys Inc., San Diego, CA) and also by a simple homemade program counting the number of identities with the consensus site in the 63 subsequent segments comprising 22 residues along the 84-bp sequence. It was found that there are three sites with ten identities. These sites are located toward the ends of the fragment: one of them starts at position 4 (α) from one end, and the other two start at positions 9 (β) and 14 (γ) from the opposite end. The average number of identities in all sites along the 84-bp fragment is five. A reference number reflecting a high binding affinity is 15 identities for the case of the natural CAP binding site in the 121-bp fragment.

The central part of "pseudo-site" α shows a rather close similarity to the consensus site (9 identities among 14 residues). At the current state of knowledge about CAP affinities to different sequences, it is hardly possible to predict binding constants at a sufficient accuracy. However, it is expected that binding constants scale with the similarity to the consensus site, and, thus, non-symmetric binding of CAP to the 84-bp fragment is consistent with this expectation.

A corresponding analysis of the 121-bp fragment did not show a clear bias of identities—the standard CAP binding site is located at the center. Thus, in this case the sequence does not suggest a basis for non-symmetric binding—in agreement with the available experimental data.

Another simple analysis demonstrates the general significance of pseudo-sites: the plasmid pUC18 bears 1 site with 15 identities (the natural lac site) and 2, 9 and 15 sites with 13, 12 and 11 identities, respectively. Thus, there is a high number of pseudo-sites, which is consistent with the analysis of Berg and von Hippel (1988). Obviously, any reasonable prediction of binding affinities should not be based on simple numbers of identities, but the identities should be weighted. A first approximate weighting procedure was presented by Berg and von Hippel (1988). More experimental data are required for reliable predictions.

Discussion

Comparison of kinetic methods

The stopped flow technique is widely used for the characterization of bio-molecular processes. Commercial instruments are available for measurements of absorbance and fluorescence. Other detection methods are also available, but are not as widely used. Information on structures during reactions has been obtained by various approaches including FRET (Tims and Widom 2007; Sugimura and Crothers 2006), small angle X-ray scattering (Arai et al. 2002; Grillo 2009) and NMR (Gomez-Hens and Perez-Bendito 1991; Van Nuland et al. 1998), but each of these has problems and limitations. For example, FRET studies require labeling of the reactants, and these labels should not interfere with the reaction. Furthermore, the labels must be located within a given distance range for sufficient energy transfer. The signal-to-noise ratio of small-angle X-ray scattering at advanced time resolutions is relatively low (Arai et al. 2002), such that high concentrations of reactants are required. Relatively high concentrations are also needed for NMR measurements. The main advantage of electro-optical techniques is the fact that information on structures in solution is provided at a particularly high sensitivity. For technical reasons the present analysis is restricted to a relatively low salt concentration. As demonstrated for the case of stationary electro-optical measurements (Porschke 2011), extensions to higher salt concentrations are possible.

Kinetics of receptor binding

The stopped flow data obtained for the reaction of CAP with promoter DNA demonstrate an unexpected complexity of processes. These processes are extended over a surprisingly broad time range. The first part of the reaction is observed within a few milliseconds and is indicated by an increase of both decay- and rise-time constants. The increase of time constants reflects attachment of protein molecules to non-specific DNA sites. During the initial part of the reaction, non-specific sites are occupied preferentially simply due to their large excess over specific sites. This conclusion is supported by the close similarity of data observed during the initial part of the reaction in the cases of the specific and the non-specific DNA fragment. In all cases investigated, the increase of the rise/decay time constants extends from the first ms of the reaction up to around 1 s after mixing. The reduction of the dichroism decay time constants expected from protein-induced bending of the double helix is observed in the time range beyond 1 s and again is extended over a broad time range—even up to the range of an hour in the case of the

data measured at pH 7 (cf. Fig. 3). It should be repeated here that the increase of rise-time constants observed for the non-specific DNA (Fig. 5) at long times has a special reason, which has been discussed separately in the Sect. "Pseudosites."

The broad time range of bending is very likely due to the requirement for transfer of protein molecules from non-specific sites to the specific one. It is remarkable that the approach to the specific complex with bent DNA is much faster at pH 8. The affinity of CAP to non-specific sites (Giraudpanis et al. 1994) at the given ion concentration of 13.5 mM is lower at pH 8 than pH 7 by a factor of $\sim$100. Thus, the lifetime of non-specific complexes at pH 8 is much shorter than that at pH 7, which is consistent with the observed difference in the approach to the specific site.

A comparison of Figs. 3 and 4 also shows that there is a much broader distribution of states and time constants for $t > 1$ s at pH 7, where the non-specific binding constant is particularly high. This broad distribution reflects the high number of non-specific binding states and the fact that transfer to the specific site may involve many individual intermediate states. Thus, the global fit to the data in Fig. 3 by a mechanism with four reactions steps is an approximation: the rate constants k_3 and k_4 represent a broad distribution of rate constants.

Translocation of protein molecules along the DNA double helix was concluded to be very fast for the lac repressor (Richter and Eigen 1974; von Hippel and Berg 1989; Gorman and Greene 2008). It may be expected that translocation rates are dependent on the salt concentration. The present data were obtained at a relatively low salt concentration. Riggs et al. (1970) found a much higher than diffusion controlled reaction of lac repressor, indicating fast translocation at a salt concentration very similar to that used in the present study. In this range the equilibrium constant for binding of the lac repressor to non-specific sites is even higher than that of CAP. Thus, the rather slow reaction of CAP indicates a difference in the mode of binding. High rates of transfer along DNA cannot be expected in general (Kleinschmidt et al. 1988; Halford and Marko 2004). The binding of CAP to DNA seems to involve slow reaction steps, e.g., slow changes of the protein conformation.

Indications for the complexity of CAP-promoter binding were found already during the first studies. Fried and Crothers (1984) found different rate constants of 6.7×10^6 and 5×10^7 M^{-1} s^{-1} for the reaction 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." In a later investigation Tworzydlo et al. (2005) used fluorescence-detected stopped measurements to measure the reaction of CAP labeled by AEDANS with various promoter DNAs labeled by fluorescein. At a salt concentration of $\sim$134 mM they found rate constants in the range of $1.1–3.4 \times 10^6$ M^{-1} s^{-1}, which are much below the diffusion controlled limit. Although these values require a further discussion, at least it can be concluded that there is no evidence for any contribution by some facilitating mechanism. All the previous investigations provide rate constants reflecting "overall" binding—without identification of the binding state.

Crystal structures of the free CAP protein in the absence of cAMP, called apo-CAP, (Popovych et al. 2009; Sharma et al. 2009), demonstrate that the DNA-binding domain is buried in this state and must be released for DNA binding. Under the conditions of the present measurements the cAMP concentration was 20 μM, leading to an occupation of $\sim$56% of the first cAMP binding site in the free protein (using a binding constant of 6.4×10^4 M^{-1}; (Lin and Lee 2002)). Binding of CAP to specific DNA induces an increase of the affinity to cAMP. Thus, the binding of CAP to specific DNA under the present experimental conditions involves some allosteric conformation changes of the protein and also some cAMP binding. The kinetics of CAP-conformation changes induced by cAMP binding has been studied for various mutants and using different indicators, showing "at least a three-step conformation change" with reaction times extending into the range of a few seconds (Fic et al. 2006, 2009; Gorecki et al. 2009). Thus, slow conformation changes of the protein are expected to slow down the reaction of CAP with promoter DNA.

DNA bending dynamics

A comparison of the time range found for CAP induced DNA bending with that observed for other cases of DNA bending supports the conclusion that bending of the double helix is not a rate-determining step for the formation of the CAP-promoter complex. DNA bending corresponding to "elastic" deformation (Diekmann et al. 1982) has been observed at times below a μs. Non-elastic bending processes, involving unstacking and/or activation barriers during rearrangements of the sugar-phosphate backbone, is expected to be slower and may be strongly dependent on the DNA sequence. The transition of λDNA from wormlike chains to toroids (Porschke 1984) with a bending radius of $\sim$25 nm was observed to be faster than a few milliseconds (corresponding to the "dead time" of the stopped flow under the special conditions used for experiments). DNA bending by EcoRV was reported to occur immediately upon EcoRV binding (Hiller et al. 2003), implying a bending time constant of less than $\sim$20 ms. Relaxation times observed for complexes of integration host factor with DNA in the range of $\sim$10 ms were attributed by Kuznetsov et al. (2006) to DNA bending. In summary,

there is sufficient evidence that bending of DNA itself is much faster than the bending processes observed upon reaction of CAP with promoter DNA. Because of the relatively slow time scale of binding + bending processes found in the present experiments, there is no evidence for a separation of specific complex formation and DNA bending on the reaction time scale, and it seems that the specific complex is formed and DNA is bent simultaneously. In summary, the rate of bending observed in the CAP experiments is controlled by the binding reactions of CAP to DNA. The critical step is the transfer of CAP from non-specific to specific sites. Part of the problem may also be the existence of pseudo-sites.

General comments

Time delays in the search of specific sites due non-specific binding must be expected when the lifetime of non-specific complexes increases into the time range for diffusional encounter of proteins and specific sites—provided that there are no contributions by translocation mechanisms. An effect of this type has been included in a model of Berg et al. (1981) in the special form of a reduced overall association rate of ligands with their specific site. Some data points obtained for lac repressor binding to plasmid DNA (Winter et al. 1981) have been considered as evidence for the existence of this effect. A direct experimental demonstration of delays due to non-specific sites has not been presented previously. "Indirect" methods for analysis of protein-DNA association like filter binding usually do not provide sufficient information. The problem cannot be solved by investigations based on simple optical techniques like standard fluorescence measurements. The electro-optical approach provides more direct information. In the present case the distinction between non-specific and specific binding is mainly based on DNA bending, implying that formation of the specific complex is equivalent with DNA bending. Although a final proof is not given, this implication is expected to be correct for various reasons. In addition to the arguments given above based on experimental DNA bending rates, the crystal structures of the specific complex (Schultz et al. 1991; Passner and Steitz 1997; Parkinson et al. 1996) show that formation of the specific contacts requires DNA bending.

It should be useful to compare the in vivo state of CAP with that of the lac repressor. The number of molecules per cell is very low for the lac repressor, whereas CAP is abundant [total CRP 2.5 μM (Zubay 1980; Guiso and Blazy 1980; Blazy and Ullmann 1986) ≡ ∼1,700 CAP molecules per *E. coli* cell]. Thus, the boundary conditions for a response to a physiological signal are quite different. Most of the CAP protein is associated with non-specific DNA—the local CAP concentration in the environment of promoters is expected to be relatively high. This may be a basis for the evolution of a difference in the kinetics.

Conclusions

Structures during binding of CAP to promoter DNA have been analyzed by stopped flow electric dichroism, providing details on the course of the reaction and the structure of intermediates. The coupling of non-specific and specific binding is resolved, revealing a delayed formation of the specific complex with bent DNA due to non-specific complexes. The magnitude of the delay observed for a DNA with its specific site at the center of a 121 base pair fragment and its strong dependence on the non-specific binding constant is remarkable. The data were obtained at a relatively low salt concentration, where non-specific binding is relatively strong. However, the kinetic data for lac repressor (Riggs et al. 1970; Winter et al. 1981; Barkley 1981), accepted as verification of sliding, were interpreted to show higher than diffusion controlled rates in a range where non-specific binding constants (Revzin and von Hippel 1977) are at even higher levels. Sliding of proteins along DNA double helices requires a special type of binding allowing motion without significant barriers. Obviously sliding is limited to special conditions—further investigations are required to establish the conditions for fast translocation of proteins along DNA.

Acknowledgments The facilities of the Gesellschaft für wissenschaftliche Datenverarbeitung mbH Göttingen were used for data processing.

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