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Solution Studies on the Structure of Bent DNA in the cAMP Receptor Protein–*lac* DNA Complex†

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ABSTRACT: Cyclic AMP receptor protein is involved in the regulation of more than 20 genes. A step in the mechanism of activation of transcription is to induce a significant bending of the DNA upon complex formation between specific DNA and the protein. The induced DNA bending and a structure of the protein–DNA complex were studied by fluorescence energy transfer in 50 mM Tris, 1 mM EDTA, and 50 mM KCl at pH 7.8 and 20 °C. The symmetry of the DNA bend was estimated by measuring the efficiency of transfer between the protein and a label on either the upstream or the downstream end of a *lac* DNA fragment. The results show that the bend, despite the asymmetry in the DNA sequence, is symmetrical, for the fragments which length ranges from 26 to 40 bp. Using fluorescence energy transfer, the extent of DNA bending was estimated by measuring the end-to-end distance of the DNA fragment which was labeled with a donor–acceptor pair on two opposite ends. Both steady-state and time-resolved measurements showed that in a 26 bp *lac* DNA fragment complexed with cyclic AMP receptor protein, the end-to-end distance is about 77 Å which corresponds to a bending angle of 80° or 100°, depending on the actual contour length between the fluorophores in the free DNA fragment. The results using longer DNA fragments show no measurable amount of energy transfer; thus, it is very unlikely that the DNA completely wraps around the CRP molecule. This study shows that the approach of fluorescence energy transfer has proven to be a versatile technique to provide useful structural information on the DNA–protein complex in potentially any solution conditions.

The formation of a complex between DNA and DNA-binding protein frequently results in a change in DNA structure. These changes include DNA unwinding (Siebenlist et al., 1980), DNA looping (Schleif, 1988), and DNA bending (Wu & Crothers, 1984). Protein-induced or -mediated changes in DNA structure most likely play an important role in the regulatory mechanism of gene expression (Wu & Crothers, 1984; Ptashne, 1986; Gralla, 1989); thus, in studying any system which involves the DNA–protein complex, it is important to consider the structure of the DNA in such a complex.

cAMP receptor protein (CRP)[1] from *Escherichia coli* is known to be involved in the regulation of transcription of more than 20 genes (de Crombrugghe et al., 1984). The mechanism of activation of transcription by CRP is still unclear. There is clear evidence that CRP binding results in significant bending of the DNA (Wu & Crothers, 1984; Schultz et al., 1991). The size of the bending domain and the bending angle were measured (Liu-Johnson et al., 1986), and sequence determinants of the bending were established (Gartenberg & Crothers, 1988). Almost all of the studies by Crothers and co-workers are based on results using gel electrophoresis. Recently, the crystal structure of a CRP–DNA complex became available, and it shows that the DNA is sharply bent (Schultz et al., 1991). Thus, DNA bending is an integral step of the mechanism of transcriptional regulation by CRP.

Recent experiments show that the role of bending can be quite complex. It was observed that the CRP-binding site can be replaced with naturally bent DNA sequences. This results in a mimicking of the CRP effect; i.e., a pronounced activation of transcription was observed (Bracco et al., 1989). A detailed study of this effect showed that the induced activation seems to be coupled with supercoiling, i.e., transduction of the superhelical stress to the promoter, thus facilitating the unwinding of the promoter during its conversion from a closed to an open complex (Zinkel & Crothers, 1991; Gartenberg & Crothers, 1991). Interestingly, synergistic effects of CRP and supercoiling were indeed observed (Meiklejohn & Gralla, 1989).

It was also suggested that the energy stored in bent DNA can be used to help melt promoter DNA and therefore promote

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[1] Abbreviations: CRP, cAMP receptor protein; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide; FM, fluorescein maleimide; ssDNA and dsDNA, single-stranded and double-stranded DNA, respectively; DMF, *N*,*N*'-dimethylformamide.

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open complex formation (Liu-Johnson et al., 1986). This is probably not the case since, as shown by Zinkel and Crothers (1991), the CRP-induced bending is maintained and even enhanced in the open complex.

Another possible role of the bending, as suggested by Zinkel and Crothers (1991), is that the energy stored in the bent DNA may be used to facilitate the escape of polymerase from CRP–promoter–polymerase complex. This proposal is based on an observation that the CRP-induced DNA bending is maintained in closed complex and enhanced in open complex but released in initiated complex (Zinkel & Crothers, 1991).

The bending can also influence transcription in an indirect way. In the araBAD operon, CRP was shown to be able to activate transcription by preventing the AraC protein induced DNA looping, the formation of which represses transcription. The activation is a consequence of the direction of the CRP-induced bent being opposite to the one imposed by AraC protein (Lobell & Schleif, 1991). On the other hand, CRP-induced DNA bending can serve a positive role by facilitating protein–protein interactions which are crucial for transcription, e.g., interaction between CRP and RNA polymerase. There is ample evidence to implicate the importance of the formation of such a supramacromolecular complex, e.g., observed complex formation between these proteins in solution (Blazy et al., 1980; Pinkney & Hoggett, 1988), pronounced stabilization of CRP–DNA interaction upon formation of open complex (Straney et al., 1989), cooperativity of binding of CRP and polymerase (Spassky et al., 1984; Ren et al., 1988; Ponnambalan et al., 1987), and dependence of CRP activity on helical phasing between CRP- and polymerase-binding sites (Straney et al., 1989; Gaston et al., 1990; Ushida & Aiba, 1990). The flexibility of DNA enables it to be bent in order to bring these two proteins into correct arrangement. A refined version of this model was proposed by Gaston et al. (1990) on the basis of results of helical phasing experiments. They showed that there are two preferred locations of the CRP-binding site with respect to the start site of transcription and that these preferred locations are characterized by different kinetic properties for RNA polymerase.

All of these studies show that CRP-induced bending is an important factor in the regulation of transcription by this protein. Understanding structure, thermodynamics, and kinetics of CRP-induced DNA bending will therefore contribute to an understanding of the mechanism of transcription activation. Up to date, almost all of the information is generated from gel electrophoresis experiments or crystallography; thus, the experimental conditions are essentially limited by the low-salt buffer required by gel electrophoresis or crystallization solvents. To obviate such limitation, a study was initiated to monitor the CRP-induced DNA bending directly by fluorescence energy transfer. This approach enables a direct measurement of the changes of distances, e.g., end-to-end distance, in DNA caused by bending. Furthermore, this approach is practically applicable in any solution conditions so that structural and functional information can be collected and quantitatively correlated under the same experimental conditions.

Experimental Procedures

Materials. T4 polynucleotide kinase was purchased from Boehringer Mannheim, while cystamine and 1-methylimidazole were from Sigma Chemical Co. *N*-[4-[7-(Diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide and fluorescein maleimide were obtained from Molecular Probes. CRP was purified from an overproducing strain of *E. coli* as described previously (Heyduk & Lee, 1989). It was 100% active in binding to specific DNA as monitored by stoichiometric titration using fluorescently labeled DNA (Heyduk & Lee, 1990).

The 26, 32, and 40 bp DNA fragments of the *lac* promoter with the following sequences were used in this study: 26 bp, 5′-ATTAATGTGAGTTAGCTCACTCATTA-3′; 32 bp, 5′-GCAATTAATGTGAGTTAGCTCACTCATTAGGC-3′; 40 bp, 5′-CAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCC-3′. For all these fragments, the center of the CRP-binding site is equally spaced from the 3′ and 5′ ends of the fragment. These fragments were synthesized and labeled specifically at the 5′ end (upstream and downstream of the CRP-binding site) with CPM or fluorescein essentially as described previously (Heyduk & Lee, 1990) with the following modification: the cystamine derivative of ssDNA was dissolved in 90 μL of TE buffer. Ten microliters of 0.2 M DTT was added, and the mixture was incubated for 30–45 min at room temperature. Excess DTT was removed on a G-25 spun column. The oligonucleotide was collected in Eppendorf tubes, each of which contained 10 μL of a 10 mM solution of CPM (or fluorescein maleimide) in DMF. Reaction was carried out for 75 min at room temperature in darkness. Excess fluorescent label was removed on a G-25 spun column. To avoid complication from secondary reactions involving the maleimide ring (Wu et al., 1976), these samples of labeled oligonucleotides were adjusted to pH 8.8–9.0 for 1 h (Ishi & Lehrer, 1986). Labeled single-stranded oligonucleotides were hybridized with their complementary strands (Heyduk & Lee, 1990) and subsequently dialyzed against 50 mM Tris/1 mM EDTA, pH 7.8, buffer. This procedure reproducibly produces labeled oligonucleotides possessing 0.8–1.0 mol of probe/mol of DNA.

Methods. Concentrations of protein, DNA, and fluorescent probes were determined by absorption spectroscopy using the following absorption coefficients: 20400 M^{-1} cm^{-1} at 278 nm for CRP monomer (Takahashi et al., 1980); 30000 M^{-1} cm^{-1} at 387 nm for CPM (Sippel, 1981); 70800 M^{-1} at 494 nm for fluorescein (Cerione et al., 1983). The concentration of ssDNA was determined spectrophotometrically using absorption coefficients which are calculated from a summation of coefficients of individual bases corrected for nearest-neighbor contributions (*CRC Handbook of Biochemistry and Molecular Biology*). The extent of DNA labeling was determined from the absorbance spectrum of a derivative in a manner analogous to that published by Heyduk et al. (1986).

All fluorescence measurements were performed with an SLM 500C or SLM 8000C spectrofluorometer equipped with a polarization accessory in 50 mM Tris, 50 mM KCl, and 1 mM EDTA at pH 7.8 at 20 °C. Fluorescence spectra were collected with excitation and emission polarizer set at "magic angles" to eliminate polarization effects (Lakowicz, 1983).

For sensitized emission measurements, the excitation spectrum of a solution of 30 nM labeled DNA in a 2-mL cuvette containing 100 μM cAMP was recorded from 260 to 310 nm, while monitoring the emission at 500 nm. A small volume of 20–30 μL of CRP with 100 μM cAMP was added. The final protein concentration in the cuvette was sufficient to bind essentially all DNA. The excitation spectrum was recorded after each addition of protein solution. Spectra were corrected for buffer scattering and sample dilution. As an apparent measurement of energy transfer, the ratio of fluorescence intensity (R) integrated in the range of 278–288 nm with and without CRP was used. This value was corrected for a change in CPM fluorescence induced by CRP binding when CPM was excited above 320 nm (i.e., where protein

Table I: Spectroscopic Properties of Fluorescent DNA Molecules[a]

sample	λ_{max} emission (nm)	quantum yield	lifetime (ns)[b]	Q_{uv}/Q_{vis}[c]	limiting anisotropy[d]
CPM–ssDNA	473	0.83	3.03	3.00	0.283
CPM–dsDNA	478	0.86	3.27	2.46	0.264
FM–ssDNA	511	nd[e]	3.58 (0.63) 6.42 (0.37)	1.73	0.088
FM–dsDNA	516	nd	4.08 (0.81) 10.20 (0.19)	1.32	0.133
CPM–βme	480	nd	1.70 2.99 (in methanol)	1.00	nd
FM–βme	511	nd	3.98 (in 0.1 N NaOH)	1.00	nd

[a] All measurements were performed on a 26 bp DNA fragment in 50 mM Tris, 50 mM KCl, and 1 mM EDTA, pH 7.8, unless stated otherwise. [b] All CPM samples showed essentially single-exponential decay while in the case of fluorescein-labeled DNA sample, the decay curve was more complicated and at least two exponentials were necessary to fit the decay data (numbers in parentheses correspond to the fractional contribution of the component with that specific lifetime). [c] Ratio of quantum yields of labeled DNA excited at 260 nm and in the visible region normalized to the same ratio for β-mercaptoethanol derivatives of fluorescent probes. [d] Obtained from the *y*-axis intercept of Perrin plots. [e] Not determined.

absorption is negligible). Depending on the specific DNA fragment, this correction amounted to 0–4% of the signal.

Anisotropy data for Perrin plots (Perrin, 1926) were obtained in 150-μL cuvettes. The viscosity of the solution was changed by adding glycerol.

Fluorescence lifetimes were determined by multifrequency phase fluorometry using an SLM 48000 lifetime spectrofluorometer. The excitation wavelength was 380 nm while emission was observed using a 465-nm interference filter (Schott). This filter allows observation of the donor fluorescence in the presence of an acceptor without any fluorescence contribution from the acceptor. Typically, 10–20 data points in the range of 1–150 MHz were collected. The data were analyzed using software supplied with the instrument.

Using fluorescence energy-transfer measurements, distances between the donor and receptor were calculated in accordance to eq 1 (Forster, 1959) where Q_{da}, Q_a, τ_{da}, and τ_d are the

$$E = 1 - Q_{da}/Q_d = 1 - \tau_{da}/\tau_d = R_0^6/(R_0^6 + R^6) \quad (1)$$

quantum yield and lifetime of a donor in the presence and absence of acceptor, respectively. R_0 is the distance where $E = 0.5$ and is characteristic for a given donor–acceptor pair and can be calculated from

$$R_0^6 = (8.79 \times 10^{-5})\kappa^2 n^{-4} Q_d J \quad (2)$$

where $n = 1.4$ (Fairclough & Cantor, 1978) and κ^2, the orientation factor, was assumed to be $^2/_3$, which is a reasonable assumption taking into account the observed values for limiting anisotropies (see Table I) (Dale & Eisinger, 1975). The quantum yield of a donor (Q_d) was measured by a comparative method (Parker & Reese, 1968) assuming a quantum yield of 0.7 for quinine sulfate (Scott et al., 1970). The overlap integral, J, was calculated from digitized, normalized donor absorption and acceptor emission spectra (Fairclough & Cantor, 1978), and it assumes a value of 1.73×10^{-15} M^{-1} cm^{-1} nm^4.

Results

Spectroscopic Properties of Fluorescently Labeled DNA Molecules. Before these fluorescently labeled DNA molecules are employed in this study, it is imperative to characterize these molecules with respect to their spectroscopic properties and the potential interaction of fluorescent probes with DNA. Table I is a summary of the spectroscopic properties of these probes employed in this study. The spectroscopic properties that are tested include λ_{max} for emission and lifetime. The maximum wavelength of emission for CPM is 480 nm, and it is blue-shifted in ssDNA and dsDNA with an observed λ_{max} value of 473 and 478 nm, respectively. The quantum yield of CPM in dsDNA is 0.86 and is higher than that in ssDNA

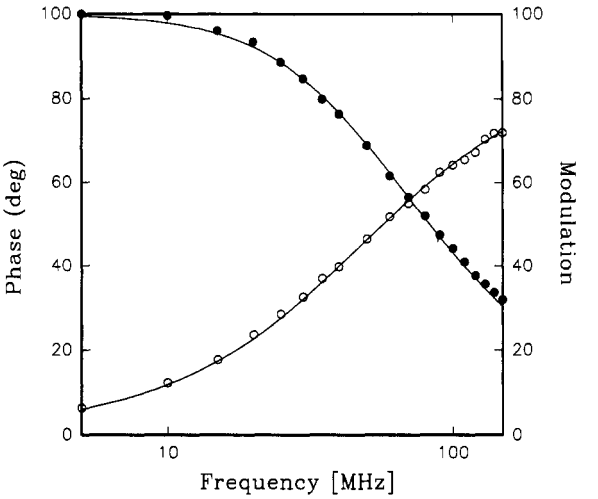

FIGURE 1: Phase and modulation data for the intensity decay of the 26 bp DNA fragment labeled with CPM. Measurements were performed on a 2 μM DNA solution in 50 mM Tris, 1 mM EDTA, and 50 mM KCl, pH 7.8. Solid lines represent the best fit to a single-exponential decay.

which assumes a value of 0.83. The lifetime of free CPM in solution or covalently linked to a 26 bp DNA fragment was monitored by phase and modulation measurements. The results for CPM on a 26 bp dsDNA fragment are shown in Figure 1. The fluorescence decay can essentially be described by a single exponential with a lifetime of 3.27 ns. The fluorescence decay of CPM–ssDNA and free CPM can also be described by a single exponential with lifetimes of 3.03 and 1.70 ns, respectively, although in methanol free CPM exists with a lifetime of 2.99 ns. Hence, it can be concluded that attachment of CPM to DNA does not change its spectroscopic properties significantly.

Fluorescence measurements of a dye in the presence of DNA may be complicated by tight binding of the dye to DNA. Two types of measurements were made to provide information about interaction of probes with DNA. One measurement is the limiting anisotropy which provides an estimation of the local mobility of a probe attached to DNA. Low mobility of a probe is characterized by high values of limiting anisotropy. Values of 0.283 and 0.264 were observed for CPM–ssDNA and CPM–dsDNA, respectively. These values suggest that CPM enjoys quite a lot of freedom of rotation. Another parameter which gives some information about interaction of probes with DNA is the normalization of the ratio of quantum yields of a probe excited in UV and in visible regions to the same ratio for the probe free in solution (Weill & Calvin, 1963). If the fluorescent probe and DNA base are in close proximity of each other, then the energy absorbed by the base

in the UV range might be transferred to the probe. However, this transfer would not be observed with an excitation wavelength in the visible range. Hence, this ratio of Q_{uv}/Q_{vis} is expected to be higher than 1 only if a dye is in close proximity to DNA bases. The range of values observed for CPM-labeled DNA is 2.46–3.0, which is characteristic for weak, nonintercalating interactions (Carrier et al., 1990).

The maximum emission wavelength for fluorescein is red-shifted from 511 to 516 nm for free probe and FM–dsDNA. The fluorescence decay behavior of fluorescein is different from that of CPM. Free fluorescein in solution exhibits a single-exponential decay with a lifetime of 3.98 ns in 0.1 M NaOH. However, when the probe is attached to DNA, a complex decay pattern was observed. At least two exponentials are necessary to describe this process satisfactorily, as summarized in Table I. The values for the limiting anisotropy, which range from 0.088 to 0.133, indicate that the probe is quite free to rotate in either ssDNA or dsDNA. These conclusions are in good agreement with that derived from the ratio of Q_{uv}/Q_{vis} which assumes values of 1.73 and 1.32 for FM–ssDNA and FM–dsDNA, respectively. It is interesting to note that the energy-transfer data are fully consistent with the anisotropy data; i.e., probes with higher values of Q_{uv}/Q_{vis}, which suggest more pronounced interactions between probes and DNA, are characterized by having higher values of limiting anisotropy, which reflect lower local mobility.

For all DNA samples used in this work, donor decay could be adequately described by a single exponential (κ^2 values in the range of 0.5–2.5) as illustrated in Figure 1. For the DNA fragments longer than 30 bp, due to their lengths there should be no significant energy transfer between donor and acceptor attached to opposite ends of the DNA fragment. Indeed, the presence of the acceptor in a 40 bp DNA fragment does not change the donor decay (not shown). This shows that most probably there are no nondipolar effects for this pair of fluorescent probes as opposed to the reported effect of rhodamine on fluorescein (Guest et al., 1991). Furthermore, this CPM–FM pair does not exhibit the non-Forster-type phenomena reported for the fluorescein–rhodamine pair (Copper & Hagerman, 1990) since no complex pattern of decay was observed for CPM, the donor, in all DNA samples.

All these results show that both probes exhibit freedom of rotation even when covalently attached to the DNA molecule. Their spectroscopic properties are not significantly altered. Combining the observation of high local mobility of both probes in this donor–acceptor pair and the fact that a rather long spacer was used to attach these probes to the DNA ends, it means that fluorescence energy transfer measurements would most likely yield information on the average position of these DNA ends. It does not provide measurement of enough precision to warrant consideration of the possible fine differences in the distance between probes that are attached to different locations in the DNA double helix as a consequence of the difference in length of DNA fragments employed.

Symmetry of DNA Bending. Symmetry of DNA bending induced by binding of CRP was studied using an approach illustrated in Figure 2. The efficiency of energy transfer between protein Trp residues and the acceptor attached to the downstream and the upstream ends of DNA is expected to be the same only if the DNA is bent in a symmetrical fashion.

The efficiency of energy transfer in this case was measured by sensitized emission, i.e., monitoring the fluorescence emission intensity at 500 nm where the acceptor fluorescence could be observed without the interference from donor fluorescence. Using this approach, the data need not be

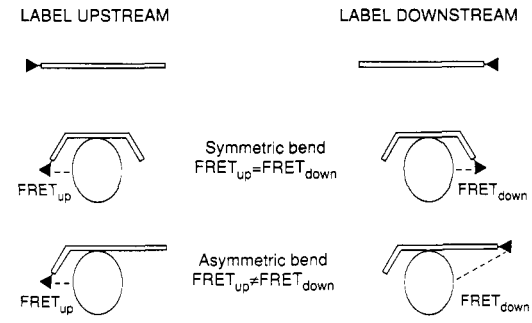

FIGURE 2: General outline of the protocol to monitor the symmetry of CRP-induced DNA bending by fluorescence energy transfer measurements. The energy transfer between protein and DNA ends is expected to be the same only if the DNA is bent symmetrically.

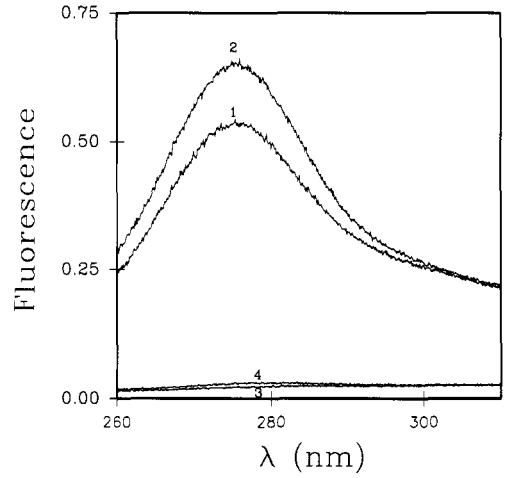

FIGURE 3: Energy transfer data of CRP and a 26 bp DNA fragment labeled with CPM at the upstream end. These excitation spectra were recorded by monitoring the emission intensity at 500 nm. The identities of samples are as follows: (1) 30 nM DNA; (2) 30 nM DNA + 260 nM CRP; (3) buffer; (4) buffer + 260 nM CRP. Buffer conditions are as described under Methods.

corrected for incomplete labeling with acceptor since only protein–DNA complexes with DNA labeled with the acceptor contribute to the fluorescence signal. A typical set of measurement is shown in Figure 3. Line 1 represents an excitation spectrum of free DNA labeled with CPM. The formation of the CRP–DNA complex results in the increase of fluorescence as shown by line 2 of Figure 3. Control experiments show that buffer scattering and fluorescence of the donor, i.e., protein Trp residues, make insignificant contribution under the experimental conditions as indicated by lines 3 and 4, respectively. Hence, the increase in fluorescence intensity of CPM–DNA in the presence of CRP is a reflection of energy transfer from CRP to the fluorophore attached to the DNA upon formation of a CRP–DNA complex. If this interpretation were correct, then the observed fluorescence enhancement should exhibit saturation behavior when a fixed concentration of DNA is titrated with an increasing concentration of CRP. The expected saturation behavior was indeed observed, as shown in Figure 4. The apparent CRP binding isotherm was further analyzed in a manner described by Heyduk and Lee (1990). An apparent binding constant of 0.83×10^8 M^{-1} was obtained and is in good agreement with a value of 1.2×10^8 M^{-1} determined by fluorescence polarization under the same experimental conditions. One can conclude, therefore, that the observed enhancement of fluorescence of an acceptor (CPM) in the presence of a donor (CRP) is due to fluorescence energy transfer which in turn reflects the distance between the protein and the labeled DNA end.

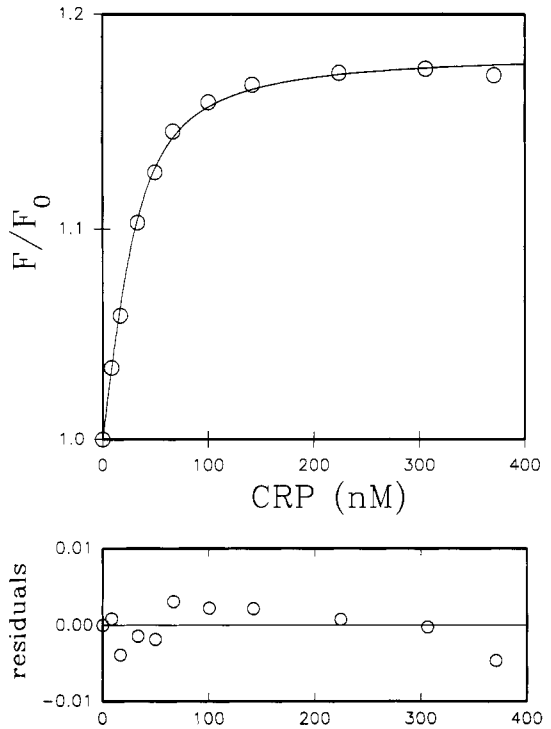

FIGURE 4: Titration of a 30 nM sample of a CPM-labeled 26 bp DNA fragment with CRP as monitored by fluorescence energy transfer. Titration was performed in 50 mM Tris, 1 mM EDTA, 100 mM KCl, 200 μM cAMP at pH 7.8 at 25 °C, and 30 nM DNA. F and F_0 represent fluorescence intensities in the presence and absence of CRP, respectively. The solid line represents the best fit to the data obtained as described by Heyduk and Lee (1990).

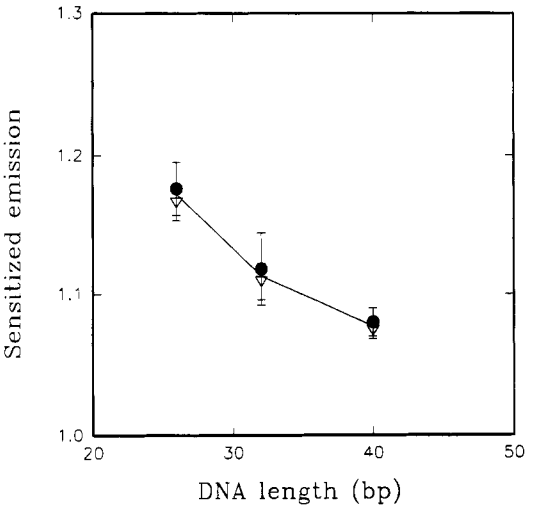

FIGURE 5: Fluorescence energy transfer between CPM-labeled DNA fragments and CRP. The symbols and positions of labeling are (●) upstream and (▽) downstream. The sensitized emission was calculated as described under Methods.

Having established the validity of this approach, fluorescence energy transfer measurements were performed to determine the symmetry of DNA bending as a function of the length of the DNA fragment. Hence, DNA fragments of 26, 32, and 40 bp were employed and labeled with CPM at the 5′ end either upstream or downstream of the CRP-binding site. Figure 5 shows the results of these experiments. Each data point is an average of at least three independent measurements. In order to eliminate the possibility of systematic errors due to small differences between preparations of labeled DNA, the measurements were also repeated using at least three different DNA preparations. The error bars in Figure 5 therefore reflect not only the precision of fluorescence measurements but also differences among several preparations of labeled DNA. The results are expressed as ratios of the integrated fluorescence intensity of the complex of CRP and DNA to that of free DNA. This number represents an apparent measurement of energy transfer. No attempt was made to calculate any distances in this case because located in different parts of each CRP subunit there are two Trp residues each of which can serve as the donor. Any distance calculation would at best be an average and would not yield any additional useful information since the purpose of this measurement is to assess if the ends of the DNA molecule are at equidistance from CRP. Hence, the empirical parameter R, as defined under Methods, is adequate for the present purpose.

The results showed that the transfer from CRP to upstream and downstream ends of DNA is the same for all three DNA fragments. Although all measurements yield a higher value for the transfer parameter when the label is located at the upstream end of the DNA fragment, this difference is statistically not significant. Another interesting observation is that the efficiency of energy transfer decreases with increasing DNA length. These results imply that with increasing DNA length the ends are positioned further away from the protein. The validity of this interpretation would be compromised if there is interaction between CRP and the fluorophores. Such an interaction may be reflected by a change in the binding affinity of CRP for the labeled and unlabeled DNA. Binding constants of CRP for the 26 bp DNA fragment were measured. For the unlabeled fragment, a binding constant of 4.05 $\times$ 10^7 M^{-1} (3.59, 4.53) was obtained. The values in parentheses correspond to 75% confidence intervals. The corresponding values for the CPM- and fluorescein-labeled fragments were 3.43 $\times$ 10^7 M^{-1} (3.22, 3.70) and 5.03 $\times$ 10^7 M^{-1} (3.96, 6.06), respectively. Hence, the presence of the fluorophores does not significantly affect the binding constant of CRP to DNA fragments. This 26 bp fragment is shorter than the thermodynamically defined CRP binding site. Thus, if no effect can be detected for this fragment, one can expect the absence of protein–fluorophore interaction in the longer fragments. A more sensitive indicator of protein–fluorophore interaction would be the measurement of the mobility of the fluorophore. Control experiments show that the limiting values of anisotropy of all CPM-labeled DNA–CRP complexes of different DNA length do not differ by more than 0.008 from the corresponding values for free DNA fragments. Therefore, there is no experimental evidence for an interaction between CRP and the fluorophore in protein–DNA complexes.

Extent of DNA Bending in Solution. Having determined the symmetry of DNA bending, it is useful to obtain direct measurements on the extent of bending in solution. The degree of bending can be estimated by measuring the end-to-end distance of the DNA fragment in the CRP–DNA complex. In order to measure the end-to-end distance, DNA duplexes were obtained with a donor molecule (CPM) on one end and the acceptor (fluorescein) on the other. Figure 6A shows the absorption spectrum of a doubly labeled 26 bp DNA fragment, and Figure 6B shows the spectral overlap between the absorption spectrum of the acceptor and the emission spectrum of the donor. The data in Figure 6B were used in calculating the overlap integral and R_0 value (50 Å). The fluorescence energy transfer measurements were performed using both steady-state and time-resolved measurements.

Table II is a summary of the results of energy-transfer experiments obtained for a 26 bp DNA fragment. For free DNA in solution, the measured transfer is very small. This is consistent with the fact that the end-to-end distance for a

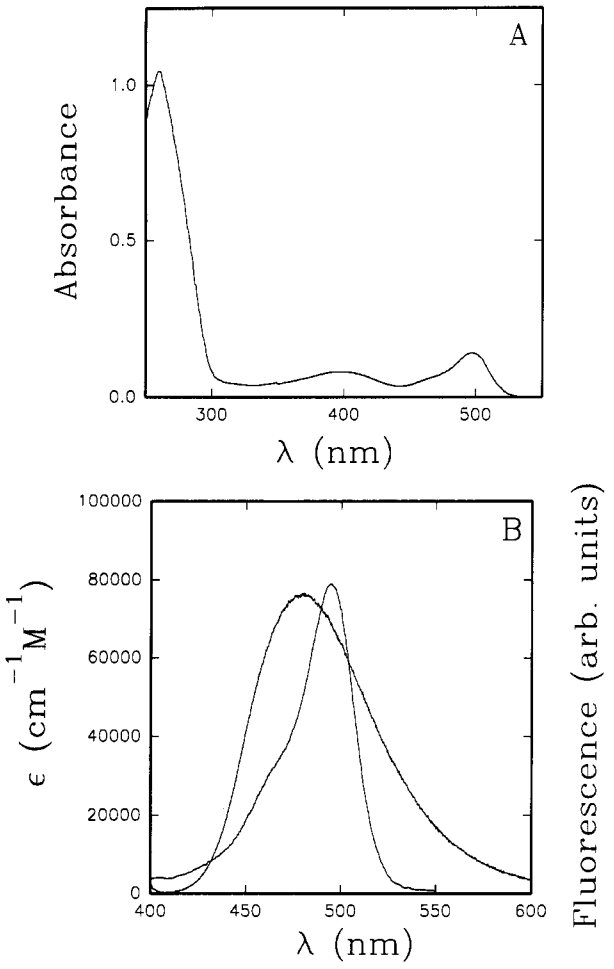

FIGURE 6: (A) Absorbance spectrum of a 26 bp DNA fragment labeled with CPM (donor) and fluorescein (acceptor). (B) Spectral overlap between the absorption spectrum of fluorescein and the corrected emission spectrum of CPM-labeled 26 bp DNA fragment.

Table II: Summary of the End-to-End Fluorescence Energy Transfer Experiment in a 26 bp DNA fragment

	E		
sample	steady-state measurement	time-resolved data	distance (Å)
CPM–DNA–FM	0.03	0.01	≥89
CPM–DNA–FM + CRP	0.08	0.06	75–79

26 bp fragment of B-DNA should be about 89 Å, and if one adds at least about 5 Å at each end to account for the distance added by flexible linkers with which the probes are attached to the DNA, then the expected distance would be around 100 Å. Thus, the end-to-end distances of the 32 and 40 bp DNA fragments are expected to be even greater. It is not surprising that for the 40 bp DNA fragment no energy transfer was observed in the free DNA samples. For the CRP–26 bp DNA complex, 6–8% of energy transfer was observed. In accordance with eq 1, this amount of energy transfer corresponds to a distance of 75–79 Å. On the basis of this distance, it is possible to calculate for the angle of bending. In these calculations, it was assumed that the bend is caused by two kinks between base pairs 5 and 6 on each side of a dyad axis and that the bending is out-of-plane by about 35° in accordance to the crystalline structure of a CRP–DNA complex (Schultz et al., 1991). If one assumes 100 Å as the contour length of the 26 bp fragment (89 Å for 26 bp B-DNA + 5 Å at each end to account for the linker in attaching the fluorophores to the DNA), it can be shown that the DNA fragment is bent by about 100°, i.e., 50° for each kink. If one assumes 89 Å for the contour length of the fragment, then the fragment is bent about 80°, accounting for two kinks of 40° each. Hence, the estimated bending angle of 80–100° represents the limiting values depending on the actual contour length between the two fluorophores.

A 32 bp DNA fragment in complex with CRP showed very marginal transfer of less than 2% (not shown). If the configuration of bent DNA observed in the 26 bp DNA fragment is preserved in the 32 bp fragment, the donor–acceptor distance should be about 93 Å; hence, an energy transfer of less than 2.5% is expected. The observed value of less than 2% is, therefore, consistent with a preservation of an 80–100° bent in the 32 bp fragment.

In the 40 bp DNA fragment complexed with CRP, essentially no transfer was observed (less than 1%). This is also consistent with the estimated end-to-end distance of about 114 Å for this fragment in complex with CRP, if DNA is bent by 80–100°.

Discussion

In the CRP system, structural asymmetry is apparently present in many configurations. One example of such asymmetry is the sequence of the DNA to which CRP binds. CRP interacts with its DNA recognition site with each subunit interacting with a half-site separated by approximately a half turn of DNA (de Crombrugghe, 1984). None of the naturally occurring CRP-binding sequences is a perfect palindrome (de Crombrugghe, 1984; Barber & Zhurkin, 1990). Therefore, most probably the two subunits of CRP are not equally contributing to the overall stability of the CRP–DNA complex. Another interesting asymmetry in the system is that a CRP dimer containing only a single cAMP molecule is sufficiently activated to bind to a specific DNA site (Fried & Crothers, 1984; Garner & Revzin, 1982; Takahashi et al., 1989). Heyduk & Lee (1989, 1990) have shown that actually the CRP–cAMP complex has higher activity for the *lac* operon than the CRP–$(cAMP)_2$ complex. Taking into account all of these asymmetric arrangements, it would not be surprising if the CRP-induced DNA bending is asymmetric as well, a proposal forwarded by Liu-Johnson et al. (1986) on the basis of results from gel mobility experiments. The results obtained in this work seem to clearly indicate that despite the asymmetry in the DNA sequence, the CRP-induced DNA bend is quite symmetric.

The results on the structural configuration of the CRP-induced DNA bend should be interpreted with caution even though multiple control experiments were conducted to rule out potential artifacts (See Results). The amount of fluorescence energy transfer is small, and, therefore, the estimation of distances can have quite a significant range of error. However, there are several conclusions which are apparent. The extent of DNA bending as observed by fluorescence energy transfer measurements (80–100°) is consistent with the bending observed in a crystal of the CRP–DNA complex (Schultz et al., 1991) and that proposed by Zinkel and Crothers (1991). The bending angle reported in this study is slightly less than the value derived from gel mobility experiments (Thompson & Landy, 1988). A potential source of the discrepancy in the estimation of the bending angle is the length of the DNA fragment employed in this study. The results derived from the 26 bp DNA fragment might best be a minimum estimation of the bending angle because the thermodynamically defined bending domain spans 28–32 bp (Liu-Johnson et al., 1986) and, therefore, some interactions important for bending might not have occurred for the 26 bp DNA fragment. If this is so, the additional bending for longer

DNA fragments cannot be very dramatic since the 32 bp fragment shows only marginal transfer, implying that it most likely assumes a configuration similar to that observed for the 26 bp fragment. Also, the 40 bp fragment does not show any energy transfer in the CRP–DNA complex; i.e., the end-to-end distance must be ≥100 Å. Thus, it is quite unlikely that the DNA molecule wraps completely around CRP since the diameter of the CRP molecule is only about 55 Å, in which case a significant amount of energy transfer would have been detected.

It is gratifying to note that the approach employed in this study can provide information on the structure of DNA in solution. These results are derived from a technique that enables a direct measurement of distance in solution. The strength of the approach is that it is not limited by the specific solvent restrictions suffered by other approaches such as low-salt buffer for gel mobility experiments or unnatural crystallizing solvent for crystallography.

All the results presented in this paper were obtained in one set of experimental conditions (salt, pH, temperature, etc.). However, with this approach, characterization of bending as a function of solution conditions is feasible and is currently under investigation.

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