Binding of the cyclic AMP receptor protein of *Escherichia coli* and DNA bending at the P4 promoter of pBR322

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The binding of the *Escherichia coli* cyclic AMP receptor protein (CRP) to its specific site on the P4 promoter of pBR322 has been studied by gel electrophoresis. Binding to the P4 site was about 40–50-fold weaker than to the principal CRP site on the lactose promoter at both low (0.01 M) and high (0.1 M) ionic strengths. CRP-induced bending at the P4 site was investigated from the mobilities of CRP bound to circularly permuted P4 fragments. The estimated bending angle, based on comparison with Zinkel & Crothers [(1990) *Biopolymers* **29**, 29–38] A-tract bending standards, was found to be ~96°, similar to that found for binding to the *lac* site. These observations suggest that there is not a simple relationship between strength of CRP binding and the extent of induced bending for different CRP sites. The apparent centre of bending in P4 is displaced about 6–8 bp away from the conserved TGTGA sequence and the P4 transcription start site.

INTRODUCTION

The cyclic AMP receptor protein (CRP) of *Escherichia coli* is a pleiotropic gene activator that, when bound to its allosteric activator cyclic AMP, stimulates transcription from a large number of catabolite-sensitive promoters [1]. The mechanism of action of CRP has been extensively investigated, and attention has focused on two principal features of the system: protein–protein interactions between CRP and RNA polymerase [2–5] and the substantial bending of DNA induced by binding of the cyclic AMP–CRP complex [6–10], which appears to increase when RNA polymerase binds [11].

The DNA bending induced by CRP clearly has a major influence on potential contacts between CRP and RNA polymerase, which need to be specifically positioned for activation [12,13], as well as having an indirect DNA-mediated effect on polymerase–promoter recognition. Intrinsically bent DNA containing appropriately phased A-tracts can substitute functionally for CRP in activation from supercoiled *lac* [14] and *gal* [15] promoters, suggesting that, under these circumstances, the induced bend itself is important.

The phenomenon of DNA bending induced by CRP was first recognized from the anomalous mobilities of protein–DNA complexes in non-denaturing gel electrophoresis [6–8]; independent evidence for DNA bending in solution comes from hydrodynamic and electro-optical studies of CRP–DNA complexes [16] and the kinetics of circularization of short DNA fragments containing CRP-bound sites [17]. The extended size of the binding site required for full binding affinity at the *lac* site (~28–30 bp) is consistent with the conclusion that the bending angle is greater than 90° [9] and that sequences flanking the central conserved region have an important role in modulating both the strength of CRP binding and the degree of bending [18,19].

Theoretical treatments of the gel-mobility anomaly imply a relationship between mobility and variation in mean end-to-end distance [20,21]; however the limitations of the theory in predicting absolute bending angles are clearly recognized [21]. For this reason empirical approaches have been developed to assess the magnitude of protein-induced bending, by relating the mobilities of protein–DNA complexes to those of standard

DNAs with independently determined bending angles. This approach was used by Thompson & Landy [22] and developed by Zinkel & Crothers [10], who used standards containing intrinsically curved A-tracts and a CRP bound with minimal effect on bending to a CRP-binding site at the end of the fragment to allow for the electrophoretic effect of the protein; the CRP-induced bend angle for the *lac* site was determined to be equivalent to 5.6 A-tracts, or about 100° on the basis that the A-tract bending angle was 18° [23].

Much of the work on CRP-induced bending relates to binding to the *lac* site, although the occurrence of bending at the *gal* and *malT* promoters was recognized in early gel-mobility studies [7]. The bend induced at several CRP sites has been investigated by Adhya and co-workers [24], who found bending to decrease in the order: *lac* ≈ *gal* > *malT* > *crp* > *ompA*; estimates of the bending angle based on Thompson & Landy's [22] A-tract results ranged from 96° for *lac* to 70° for *ompA*.

We report here the results of a study of the binding of CRP and the bending induced at the pBR-P4 (P4) promoter of pBR322. This promoter was first characterized by Queen & Rosenberg [25], who showed that the CRP-binding site was centred at about –42, overlapping the polymerase-binding site, a configuration similar to that at the *gal* promoter. DNase I footprinting results indicated that the site bound CRP only weakly, despite the fact that it shows a relatively high degree of symmetry. However, the site is very GC-rich in the region –9 to –12 from the centre of dyad symmetry adjacent to the TGTGA consensus sequence. Studies based on derivatives of the *lac* site have shown that AT richness in this region is associated with tighter binding and more pronounced bending [18,19]; however, the correlation between binding strength and extent of bending is imperfect even within this related group of sites [19], and a simple relationship between these two quantities appears not to hold when comparison is made between CRP sites from different promoters [19,24]. The purpose of the present investigation was to examine the binding and bending properties of a CRP site that was expected to be weak.

MATERIALS AND METHODS

T4 DNA ligase, the large fragment of DNA polymerase (Klenow fragment) and restriction endonucleases (except *Tth*III,

Abbreviations used: CRP, cyclic AMP receptor protein; TE, 10 mM-Tris (pH 8.0)/1 mM-EDTA.

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from New England Bio-Labs) were from Bethesda Research Laboratories. Nucleotides, cyclic AMP and tRNAs were from Boehringer, and cyclic [^3H]AMP was from Amersham International.

Purification of CRP

CRP was prepared from *E. coli* M182 Δ crp/pBS Δ crp2, which overproduces CRP [26], essentially as described by Pinkney & Hoggett [4], except that single-standard rather than double-standard DNA-cellulose chromatography was used. Samples were judged by SDS/PAGE to be more than 95% pure, and the specific activity of cyclic AMP binding was 6000 units [4]/mg. The preparation was 30% active in specific DNA binding. Stock solutions (2 mg/cm 3) were stored in 50% (v/v) glycerol, pH 7.5, at -20°C .

Preparation of DNA

Plasmid DNA was prepared by a standard alkaline-SDS-lysis procedure, followed by purification on CsCl/ethidium bromide gradients. pBR322 was digested with *AluI*, and the 281 bp fragment containing the P4 promoter region was isolated and ligated into pUR2 [27], which had been cleaved at its unique *EcoRI* site and end-filled with Klenow fragment. This ligation regenerates the *EcoRI* site. pIB1 contains one copy of the P4 fragment; in pIB2 the insert introduced into pUR2 was a tandem dimer of the P4 fragment. The P4 and 451 bp *lac* PO fragments used in the binding experiments were obtained from pIB1 by digestion with *EcoRI* (for P4) or *EcoRI*/*TaqI* (for *lac*). The fragments were purified from preparative 6% polyacrylamide gels, and DNA concentrations were determined spectrophotometrically. Circularly permuted P4 fragments used in the bending experiments were obtained from pIB2 by digestion with *HpaII*, *TthIII*, *AccI*, *RsaI*, *DdeI*, *NdeI*, *HaeII*, *MboII* or *HinfI*, followed by polyacrylamide-gel purification as described above.

DNA binding assays

Binding of the cyclic AMP-CRP complex to the P4 and *lac* fragments was monitored by the standard gel retardation procedure [28,29]. CRP and DNA were incubated for 1 h at 20°C in a buffer containing 10 mM-Tris, pH 8.0, 1 mM-EDTA, 0.1 mM-cyclic AMP, 10% (v/v) glycerol and BSA (0.1 mg/cm 3 ; 20 μl). Complexes were resolved from free DNA by electrophoresis on non-denaturing 5% polyacrylamide gels [75:1 (w/w) acrylamide/bisacrylamide] cast as a slab (15 cm $\times$ 20 cm) in 10 mM-Tris (pH 8.0)/1 mM-EDTA (TE buffer). Gels were pre-run at 20°C for 1 h at 15 V/cm in TE+0.1 mM-cyclic AMP, carefully and quickly loaded, and run for a further 90 min. The gels were stained with ethidium bromide, photographed, and the amount of DNA present in each band was quantified by densitometry; care was taken to ensure that exposures were in a range where there was a linear relationship between band intensity and DNA concentration.

DNA bending assays

CRP-induced bending of the P4 fragment was analysed by the circular permutation assay described by Wu & Crothers [8]. Circularly permuted fragments derived from pIB2 were incubated with CRP at a concentration sufficient to complex about 50% of the fragment. Free and complexed DNA were separated electrophoretically as described above, and the mobilities of the bands were determined after staining of the gels with ethidium bromide.

RESULTS

Binding affinity of cyclic AMP-CRP to the P4 site

The results of titrations using the gel retardation assay of P4

and *lac* fragments with increasing concentrations of CRP at low ionic strength are shown in Fig. 1. In the *lac* titration, band f represents free DNA, band c a 1:1 complex of CRP with its principal site on the *lac* promoter, and band d a 2:1 complex with CRP additionally bound at the weaker *lacO* site [7,30]. At the highest concentrations of CRP, some non-specific binding of CRP occurred, resulting in smearing of the specific-complex bands. The P4 titration showed similar behaviour in that three DNA bands were observed; free DNA (f) and two bands (c and d) arising from complexes. It was assumed that band c represented a 1:1 complex of CRP with its site centred at $-42/-43$ on the promoter, a supposition which was confirmed by the DNA-bending experiments described below. However, the appearance of a second sharp band at the highest concentrations of CRP was surprising. The P4 fragment does not contain any easily recognizable CRP-binding site other than that at $-42/-43$, and the footprinting studies by Queen & Rosenberg also detected only

Fig. 1. Binding of CRP to (a) the *lac* fragment and (b) the P4 fragment at low ionic strength

Conditions were: 10 mM-Tris/HCl (pH 8.0)/1 mM-EDTA/0.1 mM-cyclic AMP; 23°C . Each lane contained 40 nM-DNA and the following total concentrations of CRP: 0 (lanes 1), 40 nM (lanes 2), 80 nM (lanes 3), 120 nM (lanes 4), 160 nM (lanes 5) and 400 nM (lanes 6). Bands f, c and d represent free DNA, 1:1 CRP-DNA complex, and 2:1 CRP-DNA complex respectively.

Fig. 2. Binding of CRP to the P4 and *lac* promoters

Conditions were as in Fig. 1; for low ionic strength: $\circ$, P4; $\triangle$, *lac*. Filled symbols ($\bullet$, P4; $\blacktriangle$, *lac*) refer to data at high ionic strength with 0.1 M-KCl added.

Fig. 3. Competitive binding of CRP to P4 and *lac* at low ionic strength

Conditions were as in Fig. 1. Equimolar amounts (25 nM) of P4 and *lac* fragments were titrated with the following total concentrations of CRP in lanes 1–9: 0, 40 nM, 80 nM, 160 nM, 240 nM, 320 nM, 400 nM, 480 nM and 600 nM respectively.

Table 1. Relative binding constants of CRP to P4 and *lac* sites at low ionic strength

[CRP] (nM)*	$(I_b/I_f)_{P4}$	$(I_b/I_f)_{lac}$	$(K_{lac}/K_{P4})_{app.}$	$(K_{lac}/K_{P4})^\dagger$
0	0	0	—	—
40	0.020	1.13	55.3 ± 5	52 ± 8
80	0.111	6.69	60.2 ± 6	46 ± 8
160	0.250	19.0	76.0 ± 7	46 ± 8
240	0.887	> 50	—	—
320	2.45	> 50	—	—
400	9.00	> 50	—	—

* Refers to the total stoichiometric concentration of CRP, not the active fraction.

† Corrected for binding to the weaker *lac* operator site as described in the text.

one site [25]. Nevertheless, the sharpness of the band and its low mobility suggest that it arises from a specific complex containing more than one bound CRP molecule; we assume that it represents a 2:1 complex, with CRP additionally bound to a weaker site. The result of quantifying the bands are shown in Fig. 2. Binding to the *lac* fragment appeared to be stoichiometric, and extrapolation at the initial linear part of the curve yielded a value of 0.29 for the *lac*/CRP molar ratio at which saturation of binding occurred. This was taken to represent the proportion of active protein in the preparation, and is a value close to that found by other workers [9,10]. Binding to the P4 site appeared to be slightly weaker than to *lac*, but the process is again essentially stoichiometric. Fig. 2 also illustrates the results of similar binding experiments carried out in the presence of added KCl (0.1 M). Binding to *lac* was still tight and essentially stoichiometric, but binding to the P4 fragment was appreciably weaker. Evaluation of the binding constant on the basis that the fraction of active protein was 0.29 yielded a binding constant, K , of $1.5 \times 10^7 \text{ M}^{-1}$ ($\pm 15\%$). Comparison of this value with data for binding to the *lac* site is considered below, but it can be noted here that binding to P4 was about 40-fold weaker than to *lac* under these conditions.

Competition experiments were carried out to compare the relative affinities of the P4 and *lac* sites more directly, and the

results are shown in Fig. 3 and Table 1. Equimolar mixtures of the *lac* and P4 fragments were titrated with increasing concentrations of CRP, and the free and bound DNA were quantified as described above. In a simple competition experiment, on the basis that two fragments A and B each contain only one binding site, the ratio of the binding constants is given by the expression:

$$\frac{K_A}{K_B} = \left(\frac{I_b}{I_f} \right)_A \cdot \left(\frac{I_f}{I_b} \right)_B$$

where I_b and I_f represent bound and free concentrations respectively of the relevant fragments. Apparent affinity ratios calculated from this expression are designated $(K_{lac}/K_{P4})_{app.}$ in Table 1. The situation is complicated by the fact that the *lac* fragment also contained the weaker *lacO* site, which became progressively more occupied at higher concentrations of CRP. The 1:1 and 2:1 complexes of CRP with the fragment were incompletely resolved on the gel, and the intensity of the bound *lac* was the sum of the two species. As a consequence, the ratio $(K_{lac}/K_{P4})_{app.}$ increased with increasing concentrations of CRP; the true value of the ratio for binding to the principal *lac* and P4 sites could be obtained by extrapolating the apparent ratio to zero concentration of CRP, but allowance for secondary binding can be made as follows on the basis of the known value (17 ± 5) of the relative affinities of CRP for the *lac* promoter (K_{lacP}) and operator (K_{lacO}) sites under low ionic strength conditions [30].

With the notation that $[lacP]_b$ and $[lacPO]_b$ are the concentrations of the 1:1 and 2:1 complexes respectively, and neglecting the concentration of 1:1 complexes with CRP bound solely to the weak operator site, the total concentration of bound *lac* is given by:

$$\begin{aligned} [lac]_b &= [lacP]_b + [lacPO]_b \\ &= [lacP]_b \cdot (1 + K_{lacO}[\text{CRP}]_f) \\ &= [lacP]_b \cdot \{1 + (K_{lacP}/r)[\text{CRP}]_f\} \end{aligned}$$

where r is the known ratio K_{lacP}/K_{lacO} . The binding equilibria are all coupled through the same pool of free CRP, hence:

$$[\text{CRP}]_f = \frac{1}{K_{P4}} \cdot \frac{[P4]_b}{[P4]_f}$$

yielding the expression:

$$[lac]_b = [lacP]_b \left\{ 1 + \frac{K_{lacP}}{K_{P4}} \cdot \frac{1}{r} \cdot \frac{[P4]_b}{[P4]_f} \right\}$$

The desired ratio (designated Q below):

$$\frac{K_{lacP}}{K_{P4}} = \frac{[lac]_b}{[lac]_f} \cdot \frac{[P4]_f}{[P4]_b}$$

is related to the apparent ratio (designated R):

$$\frac{K_{lac}}{K_{P4}} = \frac{[lac]_b}{[lac]_f} \cdot \frac{[P4]_f}{[P4]_b}$$

by the equation:

$$Q = \frac{R}{1 + \left(\frac{Q}{r} \cdot \frac{[P4]_b}{[P4]_f} \right)}$$

which can be re-arranged into the following quadratic expression for Q in terms of known or observed quantities (the positive root is the desired one)

$$Q^2 + (Q - R) \cdot r \left(\frac{[P4]_f}{[P4]_b} \right) = 0$$

Fig. 4. CRP-induced bending at the P4 site

Circularly permuted fragments (≈ 40 nm) were incubated with CRP (≈ 100 nM) and subjected to electrophoresis under the conditions of Fig. 1. In (a) the fragments were generated by digestion with: lane 1, *EcoRI*; lane 2, *TthIII*; lane 3, *NdeI*; lane 4, *HaeII*; lane 5, *HinII*; lane 6, *EcoRI*. In (b) tRNA was added as a marker and the fragments were: lane 1, *HpaII*; lane 2, *AccI*; lane 3, *RsaI*; lane 4, *DdeI*; lane 5, *NdeI*; lane 6, *HaeII*; lane 7, *MboII*; lane 8, *HinII*. The *NdeI* fragment (a, lane 3, and b, lane 5) did not form a complex with CRP.

Fig. 5. Mobilities of the CRP-P4 complexes

Relative mobilities of the CRP-DNA complexes are plotted against the position of cleavage of the fragment relative to the P4 site. The location of the CRP-binding site (40 bp) and the centre of dyad symmetry ($\blacklozenge$) are indicated, together with the cleavage site *NdeI* within the CRP site. The broken lines indicate the apparent centre of bending (see the text).

Values of the affinity ratios corrected for binding to the operator site in this way are shown in Table 1. The correction does not depend heavily on the adopted value of r , and it can be seen that

the corrected ratios (48 ± 8) are independent of CRP concentration.

DNA bending at P4 induced by cyclic AMP-CRP

Fig. 4 shows the results of circular-permutation-bending experiments. Each fragment was mixed with sufficient CRP to allow both free and complexed DNA to be visible on the gel, but the concentration was kept low enough to prevent the formation of any putative 2:1 complexes. In some experiments carrier tRNA was added and used as an internal mobility reference; the carrier tRNA did not influence the binding of CRP nor the mobilities of the free and complexed DNA. The mobilities of the complexes are plotted in Fig. 5, which also indicates the location of the CRP-binding site and the restriction-enzyme cleavage sites. The mobilities show the expected pattern resulting from bending, progressively decreasing as the location of the CRP site is moved from the ends of the fragment to the middle. The *NdeI* fragment in which the centre of the CRP site is 15 bp from the end did not bind CRP. The extrapolated effective bending locus appears to be displaced some 6–8 bp from the centre of symmetry of the CRP site, away from both the TGTGA sequence and the P4 transcription start site.

DISCUSSION

Affinity and structure of the P4 site

The results of the competition experiments show that, at low ionic strength, binding of CRP to the P4 site is about $48 (\pm 8)$ -fold weaker than to the strong site on the *lac* promoter when due allowance is made for binding to the second weaker *lac* site.

Competition experiments were not carried out at higher ionic strength, but the binding constant observed for P4 under these conditions ($1.5 \times 10^7 \text{ M}^{-1}$) can be compared with existing data for other CRP sites under similar conditions. The binding of CRP to the strong *lac* promoter site on a short (40 bp) DNA fragment has been investigated by using a nitrocellulose-filter-binding assay under a range of conditions of ionic strength, pH and temperature [31]. Extrapolation of the data for *lac* binding to the conditions used in the present experiments [10 mM-Tris (pH 8.0) 0.1 M-salt; temperature $\sim 23^\circ\text{C}$] yields a value of $K = 5.7 \times 10^8 \text{ M}^{-1}$. The basis of this extrapolation, which was well within the experimentally investigated range, was to take the observed value of $3.1 \times 10^7 \text{ M}^{-1}$ at pH 8.0 (0.2 M-salt), and to estimate the effect of reducing the ionic strength using the determined number (5.8) of ion-pairs [31]. Thus, under these conditions, binding of CRP to P4 is about $38 (\pm 8)$ -fold weaker than to *lac*; this ratio is the same within experimental error as that found at low ionic strength, suggesting that a similar number of ion-pairs are formed on binding to the *lac* and P4.

In terms of the hierarchy of CRP-binding sites, P4 is clearly weak, having an affinity less than those of *malT* or *gal*, but comparable with that of the *lac* operator site [7]. The weakness of the P4 site is in some senses surprising, in view of its high degree of sequence identity with the consensus sequence in the strongly conserved regions. It possesses a TGTGA motif proximal to the transcription start and an almost symmetrical GCACA sequence at positions 4–8, reading from the dyad axis on the distal side. One feature which could account for its weakness is the absence of consensus A residues adjacent to the TGTGA sequence. These residues are strongly conserved in most CRP sites, but in the P4 site this region is very GC-rich.

Analysis and discussion of CRP-induced bending

The results of the circular-permutation assay show that cyclic AMP-CRP binding to P4 is accompanied by DNA bending, as has been observed in all specific sites so far examined [7–10,24].

Fig. 6. Dependence of R_L for A-tract bending standards on end-to-end length

The data show logarithmic plots of R_L against decrease in end-to-end length $(1-h)$ for Zinkel and Crothers' [10] A-tract bending standards. The open symbols refer to free DNA fragments with various numbers of A-tracts, and the closed symbols to the corresponding fragments with CRP bound at an end; R_L values for the latter fragments were corrected for the frictional effect of bound CRP as described in the text. The concentrations of acrylamide were 8% ($\square$, $\blacksquare$), 5% ($\circ$, $\bullet$) and 4% ($\triangle$, $\blacktriangle$).

We follow here the approaches of Thompson & Landy [22] and Zinkel & Crothers [10], which allow CRP-induced bend angles to be estimated (at least semi-quantitatively) by comparison with the electrophoretic behaviour of A-tract standards whose bending angles have been independently determined [23].

For a rigid DNA fragment of length l , bent from linearity at its centre by an angle α , the end-to-end distance is given by $l\cos(\alpha/2)$. A bend of equal magnitude located at the end of a fragment will produce only a relatively small change in the end-to-end length l , and therefore the proportional change (h) in end-to-end length in the two circumstances is given by $\cos(\alpha/2)$. Thompson & Landy's results [22] show a linear relationship between the ratio of mobilities of fragments bent at the middle and end (μ_M/μ_E) and $\cos(\alpha/2)$, and hence h , up to angles of about 120° , where this relationship breaks down (see below). The proportionality constant of the above relationship decreases with decreasing acrylamide concentration [22].

The A-tract standards used by Zinkel & Crothers [10] were similar, but were phased differently (~ 10.5 bp rather than 10 bp) and were identical with the sequences used to determine the A-tract bending angle ($18^\circ \pm 10^\circ$) [23]. The calibration molecules used to determine the CRP-induced bending angle contained a CRP molecule bound at an end to allow for its frictional effect. The mobility anomalies results are expressed as R_L values, equal to the apparent length of the molecule (derived from their

mobility relative to known length standards) divided by its actual length. Plots of R_L for the calibration molecules against the square of the number of A-tracts were fitted to an empirical polynomial expression from which the bend angle of a test DNA molecule with CRP bound centrally was evaluated.

This empirical dependence can be viewed differently to enable conclusions to be drawn more readily about the relationship between mobility and variation in end-to-end length ratios (h). R_L values for free DNA standards (without bound CRP) increase with bending angle until they level off abruptly at an angle of about 120° , which can be interpreted as evidence for a change in the mode of migration of the fragment through the gel matrix [10]. In the region where R_L increases with bending angle, the relationship between R_L and (number of A-tracts) 2 , i.e. effectively (bending angle) 2 , is non-linear. However, inspection of the data in this region shows that plots of $\log R_L$ against α^2 show excellent linear relationships for the various acrylamide concentrations used. Furthermore, it can be shown by expansion as a series that the quantity $1 - \cos(\alpha/2)$ is proportional to α^2 , with a maximum error of 1–2% when α increases to $2\pi/3$. Since $1 - \cos(\alpha/2)$ is equal to $1-h$, where h is the proportional change in end-to-end length when the bend is induced in the centre of the molecule, it follows that there is a linear relationship between the relative mobility (expressed as $\log R_L$) and the proportional decrease in end-to-end length. Fig. 6 illustrates this for acrylamide concentrations of 8, 5 and 4% on the basis of Zinkel & Crothers' [10] value for the A-tract bending angle (18°); the linearity is very satisfactory up to a value of $(1-h) = 0.5$ (corresponding to $\alpha = 120^\circ$), where the relationship fails abruptly, as mentioned above. The data for the calibration molecules containing CRP bound at an end can be treated in a similar way by first adjusting the curves so that initial R_L values are equal to 1.0 at zero bending. Logarithmic plots of adjusted R_L values against $(1-h)$ are linear, and the data virtually coincide with those for the corresponding free DNA molecules (Fig. 6). This coincidence implies that, as noted before [10], the fractional variation in gel mobility associated with a specific bend in the DNA is the same in the free DNA and DNA-CRP complex. In summary, the data of Zinkel & Crothers [10] are consistent with the view that, up to a bending angle of about 120° , there is a linear relationship between the change in mobility of a fragment, expressed as $\log R_L$ (adjusted for the frictional effect of any bound protein), and the proportional decrease in the end-to-end distance $(1-h)$. This conclusion is in essence the same as Thompson & Landy's [22] findings, although their data are expressed differently, and the failure of the relationship at high bending angles was not explicitly recognized.

For a set of circularly permuted fragments, the relationship between the end-to-end distance ratio h of a DNA molecule with a bend (α) located at a fractional distance x from the end is given by the expression:

$$h^2 = 1 + 4 \sin^2(\alpha/2) \cdot [x^2 - x]$$

which is equivalent to Thompson & Landy's [22] relation involving cosine functions. On the basis that mobility is proportional to the end-to-end-distance ratio, the form of this function around $x = 0$ suggests that linear extrapolation of data points around this region should yield satisfactory estimates of the effective bending locus and the extrapolated maximum mobility of an unbent, but CRP-bound, fragment. The dependence of h (hence mobility) on x is much shallower around $x = 0.5$, and data in this region do not specify the centre of bending so precisely.

The mobility curve for P4 is relatively well defined in this region, and extrapolation indicates an apparent bend centre at position $185 (\pm 3 \text{ bp})$, and a maximum mobility of $0.95 (\pm 0.025)$

Table 2. Analysis of the P4 bending data

x is the fractional distance from the bend centre to the nearest end. R_L is the ratio (apparent length/actual length) of the fragment. h is the ratio (end-to-end length/actual length) determined as described in the text. Abbreviation: N.D., no protein-DNA-complex band was detected.

DNA fragment	x	Mobility	R_L	h
<i>EcoRI</i>	0.349	0.60	2.47	0.682
<i>HpaII</i>	0.426	0.59	2.54	0.673
<i>TthIII</i>	0.342	0.63	2.31	0.710
<i>AccI</i>	0.250	0.72	1.88	0.788
<i>RsaI</i>	0.130	0.83	1.47	0.887
<i>DdeI</i>	0.123	0.84	1.44	0.895
<i>HaeII</i>	0.120	0.82	1.51	0.875
<i>MboII</i>	0.130	0.82	1.52	0.875
<i>HinfI</i>	0.195	0.73	1.83	0.800
<i>NdeI</i>	0.074	N.D.	—	—
DNA _f ...	—	1.00	1.000	—
Extrapolated maximum mobility...	—	0.95	1.127	1.000

Fig. 7. Dependence of predicted values of $(1 - h^2)$ against $(x - x^2)$ for CRP-induced bending of P4 fragments

Values of x , the fractional distance from the bend centre to the nearest end, and h , the predicted ratio of end-to-end length/actual length for the various P4 fragments are taken from Table 2. The line shown is the best straight line through the origin with a slope $[4 \sin^2(\alpha/2)]$ of 2.20, yielding a bending angle α of 96° .

relative to free DNA (Fig. 5). The mobilities, expressed as R_L , and values of x for the family of P4 fragments are shown in Table 2. Values of R_L , corrected for the frictional effect of the bound CRP as described above, were used to determine corresponding values of h (Table 2) from the calibration standards of Zinkel & Crothers [10] in 5% polyacrylamide (Fig. 6). According to the expression given above, a plot of $(1 - h^2)$ against $(x - x^2)$ should give a straight line through the origin, from whose slope the bending angle can be determined. Fig. 7 illustrates that the data fit well to the predicted dependence. A similar observation was reported by Thompson & Landy [22] for bending induced by *EcoRI* binding. The bend angle induced by CRP at the P4 site was found to be 96° , with an estimated uncertainty of $\pm 8^\circ$. This can be compared with Zinkel & Crothers', [10] value of $\sim 100^\circ$ for the *lac* site. Clearly the estimate for P4 is less direct than that for *lac*, but the conditions used were very similar in the two cases, as were the magnitudes of observed mobility anomalies.

The conclusion that bending of P4 and *lac* is comparable further supports the idea that there is not a simple relationship between degree of bending and strength of binding for different

CRP sites [18,24]. The P4 site has a high degree of symmetry in the consensus regions, but it lacks the conserved A residues close to the TGTGA sequence. If the weakness of the P4 site is attributable to the absence of these A residues, there must, nonetheless, be other anchoring interactions which cause extensive bending. The extent of the site size required for stable CRP binding to P4 is evident from the observation that the *NdeI*-generated fragment, which is cut 15 bp from the centre of symmetry of the site, fails to bind CRP, but the *DdeI* fragment, which is cleaved 28 bp from the centre of symmetry, binds CRP normally. These findings are consistent with the idea that the binding site spans at least 34 bp, but less than 42 bp [18].

The apparent centre of bending in the P4 fragment is displaced about 6 bp from the centre of symmetry of the CRP site away from the TGTGA sequence. A displacement of a similar magnitude and direction was observed by Wu & Crothers [8] for binding to the *lac* site, although the asymmetry was subsequently attributed to the possible distorting effect of low levels of intrinsic curvature in the free DNA [9]. Although a similar explanation may apply in the presence case, the sequences of the *lac* and P4 fragments are different, and there is no reason to suppose that they possess similar regions of intrinsic curvature which would produce the same effect. The apparent displacement in *lac*, where the CRP site is centred at -62 relative to the transcription start, is towards the promoter, but in P4, which is centred at -42 , the displacement is away from the promoter, a consequence of the different orientations of the CRP site in the two promoters. The explanation of the observed asymmetry of bending (whether as a real or 'artefactual' phenomenon) could most readily be investigated by using the bending-vector approach developed by Adhya and co-workers [24] in which different CRP-binding sites are inserted into otherwise-identical DNA fragments. The five different CRP sites investigated by using this vector appear to bend at the centre. However, the data for these sites, although they establish the magnitude of the gel anomaly and relative degree of bending very clearly, are most extensive in the region where the dependence of mobilities on position is shallow and not so well suited for precise identification of the bending site. If the observed asymmetrical binding represents a real phenomenon, such differences in promoters which are organized differently with respect to the positioning of the CRP site would have important stereochemical consequences for interactions between CRP, RNA polymerase and the promoter, and the mechanism of CRP action.

Since the present paper was originally submitted, the crystal structure of CRP complexed with a symmetrical 30 bp DNA sequence has been published [32]. These results show that the DNA is bent by about 90° , and that this bending arises predominantly from two 43° kinks that occur between the TG/CA base-pairs at positions 5 and 6 on each side of the dyad axis of the complex. In addition to these kinks, the DNA on one side of the complex is bent by a further 8° towards the protein, allowing an interaction between Lys²⁶ of the cyclic AMP-binding domain with a DNA phosphate group at ≈ 10 bp from the dyad axis, producing an overall bend of about 94° in the crystal structure. The absence of a similar bend on the other side of the DNA may be due to the effects of crystal-packing forces, and it is postulated that, in solution, the DNA may be more bent [32]; bending at this region appears to be modulated by sequence-specific distortion of DNA and may be favoured by an AT nucleotide dyad at positions 10 and 11 [18]. The observed bending at the P4 site (96°) agrees well with the crystallographic findings; the site contains TG/CA base-pairs on each side of the dyad axis, where substantial kinking is found, and AT bases at positions 10 and 11 on only one side of the dyad axis, distal to the transcription start site. Additional DNA bending in this region would have the

effect of moving the apparent locus of bending away from the dyad axis and the transcription start site.

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