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Specific DNA binding of the cAMP receptor protein within the *lac* operon stabilizes double-stranded DNA in the presence of cAMP

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The effects of varying amounts of cAMP receptor protein (CRP) in the presence and absence of cAMP on the melting and differential melting curves of a 301-bp fragment containing the *lac* control region in 5 mM Na^+ have been investigated. The native 301-bp fragment consists of three cooperatively melting thermalites. At 5 mM Na^+, thermalite I (155 bp) has a T_m of 66.4°C and the melting transitions of thermalites II (81 bp) and III (65 bp) are superimposed with a T_m of 61.9°C. The specific DNA target site for CRP and the *lac* promotor are located within thermalite II. CRP alone exerts no specific effects on the melting of the 301-bp fragment, non-specific DNA binding of CRP resulting in a progressive stabilization of the double-stranded DNA by increasing the number of base pairs melting at a higher T_m in a non-cooperative transition. The cAMP-CRP complex, however, exerts a specific effect with a region of ~36 bp, comprising the specific CRP binding site and a neighbouring region of DNA, being stabilized. The appearance of this new cooperatively melting region, known as thermalite IV, is associated with a corresponding decrease in the area of thermalites II/III. The T_m of thermalite IV is 64.4°C, 2.5°C higher than that of thermalites II/III. With two or more cAMP-CRP complexes bound per 301-bp fragment, the stabilization also affects the remaining 110 bp now making up thermalites II/III whose T_m is increased by 1°C to 62.9°C. The implications of these findings for various models of the mode of action of the cAMP-CRP complex are discussed.

Key words: CRP/cAMP/specific DNA binding/*lac* operon/thermal denaturation of DNA

Introduction

The cAMP receptor protein (CRP) is an important element in the regulation of operons of *Escherichia coli* that are subject to carbon catabolite repression, cAMP acting as an effector (Zubay *et al.*, 1970; Epstein *et al.*, 1975; de Crombrugghe and Pastan, 1978). The cAMP-CRP complex binds specifically to DNA target sites near the promoters of catabolite-sensitive operons, stimulating the initiation of mRNA synthesis (de Crombrugghe *et al.*, 1971; Majors, 1975; Taniguchi *et al.*, 1979; Odgen *et al.*, 1980). At present the mechanism by which the cAMP-CRP exerts this effect is unknown. Two models have been proposed: the first suggests that direct interaction between the cAMP-CRP complex and RNA polymerase occurs resulting in either stronger binding of RNA polymerase to the promoter or increased RNA polymerase activity (Majors, 1975; Gilbert, 1976; Simpson, 1980); the second suggests that on binding to its specific DNA target site the cAMP-CRP complex destabilizes neighbouring regions of DNA thereby facilitating the formation of an open RNA polymerase-promoter complex (Dickson *et al.*, 1975; Nakanishi *et al.*, 1974, 1975; Wells *et al.*, 1980; McKay and Steitz, 1981; Ebright and Wong, 1982). No direct experimental data, however, exists to support either model.

As regards the first model, it should be noted that although in the *lac* operon the position of the CRP binding site relative to the *lac* promoter (Simpson, 1980; Schmitz, 1981) is consistent with direct contact between the cAMP-CRP complex and RNA polymerase, the large variation in the distances between the CRP and RNA polymerase binding sites in other catabolite-sensitive operons such as *gal* (Taniguchi *et al.*, 1979), *ara* (Lee *et al.*, 1981) and *deo* (Valentin-Hansen, 1982) argues against a unique direct CRP-RNA polymerase interaction.

The second model has received particular attention recently on account of two observations. The first is that the thermal stability of the *lac* operon is correlated to the functional domain, with the CRP and RNA polymerase binding sites located at opposite ends of an 81-bp cooperatively melting region of the DNA (Hardies *et al.*, 1979; Hillen *et al.*, 1981a). This result suggests that the effect of the cAMP-CRP complex on the stability of the double helix could be extended to the RNA polymerase binding site. The second observation, based on the 2.9 Å crystal structure of the cAMP-CRP complex (McKay and Steitz, 1981), is that the molecular dyad of the cAMP-CRP complex relates a symmetrical pair of antiparallel α helices (helix F from each subunit) whose axes are 34 Å apart and so situated with respect to the remainder of the protein as to permit intimate interaction between these two helices and two adjacent major grooves of left-handed B DNA. The implication of this model for the specific interaction of the cAMP-CRP complex with DNA is that the conversion of right- to left-handed B DNA would be expected to destabilize the double helix.

We have recently shown that, with respect to non-specific DNA binding, CRP both in the presence and absence of cAMP stabilizes double-stranded DNA by increasing the T_m (by ~14°C in the case of poly[d(AT)]) and reducing the melting cooperativity (Takahashi *et al.*, 1982). Moreover, this finding is consistent with the higher affinity of CRP and the cAMP-CRP complex for double-stranded than single-stranded DNA (Saxe and Revzin, 1979; Garner and Revzin, 1981), and with the observation by electron microscopy that cooperative non-specific binding of CRP to double-stranded DNA results in the formation of a complex with regular striations at 41 Å intervals along the DNA whose length is approximately four times shorter than that of free DNA (Chang *et al.*, 1981). This latter observation has led to the proposal that non-specifically bound CRP envelopes a left-handed solenoidal coil of right-handed B DNA with the two antiparallel F helices of CRP bridging the major grooves of adjacent loops of the coil (Salemme, 1982). However, these conclusions may not be true for the interaction of the cAMP-CRP complex with its specific DNA target site.

To determine the effect of specific DNA binding of the cAMP-CRP complex on the stability of double-stranded DNA we have investigated the thermal denaturation proper-

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ties of the previously characterised 301-bp fragment containing the *lac* control region (Hardies *et al.*, 1979) complexed with varying amounts of CRP in the presence and absence of cAMP. We demonstrate that specific DNA binding of the cAMP-CRP complex results in a small but significant stabilization of a region of double-stranded DNA ~36 bp long.

Results

The location of the 301-bp fragment with respect to the genetic map of the *lac* operon and to the map of the three cooperatively melting regions as determined by thermal denaturation studies (Hardies *et al.*, 1979) is shown in Figure 1. Thermalite I consists of 155 bp, thermalite II of 81 bp and thermalite III of 65 bp. The CRP binding site is located in thermalite II close to its boundary with thermalite I. The *lac* promoter also lies in thermalite II, and the *lac* operator bridges the boundaries of themalites II and III.

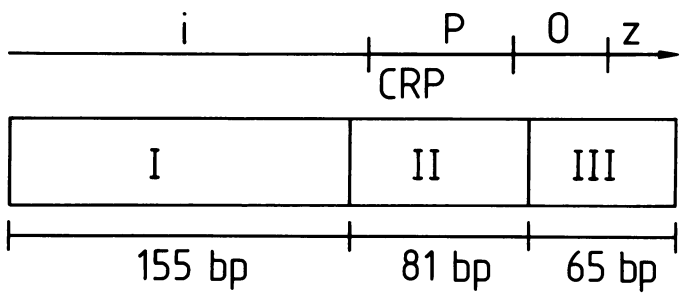

Fig. 1. Correlation of the genetic map of the *lac* operon with the map of the cooperatively melting regions of the 301-bp fragment as determined by the thermal denaturation studies of Hardies *et al.* (1979). Symbols: i, region coding for the C-terminal end of the *lac* repressor; P, *lac* promoter; CRP, specific CRP binding site; O, *lac* operator; z, region coding for the N-terminal end of β galactosidase.

Previous studies (Takahashi *et al.*, 1982; Clore and Gronenborn, unpublished results) have shown that complexes of CRP with DNA, both in the presence and absence of cAMP, are stable up to at least 70°C. Thus, in order to avoid the problems associated with denaturation of the complex of the 301-bp fragment with CRP, all experiments were conducted at 5 mM Na^+. At this concentration of Na^+, the highest melting transition, thermalite I has a T_m of 66.4°C, and the melting transitions of thermalites II and III are superimposed with a T_m of 61.9°C (Figure 2 a). Moreover, at 5 mM Na^+, the non-specific binding of CRP to DNA, both in the presence and absence of cAMP, is very tight with an equilibrium association constant K of $\sim 10^8$ M^{-1} and a cooperativity parameter ω of ~100 (Saxe and Revzin, 1979; Takahashi *et al.*, 1979). Consequently, at the concentrations of the 301-bp fragment (10^{-7} M) and CRP ($\geq 10^{-7}$ M) employed in our experiments, binding of CRP and the cAMP-CRP complex to the 301-bp fragment will be stoichiometric. This has the additional advantage that one can ensure conditions in which all the CRP present in solution is bound to the 301-bp fragment, thus completely avoiding problems associated with light scattering arising from the denaturation of free CRP which begins to occur around 50°C (Takahashi *et al.*, 1982).

Melting curves of the 301-bp fragment in the presence of varying amounts of CRP

Figure 2 shows the melting and differential melting profiles of 10^{-7} M 301-bp fragment with 0, 2, 4 and 10 CRP molecules bound per fragment. The percentage of the total melting hyperchromicity attained at 69°C, the highest temperature used in our experiments, and the areas of the thermalites, converted to base pairs, as a function of the number of molecules of CRP bound per 301-bp fragment are shown

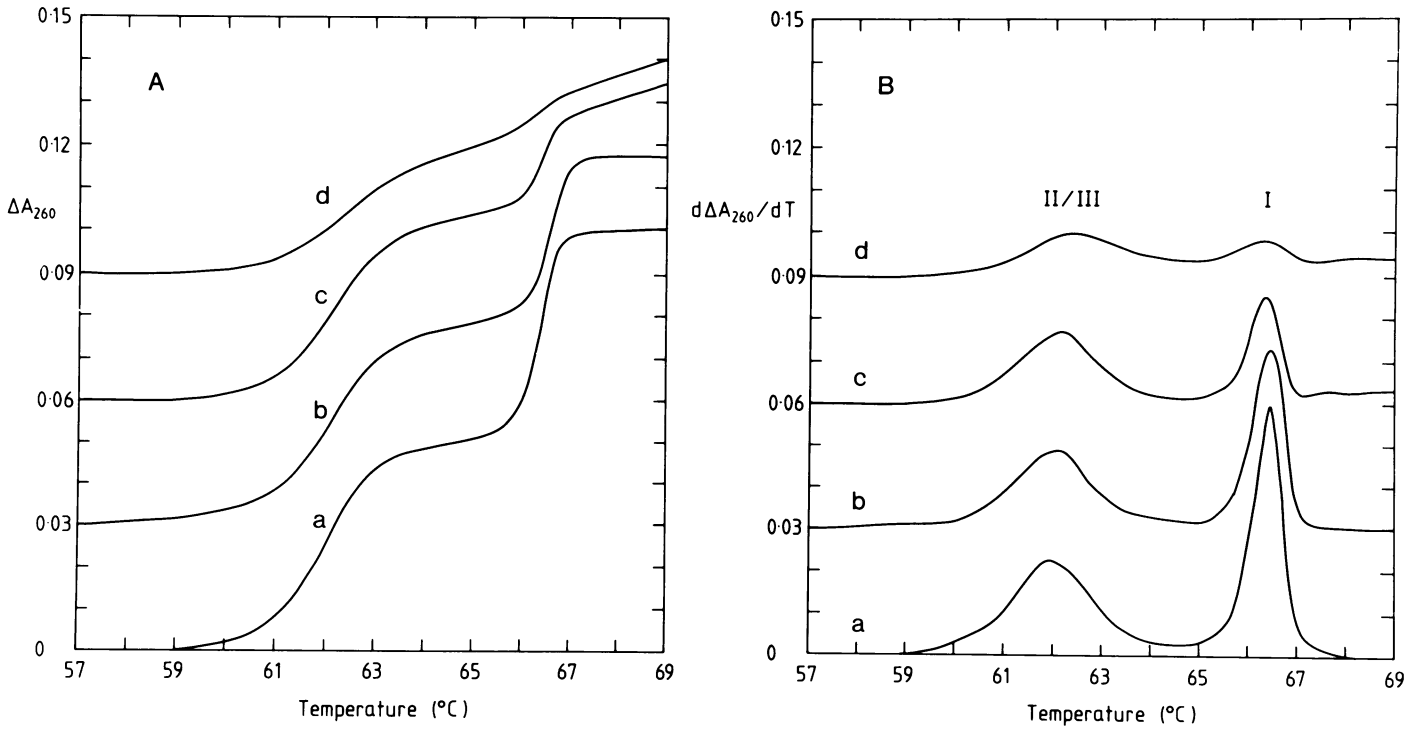

Fig. 2. Melting (**A**) and differential melting (**B**) curves of 10^{-7} M 301-bp fragment in 5 mM Na^+ with (**a**) 0, (**b**) 2, (**c**) 4 and (**d**) 10 CRP molecules bound per fragment. Curves (**b**), (**c**) and (**d**) are offset by 0.03, 0.06 and 0.09 units, respectively, on the vertical axis. Thermalite I has a T_m of 66.4°C, and thermalites II/III of 61.9°C. The total melting hyperchromicity is 0.1 absorbance units at 260 nm. The experimental details are given in Materials and methods.

in Figures 3 and 4, respectively. It can be clearly seen from Figures 2 and 3 that the hyperchromicity at 69°C decreases as the number of CRP molecules per 301-bp fragment increases, from 100% of the total melting hyperchromicity in the absence of CRP to ~55% of the total hyperchromicity when 20 CRP molecules are bound per fagment (i.e., almost complete saturation of the DNA lattice). This is associated with a reduction in the areas of the cooperatively melting thermalites (whose T_ms remain unchanged) and a corresponding increase in the area of a non-cooperatively melting transition (see Figures 2 and 4). At 20 molecules of CRP bound per 301-bp fragment, thermalite I consists of only ~5% and thermalites II/III of ~10%, whereas the non-cooperatively melting transition comprises ~85% of the total hyperchromicity (Figure 4). One can therefore deduce that the T_m of the non-coooperatively melting transition lies between 70 and 80°C. Moreover, the decrease in the area of thermalite I as a function of the number of CRP molecules bound per 301-bp fragment is more rapid than that of thermalites II/III, indicating that the cooperative non-specific binding of CRP to the 301-bp fragment occurs preferentially within the 155-bp region comprising thermalite I. If CRP alone does bind

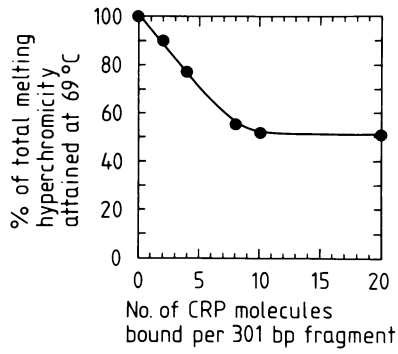

Fig. 3. Percentage of the total melting hyperchromicity attained at 69°C, the highest temperature used in our experiments, as a function of the number of molecules of CRP bound per 301-bp fragment.

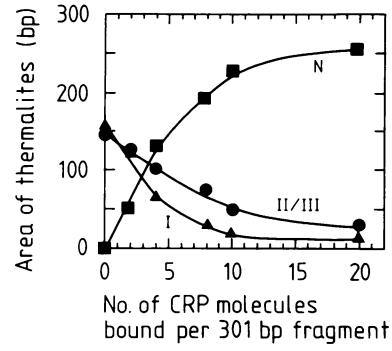

Fig. 4. Areas of the thermalites, converted to base pairs, as a function of the number of molecules of CRP bound per 301-bp fragment. Symbols: ▲, thermalite I; ●, thermalites II/III; ■, non-cooperative melting transition (N).

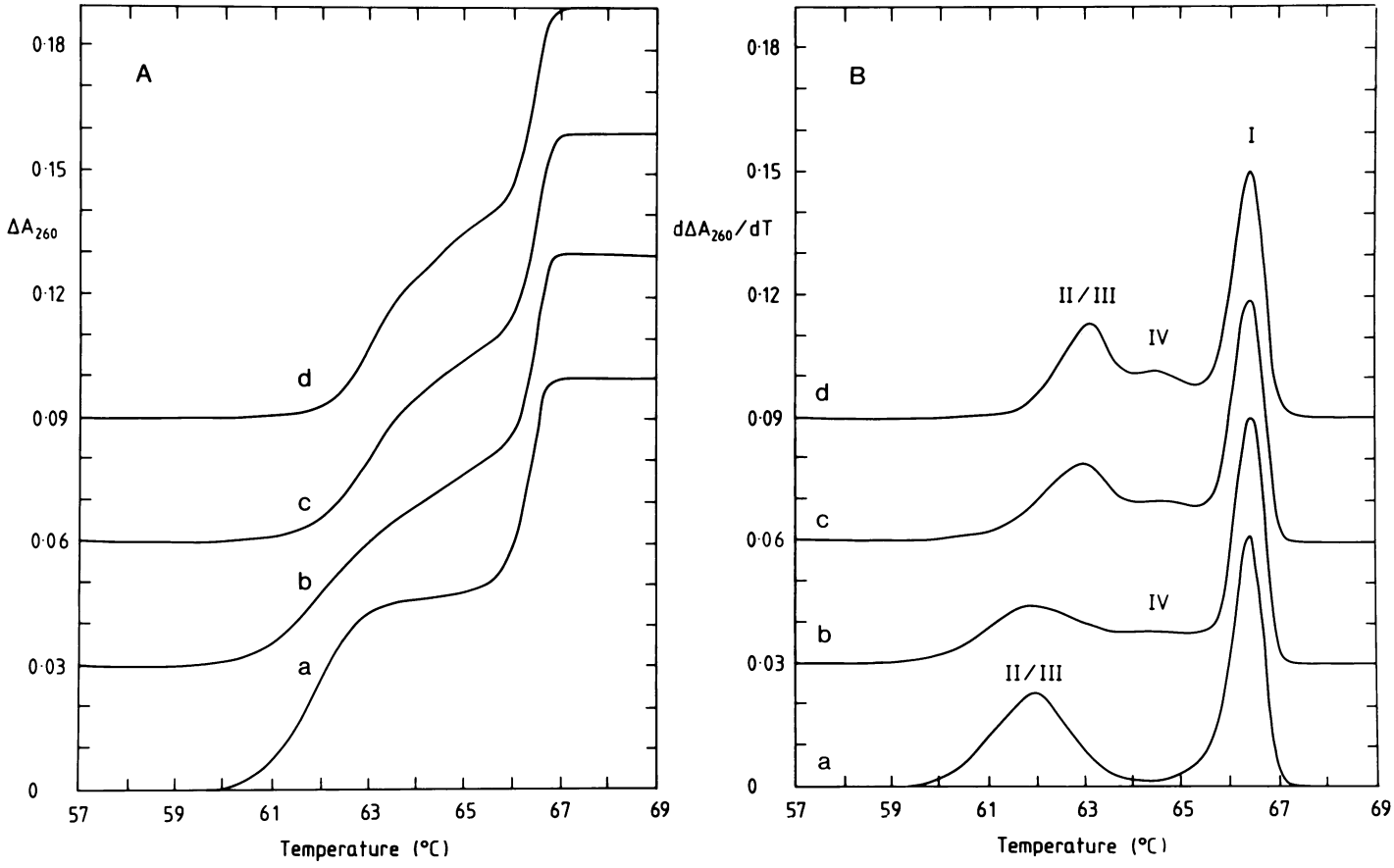

Fig. 5. Melting (**A**) and differential melting (**B**) curves of 10^{-7} M 301-bp fragment in 5 mM Na^+ and 100 μM cAMP with (**a**) 0, (**b**) 1, (**c**) 2 and (**d**) 4 cAMP-CRP complexes bound per fragment. Curves (**b**), (**c**) and (**d**) are offset by 0.03, 0.06 and 0.09 units, respectively, on the vertical axis. Thermalite I has a T_m of 66.4°C, thermalites II/III at T_m of 61.9°C with 0 and 1 cAMP-CRP complex bound per fragment and a T_m of 62.9°C with two or more cAMP-CRP complexes bound per fragment, and thermalite IV a T_m of 64.4°C. The total melting hyperchromicity is 0.1 absorbance units at 260 nm.

specifically to its target site within thermalite II, it does not appear to exert any specific effect on the melting properties of the 301-bp fragment.

Melting curves of the 301-bp fragment in the presence of varying amounts of the cAMP-CRP complex

Figure 5 shows the melting and differential melting profiles of 10^{-7} M 301-bp fragment with 0, 1, 2 and 4 cAMP-CRP complexes bound per 301-bp fragment. In these experiments, the concentration of cAMP employed was 100 μM which is sufficient to saturate $\geq$99% of all cAMP binding sites on CRP (Takahashi *et al.*, 1980). In the absence of CRP, this large excess of cAMP does not result in any change in the melting profile of the 301-bp fragment (Figure 5a), indicating that our equipment is capable of resolving the $\triangle A_{260}$ accurately at a high total absorbance ($\sim$2). The areas of the thermalites, converted to base pairs, as a function of the number of cAMP-CRP complexes bound per 301-bp fragment, are shown in Figure 6. It can be clearly seen from Figures 5 and 6 that the presence of a single cAMP-CRP complex bound per 301-bp fragment is associated with the appearance of a broad new cooperatively melting transition, thermalite IV comprising $\sim$36 $\pm$ 5 bp, with a T_m between those of thermalites I and II/III, and a corresponding decrease in the area of thermalites II/III (whose T_m remains unchanged). In addition the melting cooperativity parameter $\sigma_{1/2}$ (defined as the width corresponding to 50% of the melting transition centred about its midpoint) of thermalites II/III is increased by 0.2°C from 1.8°C in the absence of the cAMP-CRP complex to 2.0°C in the presence of the singly bound cAMP-CRP complex (i.e., the melting cooperativity is decreased). With only a single cAMP-CRP complex bound per 301-bp fragment, the melting transition of thermalite IV is too broad to be able to determine an accurate value of either its T_m or $\sigma_{1/2}$. The addition of further cAMP-CRP complexes, up to 10 per 301-bp fragment, does not result in any change in the areas of thermalites II/III and IV. However, when two or more cAMP-CRP complexes are bound per 301-bp fragment, the melting cooperativity of thermalite IV is increased so that thermalite IV becomes distinctly visible as a separate melting transition with a T_m of 64.4°C and a $\sigma_{1/2}$ of 2.5°C. In addition, the melting cooperativity of thermalites II/III is restored to its original value in the absence of the cAMP-CRP complex, and the T_m of thermalites II/III is increased by 1°C from 61.9°C to 62.9°C. No change is observed in either the T_m, $\sigma_{1/2}$ or area of thermalite I up to five cAMP-CRP complexes bound per 301-bp fragment. As the number of cAMP-CRP complexes bound per 301-bp fragment increases above five, the area of thermalite I decreases; this is associated with the appearance and corresponding increase in the area of a non-cooperatively melting transition with a T_m higher than that of thermalite I, analogous to the situation with CRP alone.

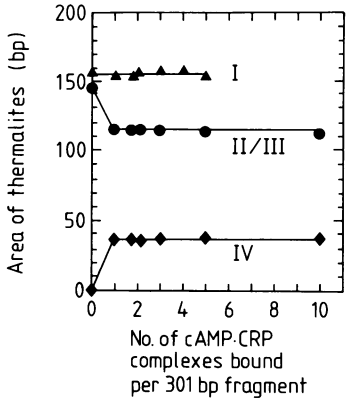

Fig. 6. Areas of the thermalites, converted to base pairs as a function of the number of cAMP-CRP complexes bound per 301-bp fragment. Symbols: ▲, thermalite I; ●, thermalites II/III; ◆, thermalite IV. The area of thermalite I was only determined up to five cAMP-CRP complexes bound per 301-bp fragment as non-specific effects, analogous to those observed with CRP alone, reduce the area of this subtransition at higher molar ratios.

Discussion

We have examined the effects of CRP and the cAMP-CRP complex on the thermal stability of a double-stranded 301-bp fragment containing the *lac* control region. No effect attributable to specific DNA binding of CRP alone could be detected on the thermal stability of the 301-bp fragment. Rather, non-specific DNA binding of CRP results in a progressive stabilization of the double-stranded DNA with an increasing number of base pairs melting at a higher T_m in a non-cooperative transition which is probably made up of the overlap of small amounts of different complex species, each having slightly different T_ms (see Figures 2 and 4). This finding is in complete agreement with our previous findings on the effect of non-specific DNA binding of CRP on the thermal denaturation of poly[d(AT)] (Takahashi *et al.*, 1980). [It should be noted that this non-cooperative transition cannot be attributed to the thermal denaturation of single-stranded DNA as CRP binds with much greater affinity to double-stranded than single-stranded DNA (Saxe and Revzin, 1979; Garner and Revzin, 1981).] In addition, the amount of conversion of thermalite I to the non-cooperative transition as a function of the number of CRP molecules bound per 301-bp fragment is greater than that of thermalites II/III (see Figure 4) indicating that non-specific binding of CRP occurs preferentially within the 155-bp region comprising thermalite I. This suggests that non-specific binding of CRP occurs preferentially to GC-rich sequences as thermalite I has a 60% GC content compared to a 47% and 43% GC content for thermalites II and III respectively (Hardies *et al.*, 1979).

In contrast, in the case of the cAMP-CRP complex, a distinct effect of specific DNA binding is observed with a double-stranded region of $\sim$36 bp being stabilized (see Figures 5 and 6). The appearance of this new cooperatively melting region, known as thermalite IV, is associated with a corresponding decrease in the area of thermalites II/III. The T_m of thermalite IV (64.4°C) is 2.5°C higher than that of thermalites II/III (61.9°C) in the native 301-bp fragment. Thermalite IV must comprise both the specific DNA target site for CRP and an adjacent region of DNA within the 81-bp region making up thermalite II in the native 301-bp fragment as: (i) the specific CRP binding site is located within this 81-bp region at its boundary with thermalite I (see Figure 1, Hardies *et al.*, 1979); (ii) the size of thermalite IV is approximately twice that of the specific CRP binding site (Simpson, 1980; Schmitz, 1981) and (iii) no change in the area of thermalite I is observed up to five cAMP-CRP complexes bound per 301-bp fragment (see Figure 6). Bearing this in mind, it is not surprising that the width of the melting transition for thermalite IV is narrower with two cAMP-CRP complexes bound to this 36-bp region than with only one bound (see Figure 5), as in the latter case one would expect the T_m of

the specific DNA target site covered by the cAMP-CRP complex to be slightly different from that of the remaining DNA within this region, resulting in an apparently broad melting transition. With two cAMP-CRP complexes bound per 301-bp fragment, the entire 36-bp region will be covered, owing to the cooperative binding of the cAMP-CRP complex to DNA (Takahashi *et al.*, 1979), so that thermalite IV will be characterised by a single T_m with the consequent reduction in the width of its melting transition. The expected width for a cooperative transition of 36 bp would be ~3.2°C (Hillen *et al.*, 1981c). The result of 2.5°C may be due to either the low ionic strength which tends to increase the cooperativity of melting (Hillen *et al.*, 1981c) or to a thermodynamic contribution from the cAMP-CRP complex binding to DNA. Moreover, once two cAMP-CRP complexes are bound per 301-bp fragment, the stabilization also affects the remaining 110 bp now comprising thermalites II/III as their T_m (62.9°C) is 1°C higher than that in the native 301-bp fragment (see Figure 5).

Our finding that the binding of the cAMP-CRP complex to its specific DNA target site in the *lac* operon stabilizes a double-stranded region of DNA ~36 bp long indicates that the cAMP-CRP complex does not stimulate transcription by destabilizing neighbouring regions of DNA and thereby facilitating the formation of an open RNA polymerase-promoter complex as has been previously suggested (Dickson *et al.*, 1975; Nakanishi *et al.*, 1974, 1975; Wells *et al.*, 1980; Ebright and Wong, 1982). Further, our findings taken together with those of Kolb and Buc (1982) that the supercoil unwinding achieved by the specific DNA binding of the cAMP-CRP complex is <0.5 turns, make the model of McKay and Steitz (1981) in which the cAMP-CRP complex converts right-handed to left-handed B DNA extremely unlikely, as such a process would be expected to destabilize double-stranded DNA and cause the removal of at least four negative superhelical turns.

Materials and methods

CRP and the 301-bp fragment containing the *lac* operon were prepared as described previously (Takahashi *et al.*, 1980; Hardies *et al.*, 1979; Hillen *et al.*, 1981b). cAMP was purchased from Sigma Chemicals Co. Ltd. All other chemicals were of the highest purity commercially available.

The experimental conditions used for the thermal denaturation measurements were: 10^{-7} M 301-bp fragment, 5 mM sodium cacodylate pH 7.0 (at 25°C), 0.1 mM EDTA, and 0–2 μM CRP. In the experiments with cAMP, the concentration of cAMP employed was 100 μM which is sufficient under these conditions of pH and ionic strength to saturate ≥99% of all cAMP binding sites on CRP (Takahashi *et al.*, 1980).

The absorption temperature profiles were recorded at 260 nm and processed and analysed as described by Hillen and Unger (1982). The heating rate did not exceed 0.07°C/min.

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