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# Mechanism for transcriptional action of cyclic AMP in *Escherichia coli*: Entry into DNA to disrupt DNA secondary structure

(cyclic AMP receptor protein/catabolite activator protein/receptor/indoleacetic acid/steroid hormones)

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**ABSTRACT** **Binding analysis with purified bacterial receptor distinguishes two structural domains in cyclic AMP (cAMP). The first, the cyclic phosphate and furanose, constitutes a binding domain. This region is bound tightly to the receptor. The rest of cAMP is not bound; the adenine moiety of cAMP is exposed. Unlike binding, activity of cAMP requires the adenine moiety. To be active, cAMP must have in domain II the base adenine—specifically, its Watson–Crick atoms N-1 and N-6. Analysis of indoleacetic acid, a compound able to replace cAMP at the L-arabinose operon, indicates a similar distinction between binding and active domains. To be active, the indole must have substitution (carboxyl or amide) electronically comparable to the cAMP N-1 and N-6. On this basis, we propose a detailed mechanism for action of cAMP (or indoleacetic acid) in *Escherichia coli*. We propose that the exposed adenine of cAMP enters into the DNA. The adenine's N-1 and N-6 form hydrogen bonds to a thymine in DNA. This interaction destabilizes the DNA. It enhances transcription. Marked similarities indicate an identical mechanism for the steroid hormones in eukaryotes.**

In bacteria, cyclic AMP (cAMP) regulates transcription at several sites—most familiar at the *lac* operon (1–8). The nucleotide binds to a soluble receptor (CRP, CAP) (2, 4–12) and is translocated to the chromosome (11–13). There, the cAMP–CRP complex binds to a specific DNA sequence adjacent to the site for RNA polymerase (14). This interaction in some manner enhances initiation by the RNA polymerase and thus transcription (4–8, 15).

The precise mechanism for this interaction is unclear. The prevalent view, however, assigns no direct role to cAMP after it has bound to CRP (16–18). Binding of cAMP is assumed to induce a conformational change in CRP (16–18), and it is this change which is believed to be responsible for the interaction of CRP with DNA and for the enhancement of transcription.

We, in contrast, are convinced of cAMP's *direct* participation, and we propose a detailed mechanism for it. Experiments with two separate classes of small molecules support this hypothesis.

## CYCLIC NUCLEOTIDES

**Structures for Binding.** (*i*) The cyclic phosphate of cAMP is essential, and sufficient, for the interaction of cAMP with CRP (Fig. 1*a*). Hence, cGMP (9,10,12), cIMP (12), cCMP, and cUMP all bind to CRP (Figs. 1 *a–c* and 2*A*). These compounds share with cAMP only the cyclic phosphate and furanose ring.

Modifications of this shared region—even minor alterations—decrease or block binding. Opening [5′-AMP (9, 12), 3′-AMP (9), adenosine] or positional isomerization (2′,3′-cAMP) of the cyclic phosphate abolishes binding altogether (Figs. 1*f–i* and 2*B*).

(*ii*) The adenine moiety of cAMP either is exposed from CRP

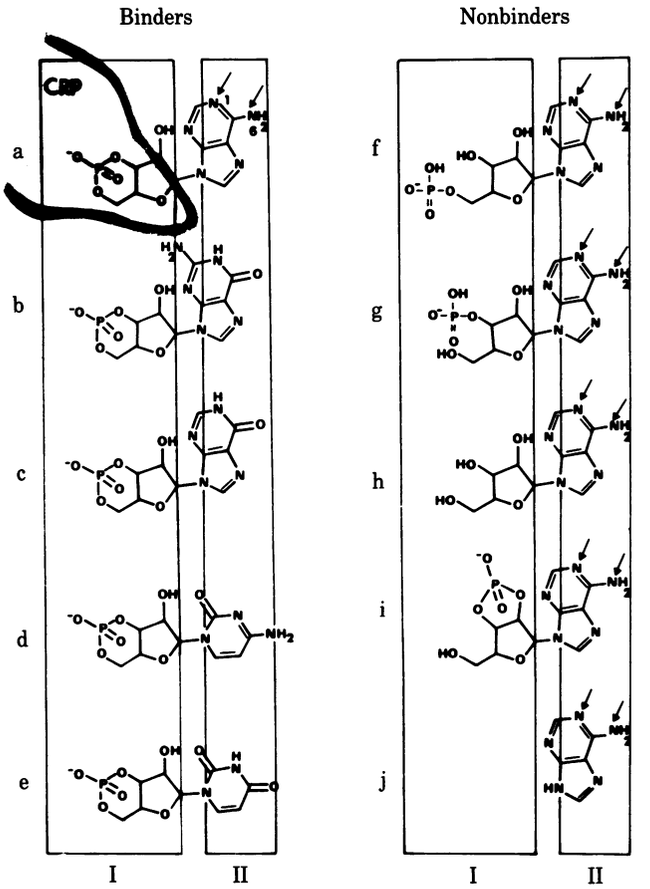


FIG. 1. Interaction of nucleotides and CRP. (*a*) cAMP's cyclic phosphate and furanose ring (domain I) are bound tightly inside CRP. The adenine moiety (domain II) is not bound; it remains exposed from CRP. (*a–e*) Binders. All nucleotides that incorporate the cyclic phosphate and furanose ring bind to CRP (Fig. 2*A*). Affinity is independent of the substitution at domain II. (*a*) cAMP; (*b*) cGMP; (*c*) cCMP; (*e*) cUMP. (*f–j*) Nonbinders. Analogs without intact cyclic phosphate and furanose (domain I) fail to bind CRP (Fig. 2*B*). Binding is not observed at concentrations as high as 5 mM. These compounds fail to bind despite their retention, as in cAMP, of adenine at domain II. (*f*) 5′-AMP; (*g*) 3′-AMP; (*h*) adenosine; (*i*) 2′,3′-cAMP; (*j*) adenine.

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cCMP, cytidine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cIMP, inosine 3′,5′-cyclic monophosphate; cUMP, uridine 3′,5′-cyclic monophosphate; CRP, cAMP receptor protein; CAP, catabolite activator protein.

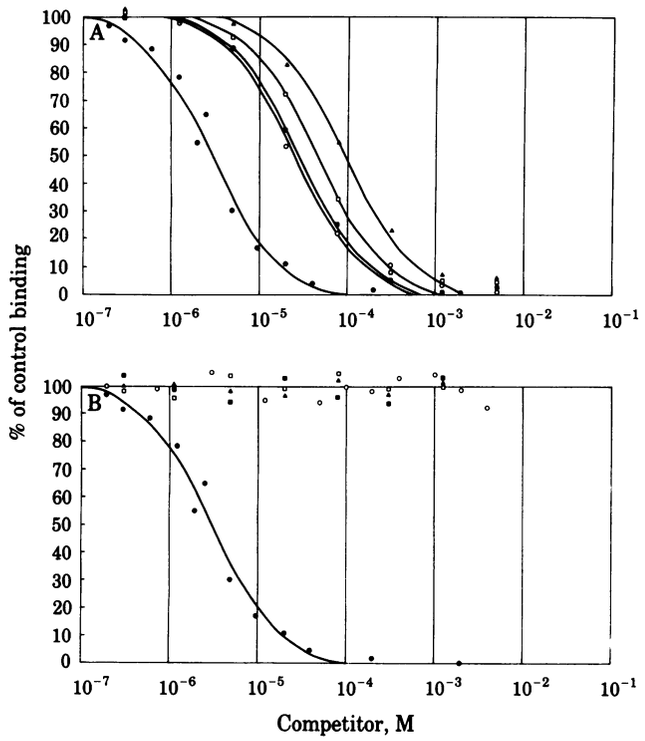


FIG. 2. Binding of cAMP and nucleotide analogs to CRP. Individual assay mixtures (12, 19) contained 1.61 μg of CRP (6460 units/mg; gift of J. S. Krakow), 1.0 μM [$^3$H]cAMP (32.3 Ci/mmol; 1 Ci = 3.7 × $10^{10}$ becquerels), 0.01 M phosphate buffer (pH 7.7), 200 μg of casein, and competitor as shown. Total volume was 100 μl. Bound [$^3$H]cAMP–CRP complex was precipitated by addition of 0.4 ml of saturated $(NH_4)_2SO_4$. Pellets (12,000 × $g_{av}$, 10 min, 0°C) were solubilized in 0.5 ml of NCS and assayed for radioactivity. Plotted values reflect correction for counting efficiency (external Compton standard). Bound counts in a trial experiment reached constant levels (equilibrium) with 10 min of incubation at 0°C. Incubations for plotted points were 15 min. This procedure gives 400,000–500,000 cpm of specific binding in the absence of competitor. Data are normalized as a percentage of this control binding. 0%, cpm with CRP replaced by pure buffer; 100%, cpm with no competitor, corrected by subtraction of the 0% binding. (*A*) Competition data for analogs that have the cyclic phosphate and furanose. These analogs compete with cAMP for occupancy of its receptor. Curves are parallel and of similar shape. ●, cAMP; ○, cGMP; ■, cIMP; □, cGMP; ▲, cUMP. (*B*) Failure of analogs without the cyclic phosphate/furanose system to interact with CRP. No depression of control [$^3$H]cAMP binding was observed, even at concentrations as high as 5 mM. ●, cAMP; ○, 5′-AMP; ■, 2′,3′-cAMP; □, adenosine; ▲, adenine.

or, if it associates, does so only loosely. Importance for binding of the adenine moiety is minimal.

Competition for CRP is independent of base substitution. At equilibrium, cAMP and cGMP compete for CRP with equal affinities (10, 19). Precipitation assay (Fig. 2*A*) confirms this. More striking, cCMP and cUMP (pyrimidine nucleotides) compete with affinities almost equal to the affinity of cAMP (Fig. 2*A*).

Tested by itself, the adenine did not interact with CRP. Adenine, adenosine, 5′-AMP (9, 12), 3′-AMP (9), and 2′,3′-cAMP do not bind to CRP, even at concentrations up to 5 mM (Figs. 1*f* and *g* and 2*B*).

Hence, one envisions a cAMP–CRP interaction whereby the cyclic phosphate/furanose complex binds tightly to CRP. The adenine, in contrast, does not bind to CRP; it remains exposed (Fig. 1*a*).

**Structures for Activity.** Activity of cAMP requires its adenine moiety (Fig. 1*a*). Despite their binding to CRP, cGMP (4, 12), and cIMP (1, 12) are inactive. Activity is absent both *in vivo*—in ability to support growth or to induce β-galactosidase (1)—and *in vitro*—in cell-free transcription systems (4, 12).

Inspection of the close analogs cAMP (active) and cIMP (inactive) defines the feature that is critical for activity. It is the N-1 and N-6 of adenine—the adenine's Watson–Crick (base-pairing) atoms (arrows in Fig. 1*a*; see Fig. 1*c*). Although without a significant effect on binding, alteration of the N-1 or N-6 entirely eliminates the compound's activity.

The compounds rendered inactive by modification of the N-1 and N-6 (e.g., cGMP and cIMP) are antagonists of cAMP (4, 11, 12). With the cyclic phosphate/furanose domain unaltered, these inert compounds can still bind to CRP. They displace active material (cAMP) from it. The net effect in biological assay is a decreased response to cAMP (antagonism).

## INDOLES

Kline *et al.* (20) have reported that certain indoles—i.e., 5-hydroxyindoleacetic acid, indoleacetic acid, indoleacetamide, and indolepropionic acid (Fig. 3)—can replace cAMP in activation of the L-arabinose operon. The effect of these indole derivatives requires CRP (20) and is site-competitive with cAMP (Fig. 4 *A* and *B*).

The activity of these derivatives lends support to the model

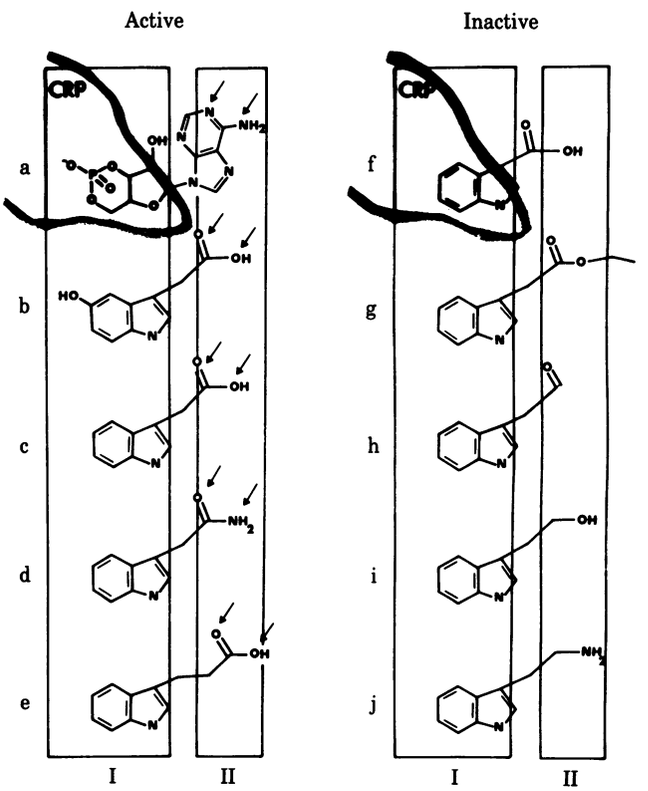


FIG. 3. Derivatives of indole that bind to CRP (*b–j*; see Fig. 4). Binding is to the site on CRP to which cAMP binds and is competitive (see Fig. 4 *B* and *D*). (*b–e*) Active indoles. These indoles are able to replace cAMP in the activation of a bacterial operon *in vivo* (20). Their domain II substitution—carboxyl or amide—presents a hydrogen bond donor/acceptor pair electronically and spatially identical to the N-1, N-6 pair of adenine. (*a*) cAMP; (*b*) 5-hydroxyindoleacetic acid; (*c*) indoleacetic acid; (*d*) indoleacetamide; (*e*) indolepropionic acid. (*f–j*) Inactive indoles. These indoles differ from the active analogs only in side-chain substitution (domain II). They are substituted so as not to constitute the required hydrogen bond donor/acceptor pair. However, because they retain the intact indole ring, these inert compounds still bind to CRP (Fig. 4*B*). (*f*) Indolecarboxylic acid; (*g*) indoleacetic acid ethyl ester; (*h*) indoleacetaldehyde; (*i*) indoleethanol; (*j*) indoleethylamine.

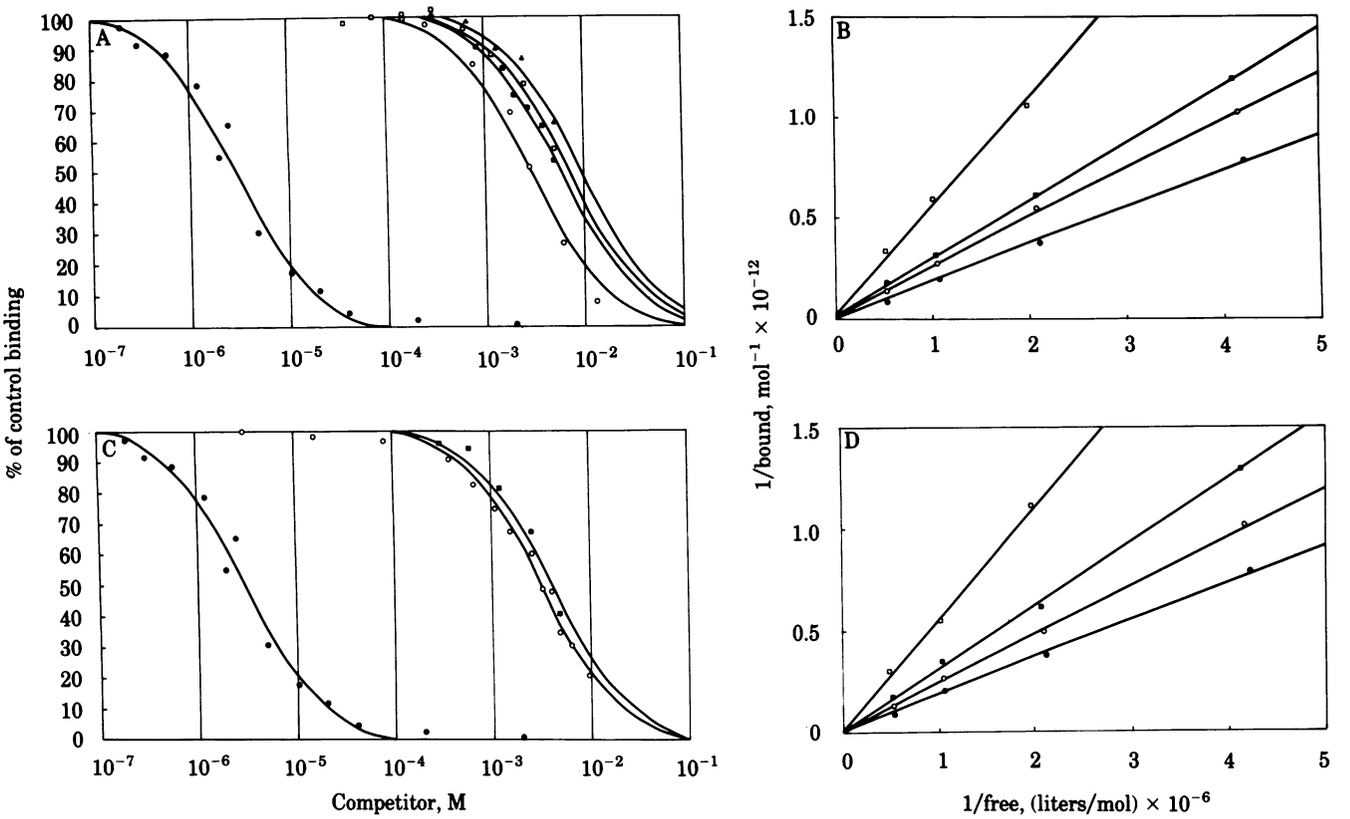


FIG. 4. Binding of active and inactive indole derivatives to CRP. (Affinities are significant; 2',3'-cAMP, 5'-AMP, 3'-AMP, adenosine, and adenine fail to compete at concentrations sufficient to give 70% maximal competition with these indoles.) Competition data were generated as in Fig. 2, with two differences. All contact with indoles was in total darkness or in the presence of only a monochromatic orange light source. Final reaction mixtures contained 10% dimethylformamide as solvent. (The presence of dimethylformamide at this level was shown in separate experiments to affect neither the position nor the shape of competition curves. This control was performed with cAMP and with indoleacetamide and indoleethanol.) Lineweaver–Burk plots were prepared by comparable methods. Each point in these represents the mean of two replicates and has been corrected for nonspecific binding. (*A*) Active indoles. ●, cAMP; ○, indolepropionic acid; ■, indoleacetamide; □, indoleacetic acid; ▲, 5-hydroxyindoleacetic acid. (*B*) Lineweaver–Burk plot for interaction of indoleacetic acid (an active indole) and cAMP–CRP, showing data for cAMP alone (●) and with three concentrations of indoleacetic acid (○, 0.555 mM; ■, 1.67 mM; □, 5 mM). All lines (least-squares fit) are linear ($r^2 \geq 0.99$) and pass through a common intercept on the *y* axis. (Common intercept is characteristic of same-site, competitive interaction.) Dissociation constant ($K_i$) for indoleacetic acid is $2.9 \times 10^{-3}$ M. (*C*) Inactive indoles. ●, cAMP; ○, indoleethanol; ■, indoleacetaldehyde. (*D*) Lineweaver–Burk analysis for indoleacetaldehyde (an inactive indole). Curves as in *B*. Dissociation constant ($K_i$) calculated from these data is $2.6 \times 10^{-3}$ M.

presented. Their simpler structure identifies exactly those features essential for binding to CRP and for action.

**Structures for Binding.** The indole moiety, with its fused, six-member/five-member ring system, resembles the fused cyclic phosphate/furanose rings of cAMP. Steric similarity of the indole and cyclic phosphate is sufficient, apparently, to permit same-site binding in CRP.

Without exception, derivatives that share the indole ring bind to CRP (Fig. 4). Binding occurs between indole and CRP irrespective of the indole's side-chain length or substitution (Figs. 3 *a–j* and 4). Thus, the side chain appears to be exposed, not bound by CRP. Interaction with CRP involves only the indole ring.

**Structures for Activity.** For activity, the side chain of the indole is critical. The indoles active in the system of Kline *et al.* (growth on arabinose, induction of arabinose isomerase) all have comparable side-chain length and substitution—specifically, a 2- or 3-carbon alkyl chain with a terminal carboxyl or amide group (20).

The active indoles incorporate in their structure a CRP-exposed hydrogen bond donor/acceptor pair (COOH or $CONH_2$). This structure corresponds to the active moiety in cAMP: the N-1 and N-6 of adenine constitute an electronically similar donor/acceptor pair ($CNNH_2$).

Indoles with modification in chain length or substitution are inactive. The ester, the aldehyde, the hydroxyl, and the amine ($COOC_2H_5$, CHO, $CH_2OH$, $CH_2NH_2$) all are inert (20).

These inert compounds, however, do retain the indole (binding) moiety. Hence, they exhibit full affinity for CRP. Their affinity is as high as or higher than that of the four active indoles (Fig. 4). Like cGMP, these inactive, yet binding, derivatives potently antagonize the actions of cAMP *in vivo* (E. L. Kline, personal communication). They bind to CRP and displace active cAMP but are themselves inactive. The antagonism observed here confirms adequate entry of these inactive compounds into the cell.

**Analysis.** The behavior of the active indoles matches that of the first ligand, cAMP. In each case, there are two structural domains: (*i*) a binding domain, and (*ii*) an exposed, active domain. For activity, the exposed domain must incorporate an electronegative substituent able to complement (to form hydrogen bonds to) a specific Watson–Crick base.

## DETAILED MECHANISM FOR CYCLIC NUCLEOTIDE (OR INDOLE) ACTION

Extensive data identify the biological response to cAMP stimulus as an increase in mRNA mediated by enhanced transcription (3–8, 11). Conflicting models have been suggested for the

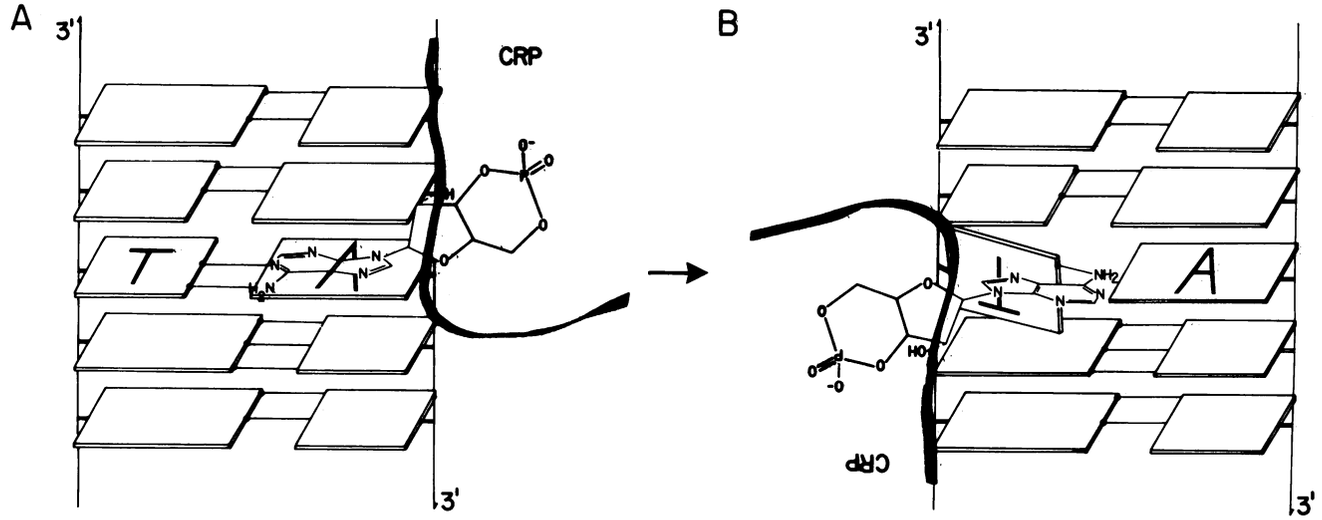


FIG. 5. Mechanism for cAMP. (*A*) cAMP binds to a specific receptor protein (CRP). Only the cyclic phosphate and furanose of cAMP are bound; the adenine is exposed. This exposed adenine inserts into the DNA helix (minor groove). Its N-1 and N-6 form two Watson–Crick hydrogen bonds to a thymine in DNA. (*B*) Torsional stresses rotate the cAMP–CRP complex to the opposite strand. Inverted repeat sequences permit opposite-strand recovery of identical CRP–DNA interaction and may promote this movement. The bound thymine is rotated and everted from its pair with DNA adenine. The structure drawn for cAMP is from its x-ray crystal coordinates (ref. 25; *anti* conformer). Sequence shown is that of the D-lactose operon −61 to −65 (14).

mechanism of that enhancement. Transcription could be increased by a direct interaction of CRP with the RNA polymerase (13) or by a local denaturation of the DNA helix (14, 15, 20–24).

**Denaturation.** We favor the denaturant mechanism. No evidence documents a direct interaction of CRP with the RNA polymerase (19, 24). In fact, such an interaction cannot be demonstrated under physiological conditions (19). By contrast, three lines of experimental evidence suggest a denaturant action for cAMP–CRP.

(*i*) cAMP can be replaced or mimicked in transcription by glycerol, a denaturant of DNA (21). Effects of glycerol and cAMP are not additive. That is, these agents act at the same site. Comparable results are obtained with low $Mg^{2+}$ concentration, with sucrose, and with dimethyl sulfoxide; each of these is known to alter DNA secondary structure and to lower the transition temperature ($t_m$) *in vitro* (15, 21).

(*ii*) cAMP–CRP shifts the transition temperature for formation of an open DNA complex (15). The shift is substantial, approximately 10°C. Its magnitude matches shifts with glycerol or with low $Mg^{2+}$ (15).

(*iii*) G·C → A·T promoter mutations relax the requirement for cAMP (14). Transition G·C → A·T occurs with net loss of one interstrand stabilizing bond. (G·C pairs form three hydrogen bonds in DNA; A·T pairs form only two.) Denaturation is enhanced at DNA sequences adjacent to the A·T pair, which is less stable (14, 22, 23). Transmitted denaturant forces suffice to obviate the requirement for cAMP (14, 23).

**Mechanism.** We propose that the denaturant activity of the cAMP–CRP complex is its most important feature. The distinction between bound and exposed structures on cAMP and the requirement for a specific hydrogen-bond pattern in the exposed structures suggest a mechanism for the denaturant activity. They suggest a direct interaction of exposed cAMP structures with the DNA.

(*i*) The cAMP–CRP complex recognizes a specific (13) DNA sequence at the controlled gene. Specificity of the complex resides in its protein component (CRP). Binding of CRP to the specific DNA sequence directs the complex to its site of action.

(*ii*) The exposed adenine of cAMP is inserted into the minor groove of the DNA helix (Figs. 5*A* and 6).

(*iii*) The hydrogen bonds involved in an adenine-thymine base pair in DNA break. The thymine of this pair forms new bonds to cAMP, specifically to its adenine N-1 and N-6 (Figs. 5*A* and 6). That is, the cAMP N-1 and N-6 replace an adenine in DNA as donor/acceptor of Watson–Crick bonds to thymine.

The reaction is an exchange or displacement with equal numbers of bonds broken and re-formed. The change in free energy, in principle, is zero.

(*iv*) Transmitted torsional forces rotate the receptor–base complex to the opposite strand; inverted repeat sequences (14, 26, 27) permit opposite-strand recovery of identical CRP–DNA binding and may promote this movement (Fig. 5*B*). (This step is not essential.)

(*v*) Local denaturation is enhanced. Base-pair breakage by

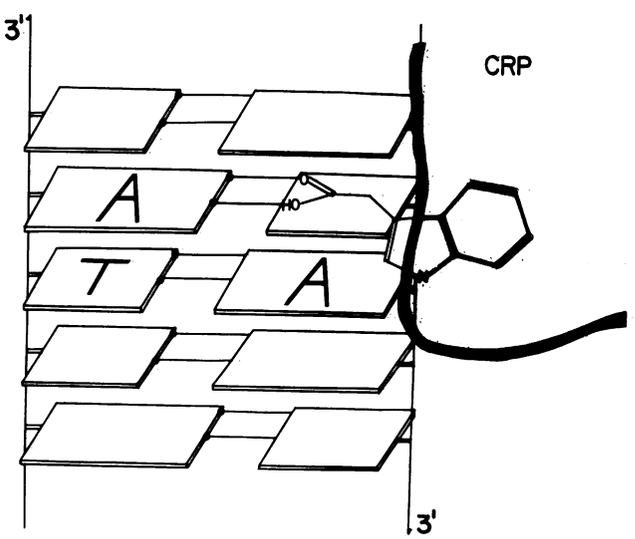


FIG. 6. Mechanism for indoleacetic acid. The indole carboxyl duplicates in its action the cAMP N-1, N-6 pair. The carboxyl's position, however, mandates its interaction with the base adjacent (3') to the thymine paired to by cAMP (Fig. 5*A*). The indole's length and hydrogen-bond character require that this base be adenine. Sequence shown is that of the L-arabinose operon −128 to −132 (26). The D-lactose operon does not respond to indoleacetic acid (20). It lacks this adenine (see Fig. 5*A*).

the nucleotide promotes denaturation at adjoining sites (14, 22, 23)—i.e., formation of an open complex. Initiation by RNA polymerase (at a site 15–100 base pairs away) is enhanced.

(*vi*) Action of the complex is terminated by reversal of the process. Energy "stored" in the cAMP–complement pair is recovered. In principle, the change in free energy for the overall process is zero.

**Analysis.** Inspection of either CPK (space-filling) or Dreiding models indicates the feasibility of this mechanism. The width of the DNA minor groove is exactly sufficient to permit cAMP entry as required. The rotation in step *iv* is possible and occurs with no distortion of helical structure. Rotation is at three bonds only (the 1′ → glycosidic linkage, 5′-phosphate, and 3′ phosphate) and is easily permissible.

Our data do not rule out a less-direct action of the cAMP adenine: e.g., the secondary establishment or maintenance of some conformational change in CRP (16–18). However, the cAMP adenine is exposed from all interaction with CRP, which makes this alternative improbable. To induce a selective conformational change, active analogs must be able to interact additionally with CRP—i.e., with affinities higher than those of inactive analogs. This prediction is inconsistent with our data. Active and inactive indoles bind to CRP with almost equal affinities; in fact, indoleacetaldehyde (inactive) demonstrates an affinity slightly higher than that of indoleacetic acid (active).

## PROSPECT

This paper describes a mechanism for the action of cAMP in the prokaryote. It predicts the exposure from CRP of a substantial part of cAMP: the adenine. The cAMP adenine, it predicts, interacts directly with DNA. This interaction enhances the initiation of RNA transcription. This pattern of action—exposure, and entry into DNA—may be common also to the steroid hormones.

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