Crystal Structure of a cAMP-Independent Form of Catabolite Gene Activator Protein with Adenosine Substituted in One of Two cAMP-Binding Sites†

Marie-Christine Vaney,‡,§,∥ Gary L. Gilliland,§ James G. Harman,⊥ Alan Peterkofsky,# and Irene T. Weber*,‡,§

NCI-Frederick Cancer Research Facility, BRI-Basic Research Program, Crystallography Laboratory, Frederick, Maryland 21701, Center for Chemical Physics, National Bureau of Standards, Gaithersburg, Maryland 20899, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061, and Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute, Bethesda, Maryland 20892

Received October 27, 1988; Revised Manuscript Received March 2, 1989

ABSTRACT: Catabolite gene activator protein (CAP) in the presence of cAMP stimulates transcription from several operons in *Escherichia coli*. A cAMP-independent variant, in which Ala-144 is replaced by Thr (CAP91), is activated by analogues of cAMP, such as adenosine, which do not activate the wild-type CAP. In order to test the effect of adenosine on the structure, a crystal of CAP91 grown as a complex with cAMP was soaked in a solution of 10 mM adenosine, and X-ray diffraction data were measured to 3.5-Å resolution. The difference Fourier map calculated with phases from the CAP91 structure showed significant negative density at the position of the phosphate of cAMP bound in one subunit of the CAP91 dimer. Adenosine was preferentially substituted for cAMP in the subunit in the "closed" conformation, while the cAMP-binding site of the "open" subunit was apparently still occupied by cAMP. The structure was refined by restrained least-squares methods to an *R* factor of 20.2%. Adenosine is not bound in exactly the same position as cAMP; instead, the 5′-OH of adenosine is in a new position that allows formation of two hydrogen bonds with Ser-83, replacing two of the three interactions of the phosphate of cAMP with Arg-82 and Ser-83.

The catabolite gene activator protein (CAP)[1] (Zubay et al., 1970), also known as the cAMP receptor protein (Anderson et al., 1972), senses the intracellular concentration of cAMP in *Escherichia coli*. When the level of cAMP increases, CAP complexed to cAMP stimulates, in the presence of inducer, transcription from several operons, including those that encode enzymes involved in the catabolism of sugars such as lactose, maltose, and arabinose (de Crombrugghe et al., 1984). Wild-type CAP does not activate transcription in the absence of cAMP. CAP is a dimer of 2 identical subunits of 209 amino acids (Aiba et al., 1982; Cossart & Gicquel-Sanzey, 1982). The crystal structure of the wild-type CAP dimer with two bound cAMP molecules has been determined at 2.5-Å resolution (Weber & Steitz, 1987; Steitz & Weber, 1984). Each subunit of the CAP dimers folds into two distinct domains which are connected by a hinge region. The amino-terminal domains bind cAMP and form the major subunit–subunit contacts, while the carboxy-terminal domains are involved in site-specific binding to DNA (Weber & Steitz, 1984). The two subunits in the CAP dimer are in different conformations. In the "closed" subunit, the two domains lie closer together, while the domains are further apart in the "open" conformation subunit.

One class of *crp* mutations in adenylate cyclase deficient cells shows a CAP* phenotype; transcription of the CAP-dependent operons is activated even in the absence of added cAMP (Sanders & McGeoch, 1973; Melton et al., 1981). The *crp* genes of several CAP* mutants including *crp*91 (Harman et al., 1986) have been cloned and sequenced (Garges & Adhya, 1985; Aiba et al., 1986). The protein, CAP91, has threonine substituted for alanine-144 of wild-type CAP. CAP91 activates transcription of the lactose operon promoter (*lac* P) in vitro in the absence of added cyclic nucleotide. This activity is dependent on CAP91 concentration and saturates at high CAP91 concentration (Harman et al., 1986). cAMP increases the apparent affinity of CAP91 for *lac* P DNA to the level observed for wild-type CAP with cAMP. Other cyclic nucleotides, such as cGMP, also stimulate CAP91-mediated transcription of the *lac* operon in vitro (Harman et al., 1986), while the wild-type protein, CAP, is not activated by cGMP. The structure of the mutant CAP91, complexed with two cAMP molecules, has been determined at 2.4-Å resolution (Weber et al., 1987a). The relaxed specificity of CAP91 for cyclic nucleotide is useful in studying the effect of cAMP analogues on the structure.

We have investigated the effect of adenosine on CAP91. Studies on the binding of analogues of cAMP to wild-type CAP suggest that stimulation of activity requires a negative charge on the phosphate (Anderson et al., 1972; Scholubbers et al., 1984). The crystal structures of CAP (Weber & Steitz, 1987) and CAP91 (Weber et al., 1987a) show that a salt bridge occurs between the side chain of Arg-82 and the phosphate of cAMP, which is consistent with the results of analogue binding experiments. We report that adenosine, although it lacks the phosphate of cAMP, stimulates *lac* P activity in transcription assays that contain low levels of CAP91. In order to understand how adenosine binds to CAP91 in the absence of the important charged phosphate,

† Research sponsored by the National Cancer Institute, DHHS, under Contract N01-CO-74101 with Bionetics Research, Inc.

* Address correspondence to this author at the NCI-Frederick Cancer Research Facility.

‡ NCI-Frederick Cancer Research Facility.

§ National Bureau of Standards.

∥ Present address: Laboratoire de Mineralogie-Cristallographie, Tour 16, Université Pierre et Marie Curie, 4 place Jussieu, 75230, Paris Cedex 05, France.

⊥ Texas Tech University.

National Heart, Lung and Blood Institute.

[1] Abbreviations: CAP, catabolite gene activator protein; CAP91, CAP mutant crystallized with two molecules of cAMP [CAP91-(cAMP)$_2$]; CAP91A, CAP91 crystal soaked in adenosine (CAP91-cAMP-adenosine).

0006-2960/89/0428-4568$01.50/0

adenosine has been substituted for cAMP in the crystals of the CAP91 variant. The differences in the observed cAMP or adenosine interactions with CAP91 are discussed in relation to the ligand concentration requirements in CAP91-mediated transcription.

Experimental Procedures

Transcription Assays. Transcription assays were carried out in 1.5-mL polypropylene microfuge tubes in a volume of 0.025 mL. Reactions contained (per liter) the following: 30 mmol of Tris-HCl, pH 8.0 at 25 °C, 2.5 mmol of $MgCl_2$, 0.1 mmol of EDTA, 0.1 mmol of DTT, 0.5 mg of RNase-free BSA, 100 mmol of KCl, 0.25 mmol each of ATP, GTP, and CTP, 0.005 mmol of [α-^{32}P]UTP (20 Ci/mmol), and 1.4 mmol of supercoiled pKL201 DNA. RNA polymerase and CAP were added at 20 and 50 nM final concentration, respectively. Reaction mixtures lacking UTP, CAP, and RNA polymerase were prepared and placed on ice. Aliquots of CAP and RNA polymerase were added to the reaction tubes, and the tubes were incubated at 37 °C for 15 min. Polymerization was initiated with the addition of [α-^{32}P]UTP, followed 30 s later by 0.003 mg of rifampicin (neutralized with NaOH). The elongation reaction proceeded for 15 min at 37 °C and was terminated with the addition of 0.1 mL of ice-cold stop solution that contained (per liter) the following: 2.125 mol of ammonium acetate, 2 mmol of EDTA, and 0.4 g of *E. coli* tRNA. The RNA was precipitated in 2.5 volumes of ethanol, collected by centrifugation, washed in ethanol, and resolved by electrophoresis on 8.0% polyacrylamide gels that were 8.0 M in urea. Transcripts were visualized in wet gels at room temperature by autoradiography using Kodak XAR-5 film. Signals on the autoradiogram were quantitated by densitometry on a Shimadzu CS-930 TLC scanner.

X-ray Data Collection. A CAP91 crystal grown in the presence of cAMP (Weber et al., 1987a) was soaked for 4 months in 10 mM adenosine dissolved in 50 mM phosphate buffer, pH 8.0. It was not possible to use much higher concentrations of adenosine since adenosine precipitates under those conditions. X-ray diffraction data were collected at Genex Corp. with a Nicolet imaging proportional counter (IPC), an electronic area detector, mounted on a modified Supper oscillation camera controlled by a Cadmus 9000 microcomputer (Howard et al., 1987). The IPC chamber was positioned 12 cm from the crystal with the carriage angle set to 0°, intercepting data from infinity to 3.5-Å resolution. Diffraction data were collected at room temperature as described previously (Weber et al., 1987a).

The raw data frames were transferred to a Digital Equipment Corp. MicroVax II computer for processing. The determination of crystal orientation and the integration of reflection intensities were performed with the XENGEN program system (Howard et al., 1987). The measured X-ray diffraction data included 16 570 observations giving 4029 unique reflections out of 6467 possible reflections at 3.5-Å resolution. the 3826 reflections with $I > 2\sigma(I)$ gave a weighted least-squares R factor of 3.7% on intensity. A difference Fourier map was calculated with the phases from the CAP91 structure using the program PROTEIN (Steigemann, 1974). A peak was seen at the phosphate of the cAMP in the "closed" subunit, indicating the substitution of adenosine for cAMP at that site.

Structure refinement was performed with the restrained least-squares program PROLSQ of Hendrickson and Konnert (1980). The coordinates of CAP91 (Weber et al., 1987a) provided a starting model after the phosphate group of the cAMP molecule in the "closed" subunit was removed to model adenosine. Refinement was alternated with manual adjustments with the aid of the $2F_o - F_c$ and $F_o - F_c$ difference maps, using the model-building program FRODO (Jones, 1978) running on an Evans and Sutherland PS300 graphics system attached to a DEC MicroVax II. The model coordinates were refined with an overall thermal factor of 60. The final crystallographic R factor was 20.2% for 3600 reflections from 20- to 3.5-Å resolution. During the course of refinement, solvent molecules were kept in the same positions as in the native structure of CAP91. One water molecule near the cAMP-binding site of the "closed"subunit of the starting model coordinates of CAP91 was deleted, since there was no clear electron density for it. The new dimer structure containing adenosine in the "closed" subunit and cAMP in the "open" subunit was desginated CAP91A.

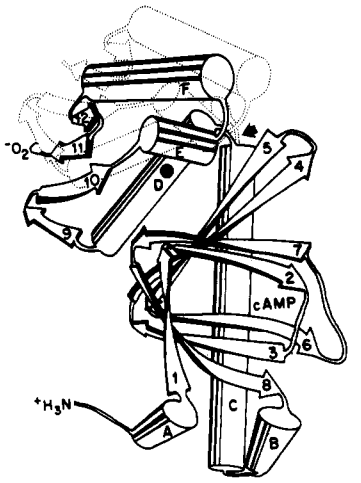

FIGURE 1: Schematic diagram illustrating the CAP subunit. Solid lines show the subunit in the "closed" conformation with lettered cylinders as α-helices and numbered arrows as β-strands. Cyclic AMP is shown in the amino-terminal domain. The arrow points to the hinge, residues 130–139, between the amino- and carboxy-terminal domains. The site of mutation in CAP91, Ala-144 to Thr, is indicated by a black circle on the D helix. The dotted lines show the orientation of the carboxy-terminal domain of the "open" subunit when the two amino-terminal domains are superimposed.

Results and Discussion

Effect of cAMP Analogues on CAP and CAP91. The binding of cAMP to wild-type CAP induces a conformational change, as shown by fluorescence experiments (Wu et al., 1974; Wu & Wu, 1974), which increases the affinity of CAP for specific DNA sequences. CAP is protease resistant in the absence of cAMP, while in the presence of cAMP, but not cGMP, it is digested to a stable amino-terminal core fragment (Eilen et al., 1978). In contrast, the variant CAP91, in the absence of cyclic nucleotide, is digested by subtilisin to yield a core fragment similar in size to the wild-type core (Harman et al., 1986). This suggests that CAP91 in the absence of cAMP has a similar conformation to wild-type CAP with cAMP.

The structures of CAP and CAP91, both complexed with two cAMP molecules, have been compared (Weber et al., 1987a). No structure is available for either protein in the absence of cAMP. The mutated residue, Thr-144 in CAP91, is located in the D helix of the carboxy-terminal domain, close to the hinge between the two domains (Figure 1). Thr-144 is about 12 Å from the closest DNA atom in the modeled complex of CAP and DNA (Weber & Steitz, 1984), and 20–30 Å from the cAMP, so that the effect of the mutation on cyclic nucleotide specificity is presumed to be indirect. The D helix may be stabilized by hydrogen bond interactions between the hydroxyl of Thr-144 and the carbonyl oxygens of

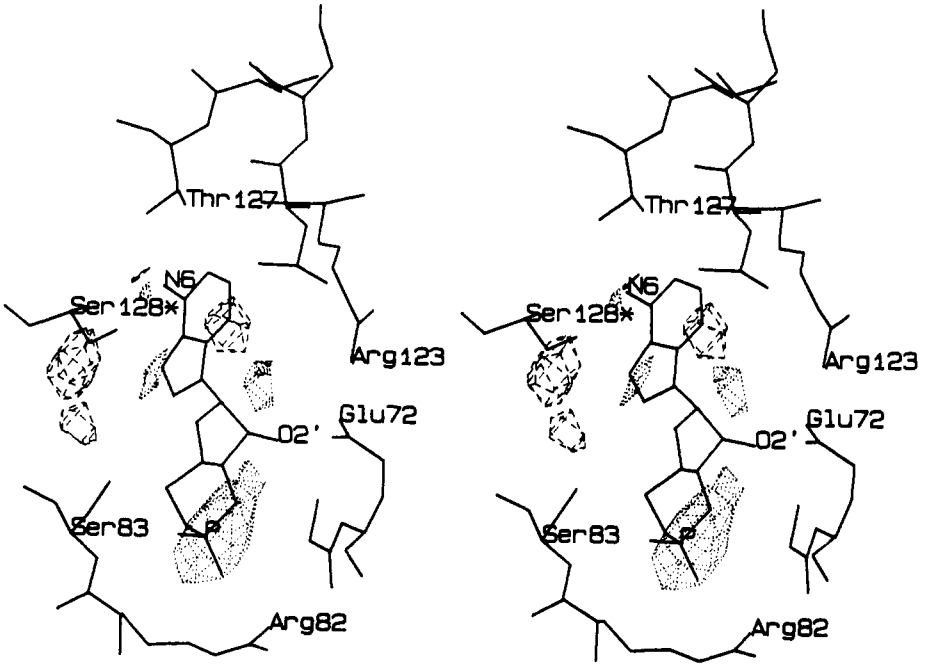

FIGURE 2: Stereoscopic view of the difference density for $F_{CAP91A} - F_{CAP91}$ with phases from CAP91 showing the cAMP-binding site in the "closed" subunit. The electron density is contoured at 3σ level with dashed lines for positive and dotted lines for negative difference density, and cAMP is shown here with the nearest atoms of CAP91. The negative peak is located on the phosphate of cAMP which indicates the substitution of adenosine for cAMP in this subunit.

residues 140 and 141, which cannot occur with the wild-type Ala-144. Small conformational differences were observed on comparing the structures of CAP and CAP91. These differences occur in the hinge and DNA-binding domain of the "closed" subunit, and residues 52–55 of the "open" subunit. The significance of these changes with respect to the altered phenotype is not understood at present.

The only analogues of cAMP which have been observed to fully activate the wild-type CAP to stimulate transcription are 7-deazaadenosine 3′,5′-monophosphate and (R_p)-adenosine 3′,5′-monothionophosphate, which have minor substitutions of C for the N7, and sulfur for the equatorial exocyclic oxygen of cAMP (Anderson et al., 1972; Scholubbers et al., 1984). Analogues with other modifications of the cyclic phosphate are inactive with wild-type CAP, and experiments suggest that a negative charge on the cyclic phosphate is required for the CAP–cAMP complex to be effective in stimulating transcription. In contrast, the CAP* variant CAP91, in which Ala-144 is replaced by Thr, is activated in the absence of added cyclic nucleotide and by a variety of analogues, some of which are shown in Table I. Both cGMP and cAMP stimulate transcription promoted by CAP91 (Harman et al., 1986), although cGMP does not activate wild-type CAP. In fact, CAP91 exhibits half-maximal activation by 28 nM cAMP, a lower concentration than the 1.2 μM cAMP required for half-maximal activation of CAP. Also, at a higher concentration, adenosine will stimulate the activity of CAP91 but not wild type; 1 mM adenosine is almost as effective as 0.1 mM cAMP in stimulating CAP91-dependent *lac* transcription. Since adenosine lacks the phosphate of cAMP, this suggests that CAP91, unlike wild-type CAP, does not require the ionic interaction with the phosphate of cAMP for effector-mediated activation. However, this ionic interaction appears to be important in determining the affinity of the ligand for CAP.

Initial Difference Fourier Map. The effect of adenosine on the structure of CAP91 has been tested by adding 10 mM adenosine to CAP91 which had been crystallized in 0.5 mM cAMP (Weber et al., 1987a). A refined structure was

Table I: Effect of Adenosine and Cyclic Nucleotide Monophosphates on in Vitro *lac* Transcription

compd	concn (mM)	*lac/rep* transcription ratio (% cAMP control)[a]	
		CAP	CAP91
adenosine	1.0	4	78
cAMP	0.1	100	100
cGMP	0.1	4	67
none (water)		2	4

[a] The ratio of the *lac/rep* transcription obtained with cAMP is set at 100. The synthesis of *rep* RNA is unaffected by CAP and serves as an internal control.

available for CAP91 complexed with cAMP (Weber et al., 1987a). The difference in X-ray amplitudes after scaling the two data sets, CAP91A and CAP91, was 21.2%. An initial difference electron density map was calculated between the CAP91A and CAP91 data sets with the phases from CAP91. This difference map showed a major negative peak at -5.3σ centered on the phosphate of the cAMP molecule in the "closed" subunit, which clearly indicates the substitution of adenosine for cAMP as shown in Figure 2. No large differences were seen in the cAMP-binding site in the "open" subunit, suggesting that cAMP bound at this site was not displaced by adenosine. No other significant differences were observed within the protein dimer.

The preferential substitution of adenosine in one of the two cAMP-binding sites of the CAP91 dimer is unusual. The presence of adenosine in the "closed" subunit and cAMP in the "open" subunit may be due to the different crystal packing interactions of the nonequivalent subunits in the dimer, or it is possible that cAMP binds more tightly to the subunit in the "open" conformation than to the "closed" subunit. In the crystal structure, the cAMP molecules are deeply buried within the CAP dimer between the β-roll and the C helix (Figure 1), and only the C8 atom is visible from the surface of the protein. It is not obvious how the cAMP enters or leaves the binding

Table II: Root Mean Square Displacements (Å) between CAP91A and CAP91 Structures and between CAP91 and CAP Structures[a]

	CAP91A/CAP91		CAP91/CAP (wild type)	
	"closed" (10–200)[a]	"open" (10–200)	"closed" (10–200)	"open" (10–200)
main chains	0.49	0.50	0.45	0.37
all atoms	0.76	0.74	0.70	0.64
cAMP		0.67	0.14	0.24
adenosine[b]	0.65			

[a] Range of amino acids in subunits. [b] The value for adenosine is for all the common atoms of adenosine and cAMP.

site; however, this would probably necessitate either separation of the two subunits of the dimer or else movement of the β-roll residues away from the C helix. The β-roll of the "closed" conformation subunit is more exposed to solvent in the crystal lattice than is the β-roll of the "open" subunit. Therefore, the crystal packing is consistent with the observed preferential substitution of adenosine for cAMP in the "closed", rather than the "open", subunit. The affinity of the mutant CAP91 for cAMP [apparent affinity = 28 nM (Harman et al., 1986)] is 20 000 times greater than for adenosine [apparent affinity = 600 μM (Harman and Peterkofsky, unpublished results)], which may make the complete substitution of adenosine for cAMP in the crystal more difficult.

Refined Structure of CAP91 with Adenosine and cAMP. The CAP91A coordinates with adenosine in the "closed" subunit and cAMP in the "open" subunit were refined to a crystallographic *R* factor of 20.2% using data from 20- to 3.5-Å resolution. The final model had deviations from ideality of 0.012 Å in bond lengths, and the geometry was comparable to the previously refined structure of CAP91 (Weber et al., 1987a). It is not straightforward to estimate the errors in atomic positions for structures refined by least-squares methods. The method of Luzzati (1952) gives an estimate of 0.35–0.40-Å overall mean error in the atomic positions of CAP91A, compared to better than 0.25 Å for CAP91; however this method is generally regarded as producing an overestimate of the true error in the coordinates. Table II summarizes the overall change between the two structures. Most of the differences observed between the CAP91A and the CAP91 structures are of the same magnitude as the differences between CAP91 and wild-type CAP (Weber et al., 1987a). The protein atoms show displacements of about 0.7 Å in both comparisons. Detailed analysis of the small differences in conformation between CAP91 and CAP91A was not warranted due to the lower resolution (3.5 Å) and higher error of the CAP91A data. Adenosine and cAMP in CAP91A show relatively large displacements from the positions in CAP91, compared to the change in position of cAMP bound in CAP or in CAP91. The cAMP in the "open" subunit of CAP91 is translated by about 0.67 Å relative to its position in CAP91. When the molecules of cAMP and adenosine in CAP91A are superimposed on the two cAMP molecules in CAP91, both the adenosine and cAMP appear to have rotated away from the two C helices in the center of the dimer and perpendicular to the small domains.

A test was run in which the coordinates were refined after removing the atoms of both adenosine and cAMP. This refinement converged after 14 cycles to an *R* factor of 20.7%. A difference Fourier was then calculated, and Figure 3 illustrates the difference density for adenosine and cAMP in the two subunits of CAP91A. This difference density is in agreement with the presence of adenosine in the "closed" subunit and of cAMP in the "open" subunit, although we cannot rule out the presence of adenosine at low occupancy alternatively with cAMP in the "open" subunit.

cAMP-Binding Sites. Four different binding sites can be described for CAP91: the two cAMP-binding sites in CAP91 and the cAMP- and adenosine-binding sites seen in CAP91A. There are approximately the same number of interactions between protein and ligand in all the sites. The cAMP-binding site of CAP is illustrated in Figure 4. Many of the interactions observed between cAMP and the protein are maintained when adenosine is bound to CAP91, as shown in Table III. The ribose 2′-OH forms hydrogen bonds with the amide of Gly-71 and the side chain of Glu-72 in all sites. The Thr-127 OH is positioned to form a hydrogen bond to N6 of the adenine ring in all four binding sites. Arg-82 forms an ionic interaction with the phosphate of cAMP in each of the three sites with bound cAMP. The two cAMP-binding sites in CAP91 are nearly identical; however, the cAMP bound to CAP91A appears to have moved slightly. In CAP91A, the ribose O5′ is within hydrogen bonding distance of the Ser-83 NH, and O3′

Table III: Interactions between CAP91 and cAMP or Adenosine[a]

protein atom	ligand atom	CAP91A distances (Å) "closed" subunit	CAP91A distances (Å) "open" subunit	cAMP atom	CAP91 distances (Å) "closed" subunit	CAP91 distances (Å) "open" subunit
			Phosphate Oxygens			
Arg-82 NH	O7		3.5	O7	3.0	3.4
Ser-83 OH	O6		(3.6)	O6	3.3	2.6
Ser-83 NH	O6		2.9	O6	2.9	3.2
			Ribose Oxygens			
Ser-83 OH	O5′	2.9	(5.1)	O5′	(5.0)	(4.6)
Ser-83 NH	O5′	3.1	3.4	O5′	(4.0)	(4.0)
Gly-71 NH	O2′	2.6	2.5	O2′	2.7	3.1
Glu-72 OE2	O2′	2.5	2.6	O2′	2.3	2.9
Glu-72 NH	O2′	(3.8)	3.1	O2′	(3.9)	(4.0)
Gly-71 NH	O3′	(3.6)	(3.7)	O3′	(3.7)	(3.6)[b]
Glu-72 NH	O3′	3.5	3.2	O3′	(3.8)	(3.8)
Leu-73 NH	O3′	(3.6)	3.3	O3′	(4.1)	(3.9)
			Adenine Rings			
Ser-*128* OH	N7	(3.6)	(3.7)	N7	3.2	(3.8)[b]
Ser-*128* OH	N6	3.0	(3.9)	N6	2.6	3.0
Thr-127 OH	N6	3.4	3.3	N6	3.1	3.0

[a] The distances are given between atoms of adenosine or cAMP and the closest atoms of the protein in the CAP91A and CAP91 structures. The ligand is adenosine in the "closed" subunit of CAP91A, and cAMP elsewhere. The values indicate the possible hydrogen bonds between pairs of atoms with a distance separation of 2.3–3.5 Å. Larger distances are given in parentheses. Ser-*128* is from the adjacent subunit in the CAP91 dimer. [b] These distances are also long in the wild-type CAP structure, indicating weak hydrogen bonding.

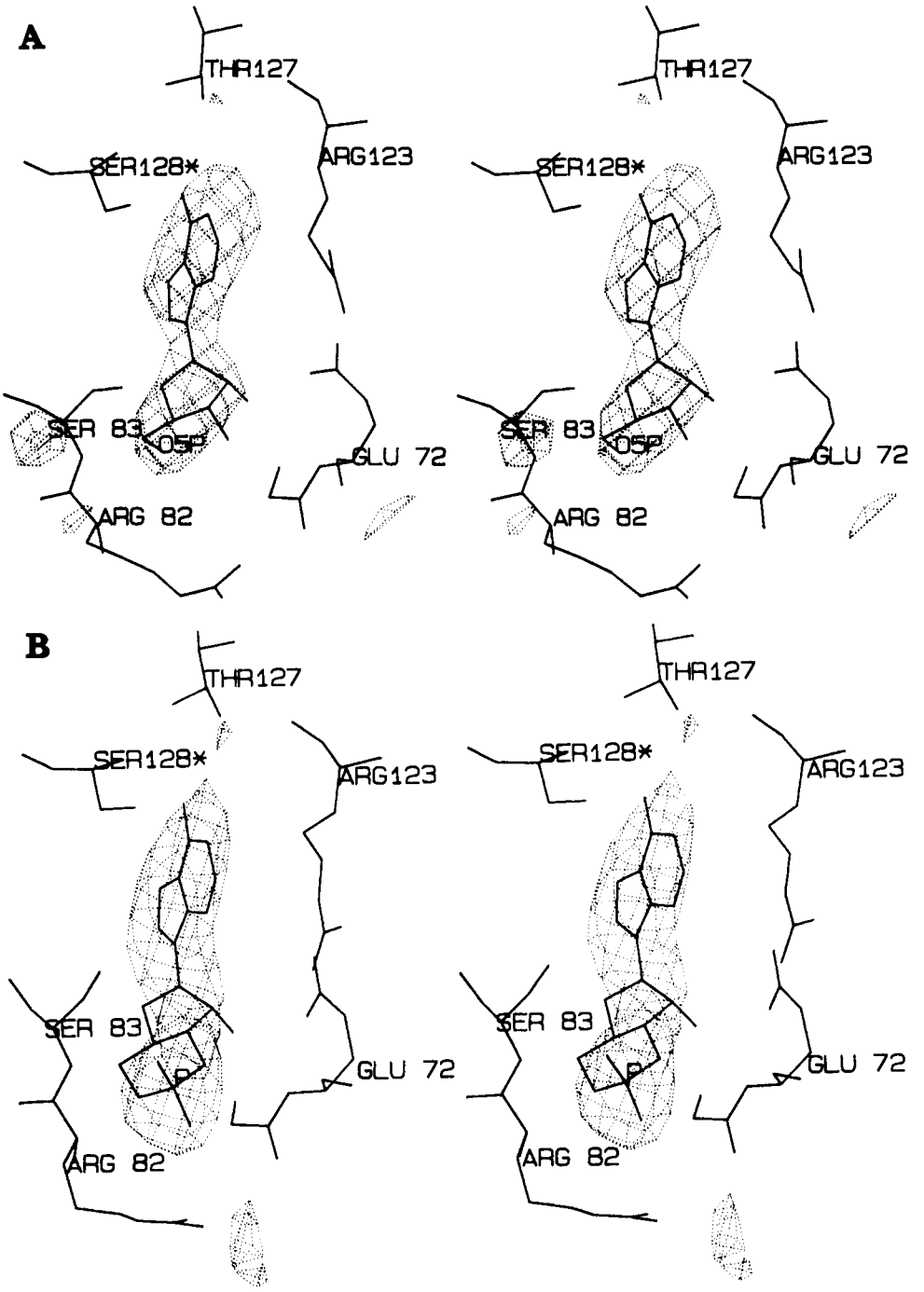

FIGURE 3: Stereoscopic views of the $F_o - F_c$ maps after refinement excluding the atoms of the cAMP and adenosine molecules in the CAP91A structure. The positive density is contoured at 3σ level. These views show the anti conformation of adenosine in the "closed" subunit (A) and cAMP in the "open" subunit (B).

is close to the Glu-72 NH and the Leu-73 NH. Ser-128 OH from the other subunit of the dimer forms a hydrogen bond with N6 of cAMP in both cAMP-binding sites of CAP91. In CAP91A, the N6 of cAMP is further from the Ser-128 OH, although adenosine in the "closed" subunit still forms a hydrogen bond interaction with the Ser-128 OH. Both adenosine and cAMP in CAP91A show potential hydrogen bonding between the protein and the ribose O3′ and O5′, which is less likely in CAP91. It was observed previously that wild-type CAP shows a weak hydrogen bond interaction with O3′ (Weber & Steitz, 1987). The ribose 2′-OH, O3′, and O5′, the adenosine N6 position, and the charged phosphate are implicated in significant interactions by binding experiments with analogues of cAMP (Anderson et al., 1972; Scholubbers et al., 1984), which is consistent with the crystal structure. Similar interactions to those shown in Figure 4 are predicted to occur in the cAMP-binding sites of mammalian cAMP-dependent protein kinases (Weber et al., 1987b).

The interactions of adenosine with CAP91A are illustrated in Figure 5. The refined coordinates of CAP91A show the potential for new hydrogen bonds between the 5′-OH of adenosine and Ser-83, instead of the hydrogen bonds between the axial exocyclic phosphate oxygen O6 of cAMP and the NH and OH of Ser-83 seen in the CAP91 structure. The ionic interaction between the other exocyclic phosphate oxygen (O7) of cAMP and Arg-82 cannot occur when adenosine is bound, since adenosine lacks a phosphate moiety. This suggests that the interactions of the phosphate with Arg-82 and Ser-83 can be partially replaced by the interactions of 5′-OH of adenosine with Ser-83, as illustrated in Figure 6.

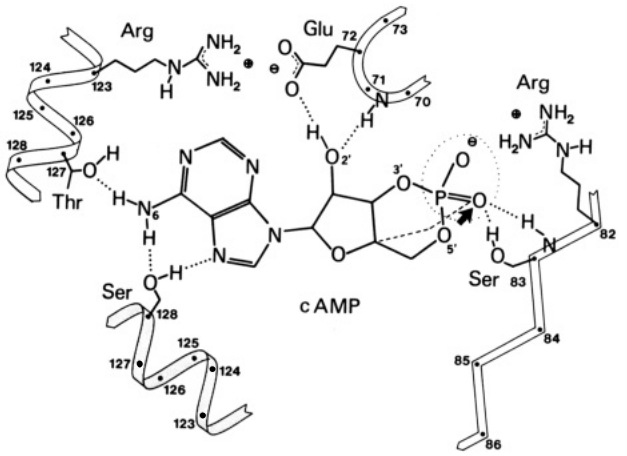

FIGURE 4: Interactions of adenosine or cAMP in the binding site of CAP are illustrated schematically. Several atoms of cAMP are labeled. Amino acid residues are numbered, and the shaded helix is from the other subunit of the dimer. Only the side chains that interact with cAMP are shown. Hydrogen bonds are indicated by dotted lines for cAMP in CAP91. The arrow indicates the new position of O5′ in adenosine, and the phosphate which is absent in adenosine is circled by a dotted line.

CAP91 requires 28 nM cAMP for half-maximal activation of *lac* transcription (Harman et al., 1986). In contrast, 600 μM adenosine is required for half-maximal activation of CAP91-mediated *lac* transcription. Assuming these values accurately reflect the apparent binding constants, we can calculate an approximate difference in the free energies of binding of adenosine and cAMP. If we assume that this difference is due to the phosphate which is present in the cAMP molecule and absent in adenosine, then

$$\Delta G_p \approx \Delta G_{cAMP} - \Delta G_{Ado}$$

This difference, ΔG_p, was calculated to be 5.8 kcal/mol from the binding constants given above.

Experimental measurements on substrate binding to tyrosyl-RNA synthetase have suggested that an ionic hydrogen bond provides 3.5–4.5 kcal/mol of binding energy and 0.5–1.5 kcal/mol is provided by a nonionic hydrogen bond (Ferscht et al., 1986). Inhibitor binding to thermolysin shows a difference of 4 kcal/mol between phosphonamidate analogues ($-PO_2^-$–NH–) and phosphonate analogues ($-PO_2^-$–O–) due to a difference of one hydrogen bond (Bartlett & Marlowe, 1987) in virtually identical structures (Tronrud et al., 1987). In both these cases, the crystal structures showed no rearrangement of atoms in the binding sites. The ΔG_p of 5.8 kcal/mol estimated for CAP91 is higher than expected for removal of one ionic hydrogen bond; however, in this case, the interaction with cAMP involves two charged groups, the phosphate of cAMP and the Arg-82 side chain, so there is no directly comparable experimental measurement.

The difference in binding energy deduced from the CAP91 crystal structures is due to loss of an ionic interaction, and some rearrangement of O5′ and Ser-83 (Figure 6). It is not clear what is the contribution due to the rearrangement which results in the formation of hydrogen bonds between 5′-OH of adenosine and Ser-83. There will also be contributions due to the differences in solvation of the free cAMP and adenosine, and entropic effects which are difficult to estimate. The major change is due to the absence of the phosphate in adenosine, which results in loss of an ionic interaction, and this probably

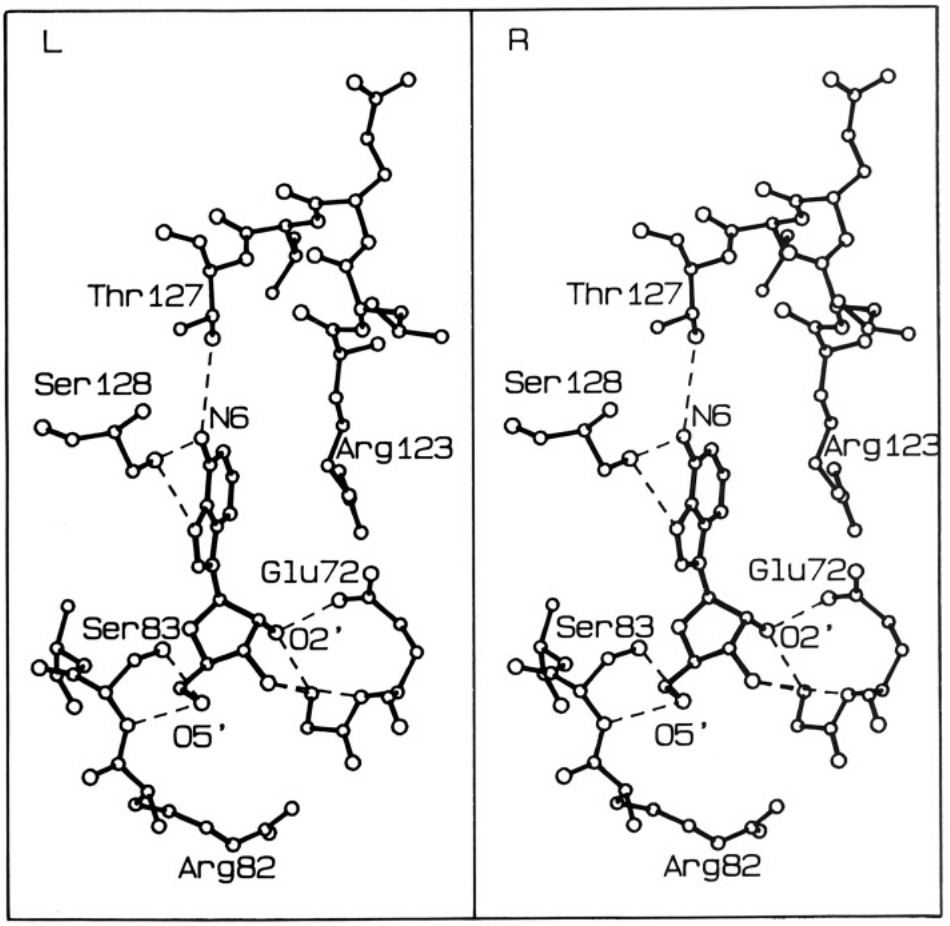

FIGURE 5: Stereoscopic view of adenosine with the closest amino acids of the binding site in the "closed" subunit of CAP91. Hydrogen bonds are indicated by dashed lines. Residues that do not make ionic or hydrogen bond interactions with cAMP or adenosine have been omitted for clarity.

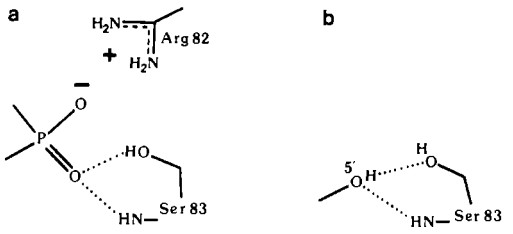

FIGURE 6: Major differences observed between the interactions of CAP91 with cAMP and adenosine. Charged residues are indicated (+) or (−), and hydrogen bonds are indicated by dotted lines. (a) Interactions of the phosphate of cAMP with CAP91, which are not possible for adenosine; (b) interactions seen in CAP91A between the ribose 5′-OH of adenosine and the protein.

contributes much of the measured free energy difference of 5.8 kcal/mol.

We have observed preferential substitution of adenosine in one out of two cAMP-binding sites in the dimeric CAP91. Also, small changes in the conformation of the ligand, or of the protein upon ligand binding, allow more interactions and a lower difference in binding energy than predicted by a direct substitution of one analogue for the known ligand. This suggests that results of analogue binding experiments in other systems should be treated with caution in the absence of direct structural data.

REFERENCES

Aiba, H., Fujimotot, S., & Ozaki, N. (1982) *Nucleic Acids Res. 10*, 1345.

Aiba, H., Nakamura, T., Mitani, H., & Mori, H. (1986) *EMBO J. 4*, 3329–3332.

Anderson, W. B., Perlman, R. L., & Pastan, I. (1972) *J. Biol. Chem. 247*, 2717–2722.

Bartlett, P. A., & Marlowe, C. K. (1987) *Science 235*, 569–571.

Cossart, P., & Gicquel-Sanzey, B. (1982) *Nucleic Acids Res. 10*, 1363.

de Crombrugghe, B., Bushby, S., & Buc, H. (1984) *Biol. Regul. Dev. 38*, 129–167.

Eilen, E., Pampeno, C., & Krakow, J. S. (1978) *Biochemistry 17*, 2469–2473.

Ferscht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1986) *Nature 314*, 235–238.

Garges, S., & Adhya, S. (1985) *Cell 41*, 745–751.

Harman, J. G., McKenney, K., & Peterkofsky, A. (1986) *J. Biol. Chem. 261*, 16332–16339.

Hendrickson, W. A., & Konnert, J. H. (1980) in *Biomolecular Structure, Function, Conformation and Evolution* (Srinivasan, R., Ed.) p 43, Pergamon Press, Oxford.

Howard, A. J., Gilliland, G. L., Finzel, B. C., Poulos, T. L., Ohlendorf, D. H., & Salemme, F. R. (1987) *J. Appl. Crystallogr. 20*, 383–387.

Jones, T. A. (1978) *J. Appl. Crystallogr. 11*, 268.

Luzzati, V. (1952) *Acta Crystallogr. 5*, 802–810.

Melton, T., Snow, L. L., Freitag, C. S., & Dobrogosz, W. J. (1981) *Mol. Gen. Genet 182*, 480–489.

Sanders, R., & McGeoch, D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1017–1021.

Scholubbers, H.-G., van Knippenberg, P. H., Baraniak, J., Stec, W. J., Morr, M., & Jastorff, B. (1984) *Eur. J. Biochem. 138*, 101.

Steigemann, W. (1974) Ph.D. Thesis, Technische Universitat, Munchen.

Steitz, T. A., & Weber, I. T. (1984) in *Biological Macromolecules and Assemblies* (Jurnak, F., & McPherson, A., Eds.) Vol. 2, p 290, Wiley, New York.

Tronrud, D. E., Holden, H. M., & Matthews, B. W. (1987) *Science 235*, 571–574.

Weber, I. T., & Steitz, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3973–3977.

Weber, I. T., & Steitz, T. A. (1987) *J. Mol. Biol. 198*, 311–326.

Weber, I. T., Gilliland, G. L., Harman, J. G., & Peterkofsky, A. (1987a) *J. Biol. Chem. 12*, 5630–5636.

Weber, I. T., Steitz, T. A., Bubis, J., & Taylor, S. S. (1987b) *Biochemistry 26*, 343–351.

Wu, C., & Wu, F. (1974) *Biochemistry 13*, 2573–2578.

Wu, F., Nath, F., & Wu, C. (1974) *Biochemistry 13*, 2567–2572.

Zubay, G., Schwartz, D., & Beckwith, J. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 104–110.