Crystal Structure of a Cyclic AMP-independent Mutant of Catabolite Gene Activator Protein*

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The Escherichia coli catabolite gene activator protein (CAP)1 (1), also known as cAMP receptor protein (2), forms a complex with cAMP which regulates transcription from several operons, including those that encode enzymes involved in the catabolism of sugars such as lactose, maltose, and arabinose (3). In the absence of cAMP, CAP has a lower, sequence-independent affinity for DNA and does not regulate transcription. In adenylyl-cyclase-deficient mutants, added cAMP is normally required for CAP to function. One class of mutants which also lack adenylyl-cyclase activity, has a CAP* phenotype; in the absence of cAMP it is able to express genes that normally require cAMP. CAP91 has been purified and crystallized with cAMP under the same conditions as to crystallize the wild type CAP·cAMP complex. X-ray diffraction data were measured to 2.4-Å resolution and the CAP91 structure was determined using initial model phases from the wild type structure. A difference Fourier map calculated between CAP91 and wild type showed the 2 results from the base change C to T in the second position of codon 144 which substitutes threonine for the alanine of wild type CAP. The NCR91 mutation produces the protein which is designated CAP91.

CAP91 has been characterized in vitro; in the absence of added cyclic nucleotide, CAP91 activates the lacP1 promoter contained on a supercoiled DNA template (8). The addition of cAMP increases the apparent affinity of CAP91 for lacP1 DNA to the same level as wild type CAP with cAMP. The mutant shows related specificity for cyclic nucleotide since either cAMP or cGMP will activate CAP91 while wild type CAP is very specific for cAMP.

CAP is a dimer with subunit molecular weight of 22,500. The sequence of 209 amino acids has been determined from the DNA sequence of the cloned crp gene (9, 10). The crystal structure of the wild type CAP dimer with bound cAMP has been determined (11, 12). Each subunit in the CAP dimer folds into two domains which are connected by a hinge region. The amino-terminal domain contains a helix between β-strands 4 and 5. The mutation at residue 144 apparently causes changes in the position of some protein atoms that are distal to the mutation site.

EXPERIMENTAL PROCEDURES

The gene for the mutant CAP91 of E. coli strain NCR91 has been cloned and sequenced (8). A single base change of C to T in codon 144 causes the substitution of threonine for alanine. CAP91 was purified to homogeneity from a strain of E. coli harboring a plasmid (pCRP91-37) in which the crp gene was under the control of the thermorepressible PL promoter. Crystals of CAP91 were grown in the presence of cAMP under the same conditions as the wild type protein: 0.5 mM cAMP, 50 mM phosphate at pH 8.0, and room temperature (14). These crystals are isomorphous with the wild type CAP crystals with unit cell dimensions of a = 46.5, b = 96.7, and c = 105.3 Å and space group P2₁2₁2₁.

A single crystal measuring about 0.3 × 0.4 × 1.0 mm³ was used for x-ray data collection at room temperature. Diffraction data were measured on a Nicolet/Xenographics Imaging Proportional Counter which was positioned 12 cm from the crystal with the carriage angle set at 20°. In this configuration, data ranging from infinity to 2.3-Å resolution were intercepted. Diffraction data were collected as a series of discrete frames or electronic images (15), each comprising a 15-min oscillation. The individual frames were contiguous in that the start of each small oscillation range coincided with the end of the previous range. The raw data frames were transferred to a Digital Equipment Corp. VAX 11/780 computer for subsequent processing. The crystal orientation and integration of reflections were performed with the XGENEN program system.2 A total of 61,342 observations were recorded which merged to give 19,328 unique reflections out of the 24,435 possible at 2.3-Å resolution. The 12,447 reflections with

2 A. J. Howard, G. L. Gilliland, B. C. Finzel, T. L. Poulos, D. H. Ohlendorf, and F. R. Salemme, unpublished data.

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amplitudes above 3.0 σ gave a symmetry R factor of 4.7% on intensity. The refined coordinates of CAP were used for initial phasing of the CAP3 structure. The difference in x-ray amplitude between the data for CAP91 and CAP was 14.4% and includes both differences in the methods of data collection and in the crystal structures. A difference Fourier was calculated using the program, PROLSQ (20), between CAP91 and CAP (wild type) with the phases of the known structure. The difference Fourier was examined using FRODO (19) running on a PS330 computer graphics system attached to an IBM 381. The difference map clearly indicated the sequence change and also a movement of 1 Cys-178 and these changes were made to the coordinates to provide a starting model for refinement. The model coordinates were refined by restrained least squares procedures using the program, PROFFIT (21), and a fast Fourier version, PROFFT (22). This was alternated with manual adjustment using 2F0-FC, and F-FC maps as a guide for repositioning the model coordinates. Initially, water molecules were positioned as in the original CAP structure. Several waters were removed during refinement and new waters were added at peaks in the 2F0-FC or F-FC density that were close to possible hydrogen bonding groups. When the refinement had converged and no further adjustments were deemed necessary the refined coordinates were analyzed and compared to the coordinates of the wild type structure. The difference in position of equivalent atoms in the two structures was computed and correlated motions were located by a vector averaging technique that minimizes random errors.

RESULTS AND DISCUSSION

Initial Difference Fourier Map—The structure of the CAP subunit is shown in Fig. 1 and the site of mutation from Ala-144 to Thr is indicated. A difference Fourier map was calculated for CAP91-CAP(wild type) data with phases from the wild type structure. Difference density appeared as expected at the site of the mutation, residue 144, in the D helix: a peak at 5.5 σ occurs in the open subunit (Fig. 2a) and at 5.1 σ in the closed subunit (Fig. 2b). This corresponds to the substitution of alanine 144 by threonine. There are also smaller differences occurring along the D helix. The highest peak in the difference map occurs near Cys-178 in the open subunit (Fig. 3a). This peak is at 7.0 σ and corresponds to a movement of the side chain of Cys-178 which lies in the turn between α helices E and F. There are no large differences around Cys-178 in the closed subunit, as shown in Fig. 3b. The negative density gave the largest peak of 5.5 σ near the side chain of Thr-144, a peak at 5 σ at Cβ of Cys-178 (both in the open subunit), and one at 4.8 σ near Gly-33 in the closed subunit. Smaller differences are also observed (4.5 σ) at the end of the hinge in the closed subunit.

This initial difference Fourier confirms the site of the mutation, indicating density for the difference corresponding to the additional methyl and hydroxyl of threonine compared with alanine. It also shows an asymmetrical change: Cys-178 moves in the open subunit only. It is noteworthy that the distance between the closest atoms of Thr-144 and Cys-178 is 13 Å so that the motion of Cys-178 cannot be due to direct proximity to the mutation. Residues from the hinge of the adjacent subunit in the CAP dimer (Ala-135 and Phe-136) lie close to Cys-178 (Fig. 3) as do residues near the mutation in the D helix (Arg-142 and Ile-143); but there is no direct route for transmitting a change from the mutation at 144 to Cys-178. The predicted binding site for DNA in the model complex with CAP (21) includes Cys-178 in the turn between the E and F helices. In the modeled complex of CAP and DNA, Cys-178 lies about 4 Å from a phosphate oxygen of the DNA so that a movement of this residue might tend to alter the affinity of CAP for DNA. Alternatively this Cys may be oxidized and the different positions of the SH1 may be due to unknown differences in the solutions used to crystallize CAP91 or CAP. Overall the peaks in the difference Fourier are clustered in the small domains near the hinge region and the site of mutation.

Comparison of the Two Refined Models—The model coordinates of CAP91 were refined to an R factor of 18.6% for the reflections above 3 σ and from 7.0- to 2.4-Å resolution. A total of 105 water molecules were included in the final model. The amino terminus of the open subunit was recut to what is probably an alternative position since the first 6 residues have high temperature factors. The geometry of the protein model had a root mean square error of 0.023 Å in bond lengths, 0.22 in planar restraints, and the overall thermal factor was 19.16. The thermal factors are plotted against sequence number in Fig. 4. The four termini have high temperature factors as do residues around 55 and 150-160 in the closed subunit. The termini are probably flexible and no density was visible for 209 in the closed subunit and 206-209 in the open subunit for both CAP and CAP91. Similarly, no density appears in the 2F0-FC map for residues 159 and 160 in the closed subunit which occur at a surface turn. Residues 52-56 form a surface hairpin turn between β4 and -5.

The refined coordinates of the mutant, CAP91, were compared with the refined coordinates of the wild type CAP (R = 20.5%). The overall change between the two structures was evaluated by calculating the root mean square differences in atomic positions. The terminal residues were omitted from the comparison which is summarized in Table I. The root mean square distances calculated for α carbon atoms are slightly smaller than for the side chains which tend to have more torsional freedom. Note that the two Camp molecules are displaced less than main chain or α carbon atoms. This can be compared to results from other refined protein structures at high resolution. The root mean square displacements listed in Table I are larger than seen in a comparison of a
mutant and wild type subtilisin\(^*\) which had an overall root mean square displacement of 0.07 Å for α carbon atoms and 0.10 Å for all atoms. The major difference in subtilisin is located at the altered amino acid where the backbone atoms moved by 0.3 Å. The overall differences between CAP91 and CAP are more similar to those between two independently

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refined models of ribonuclease A (22) which gave root mean square deviations of 0.22 Å for main chain atoms and 0.80 Å for all atoms; and two crystal forms of bovine pancreatic trypsin inhibitor with root mean square differences of 0.40 Å for main chain atoms and 1.53 Å for side chain atoms (23).

The root mean square differences between CAP91 and CAP can also be compared to the differences between the two subunits in the CAP dimer.\(^{5}\) The 2 amino-terminal domains have root mean square deviations of 0.82 Å for main chain

FIG. 2. Stereo view of the difference density for \((F_{\text{CAP91}} - F_{\text{CAP}})\) on CAP phases. The positive density is contoured at 3.0 \(\sigma\) in solid lines and the negative density at \(-3.0 \sigma\) in dashed lines. Residues in the open subunit are labeled with an asterisk. a, The open subunit showing Thr-144 at the site of the mutation. b, The closed subunit showing Thr-144 in a similar orientation.
FIG. 3. Stereo view of the difference electron density as described in the legend to Fig. 1 with contour levels of +4.0 and −4.0 σ. Residues in the open subunit are marked with an asterisk. Amino acids 135 and 136 from the hinge of the adjacent subunit are seen in these views. a, the open subunit showing Cys-178. b, the closed subunit, here Cys-178 has no large differences.
terminal domains show more change in both subunits. The magnitude of the vector differences is 0.13 Å for the closed subunits. These two plots show similar changes in subtilisin.6

There is a relatively large shift of the surface loop between residues 205-209, were not included in this analysis. The average magnitude of the vector differences is 0.13 Å for the closed subunit and 0.15 Å for the open subunit. The two CAP subunits have changes in different regions. The region from 155 to 160 in the closed subunit has a large change in position (peak b of Fig. 5a) and the atoms also have higher thermal factors (Fig. 4). Significant change is centered on residues 139 and 179 (peaks a and c) in the closed subunit and on residues 55 (peak d) and 125 in the open subunit. The carboxyterminal domains show more change in both subunits. The largest differences are similar in magnitude to the 0.3-Å average shift of the backbone atoms of the mutated residue in subtilisin.8

These small motions are more readily examined in three dimensions: the vector between α carbon atoms of CAP and CAP91 is shown in Fig. 6a and the average vector difference is displayed in Fig. 6b. These two plots show similar changes in the structures. The amino-terminal domains show smaller changes. Residues, 29-33 and 82-84, of β strands 2 and 7 in the cAMP-binding pocket of the closed subunit move together, as do residues, 18-23 and 87-94, of β strands 1 and 8 in the open subunit. Most of the residues forming the cAMP-binding site do not alter position substantially, however, there is a small shift of the cAMP bound in the open subunit in a direction that correlates with the motion of residues 117-127 in the C helix. This may be due to the hydrogen bond interaction between Thr-127 and the 6-amino group of cAMP. There are large motions are possible for the F helices since these form crystal lattice interactions with other CAP dimers.

The single sequence change occurs at residue 144 which is 20-30 Å from the cAMP molecule bound in the same subunit and about 12 Å from the closest phosphate oxygen of the DNA in the specific model complex (21). Residue 144 is also at the surface of the protein so there is little steric hindrance for the change from Ala to Thr. It is not immediately obvious how a change in a residue distant from the cAMP can alter the properties of the protein so that it acts in an allosteric manner and is also activated by cGMP unlike the wild type. The changes that occur distal to the site of mutation have not been observed in the structure of subtilisin mutants; however, such changes are expected in an allosteric protein. Residue 144 is in the D helix and connected via the hinge region with the C helix that has hydrogen bond interactions with cAMP at residues Thr-127 and Ser-128. This provides one possible route for transmission of a conformational change that would alter the effect of cAMP or cGMP binding to CAP. The change in position of Cys-178 in the open subunit shows that conformational changes could result at distances up to 13 Å from the site of mutation and indicates that residues on the DNA-binding surface of CAP are altered in the CAP91 mutant.

The differences between the two crystal structures have been analyzed in terms of small conformational changes or concerted movements of segments of the structure. The binding of cAMP produces an allosteric conformational change in CAP (24) which increases the affinity for DNA (25, 26).
Structure of CAP Mutant

Fig. 5. a, the vector average of the differences in atomic position is plotted against sequence number for the closed subunit. The average is taken over 11 residues. The peaks marked are: a, residues 133-142 including the hinge; b, surface loop residues 160-163; and c, residues 175-195. b, the local vector average for the open subunit, plotted as in a. The peak labeled d is at the hairpin turn between β4 and -5, residues 52-55.

Fig. 6. a, the displacement between α carbon atoms of CAP91 and CAP is shown as a vector from each α carbon in a stereo diagram of the CAP dimer structure. The labels O and C indicate the open and closed subunits, respectively. The vector between equivalent α carbon atoms has been scaled by a factor of 3.0. b, the vector average is plotted on a stereo α carbon representation of the CAP dimer. The local vector averaged over all atoms of 11 residues is shown extending from the central α carbon atom. The vector is scaled by a factor of 5.
Experiments in solution show that CAP91 in the absence of cAMP appears to have a similar conformation as the wild type CAP in the presence of cAMP (8). This has also been shown for other CAP* mutants (17). However, CAP91 exhibits half-maximal activation of transcription at a concentration of cAMP that is approximately 40-fold lower than required by wild type (8); so that, even in the absence of cAMP, there are differences between CAP and CAP91. The comparison of the structures of CAP91 and CAP, both crystalized with cAMP, show many small changes associated with the DNA-binding domains. The most distinct changes in the wild type compared to CAP91 correspond to a motion of the hinges away from the central axis of the dimer; the β4 to -5 loop of the open subunit moves towards the center and the turn between the E and F helices moves in different directions in the two subunits.

Location of Other CAP* Mutations—The mutations producing the CAP* phenotype occur in widely separated parts of the molecule; substitutions have been reported in residues from 53 to 195 in the amino acid sequence (6-8). None of these crp* mutations lie in the E and F helices that are expected to bind DNA (21), however, many occur in the D helix (residues 140–151). Mutations have been observed in residues 141, 142, 144, and 148, so this helix is apparently important for activation of CAP by cAMP. The D helix follows the hinge between the two domains and lies close to the F helix that binds DNA. The location near the hinge also suggests that the D helix plays a role in the allosteric change that occurs when cAMP binds to CAP, since cAMP forms hydrogen bonds to Ser-128 and Thr-127 in the C helices that precede the hinge.

Two mutations occur in the β strands 4 and 5 (7); these have different phenotypes since the mutation with Phe-62 is not activated by cGMP unlike the mutation with His-53. The structure provides an explanation since residue 53 is on the protein surface and lies close to the E helix whereas residue 62 is normally serine and lies next to the adenine ring of the bound cAMP. Therefore changes at position 62 are likely to disrupt the binding of cAMP, especially a change to the large Phe side chain which might sterically interfere with the binding of cyclic nucleotides.

Two other mutations are located in the cAMP-binding pocket (6, 8). One is in residue 127 which in CAP is Thr and forms a hydrogen bond with the 6-amino group of cAMP. Another result in the substitution of Ala for Glu-72 as part of a double mutation which would eliminate the hydrogen bond between Glu-72 and the ribose 2'-OH of cAMP. Therefore a CAP* phenotype may involve alteration or elimination of interactions between CAP and cAMP.

Conclusions—The refined structures of wild type CAP and the CAP91 mutant protein have been compared, revealing that the two structures with bound cAMP are very similar overall. However, small concerted movements occur in the residues of the hinge between the two domains of the closed subunit, in the adjacent loop of β4 to -5 in the open subunit, and in the turn between the DNA-binding E and F helices in both subunits. These changes are distal to the site of mutation at residue 144, unlike the changes observed in the structures of substituton mutants which are seen only in close proximity to the mutated residue. These small changes are seen even though CAP91 and CAP are both crystalized in a complex with cAMP, however, in solution, CAP and CAP91 show different affinities for cAMP and other cyclic nucleotides. The structures of other mutants or of CAP crystalized in the absence of cAMP will probably be required to understand the small conformation changes due to the Ala-144 to Thr mutation described here.

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