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2.0 Å Refined Crystal Structure of the Catalytic Subunit of cAMP-Dependent Protein Kinase Complexed with a Peptide Inhibitor and Detergent

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Abstract. A mutant (Ser139Ala) of the mouse recombinant catalytic (*C*) subunit of cAMP-dependent protein kinase was co-crystallized with a peptide inhibitor, PKI(5-24), and MEGA-8 (octanoyl-*N*-methylglucamide) detergent. This structure was refined using all observed data (30 248 reflections) between 30 and 1.95 Å resolution to an *R* factor of 0.186. R.m.s. deviations of bond lengths and bond angles are 0.013 Å and 2.3°, respectively. The final model has 3075 atoms (207 solvent) with a mean *B* factor of 31.9 Å^2. The placement of invariant protein-kinase residues and most *C*:PKI(5-24) interactions were confirmed, but register errors affecting residues 55-64 and 309-339 were corrected during refinement by shifting the affected sequences toward the C terminus along the previously determined backbone path. New details of *C*:PKI(5-24) interactions and the Ser338 autophosphorylation site are described, and the acyl group binding site near the catalytic subunit NH_2 terminus is identified.

Introduction. Reversible protein phosphorylation is a widespread biological regulatory mechanism. In eukaryotes the phosphorylation step is catalyzed by a family of homologous protein kinases (Hanks, Quinn & Hunter, 1988). Study of the cAMP-dependent protein kinase (cAPK) has provided many general insights into this family (Taylor, 1989). A crystal structure of the mouse recombinant cAMP-dependent protein-kinase catalytic (C_α) subunit complexed with the substrate-analog peptide inhibitor PKI(5-24) (Cheng, van Patten, Smith & Walsh, 1986), revealed the structural framework of the protein-kinase catalytic domain and identified the peptide substrate binding site and nucleotide binding site (Knighton, Zheng, Ten Eyck, Ashford *et al.*, 1991; Knighton, Zheng, Ten Eyck, Xuong *et al.*, 1991). In the present work the resolution of the initial 2.7 Å model of the recombinant *C* subunit has been extended to 2.0 Å, correcting the sequence registration in the electron density for residues 55-64 and 309-339, and identifying a detergent binding site near the NH_2 terminus.

Experimental methods. A triply phosphorylated isoform of the mouse recombinant C_α subunit, with Ser139 mutated to Ala to remove the Ser139 autophosphorylation site, was purified to homogeneity using Mono S chromatography (Herberg, Bell & Taylor, 1993; Slice & Taylor, 1989). The *C* subunit used for previous studies represented a mixture of isoforms. Crystals were grown using previously described methods (Zheng *et al.*, 1992), but with 0.4% (*w*/*v*) of the detergent MEGA-8 (octanoyl-*N*-methylglucamide) present in the hanging drop. The improved purification and inclusion

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of detergent together improved the diffraction from 2.7 to 2.0 Å. Diffraction data were acquired from a single $P2_12_12_1$ crystal of cell dimensions a = 73.84, b = 75.76, c = 81.01 Å at 277 K using graphite-monochromated Cu $K\alpha$ X-rays from a Rigaku RU-200 diffractometer equipped with Xuong-Hamlin area detectors (Hamlin *et al.*, 1981; Xuong, Sullivan, Nielsen & Hamlin, 1985). Data reduction was performed with UCSD area-detector programs (Howard, Nielson & Xuong, 1985). The R_{sym} on intensities for 30 428 reflections (90% complete to 1.95 Å) was 5.61% with each reflection being measured 3.4 times on average.

Refinement was started with the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977) entry 1CPK, using *X-PLOR* simulated-annealing and conjugate-gradient refinement (Brünger, Kuriyan & Karplus, 1987). All *X-PLOR* calculations used data from 20 to 2.0 Å. The initial model refined to an R factor of 0.25. The model was then rebuilt using 36 omit maps. For each map a ten-residue stretch, plus all residues within 6 Å of the ten residues, was omitted from the annealed model and the resulting model subjected to 20 cycles of *X-PLOR* conjugate-gradient refinement of positional parameters. Maps were calculated using model phases and amplitudes of $mF_o - DF_c$ or $2mF_o - DF_c$ from the program *SIGMAA* (Read, 1986), in which m is a Sim weight and D is a weighting factor based on expected coordinate errors. The model was manually adjusted using the program *TOM/FRODO* (Cambillau & Horjales, 1987; Jones, 1978). The registration errors mentioned previously were corrected during this process. 34 surface side chains were found to have no supporting electron density and were truncated at usually C^β or C^γ, deleting 130 atoms from the model.

Solvent molecules were added to the model by a program which placed candidate molecules on peaks greater than 3σ in $mF_o - DF_c$ maps. Candidates were rejected if they were closer than 2.5 Å to any atom in the model, were not symmetry unique, or were close to the possible position of a truncated side chain. The edited model gave an R of 0.22 after conjugate-gradient refinement of coordinates followed by unconstrained B refinement; the r.m.s. deviations of bond lengths and angles from ideal values were 0.016 Å and 3.2°. At this point, the model was refined with *TNT* (Tronrud, Ten Eyck & Matthews, 1987) using the conjugate-direction algorithm (Tronrud, 1992), a bulk solvent correction, and all 30 428 reflections from 30 to 1.95 Å resolution. Coordinates and B factors were refined together. The *TNT* model gave an R factor of 0.186 with r.m.s. deviations from ideal bond lengths and angles of 0.013 Å and 2.26°. All residues are within the allowed regions of the Ramachandran plot. During the refinement, 125 atoms hit the upper program limit on B's of 100 Å^2. Seven of these were solvent; the side chains of Ser10, Glu11, Asp276, Arg336, and 10 of the 19 atoms at the C-terminal dipeptide of the inhibitor are essentially invisible. The main chain has high B factors in residues 51-55, which are part of the flexible glycine loop, at Gly253, and in a surface strand at residues 319-322. The remainder of the very high B factors are in surface side chains. These atoms have been left in the model as internal-reliability indicators. Including these atoms, the mean main- and side-chain B factors are 27.8 and 33.8 Å^2; for solvent, 48.1 Å^2. The final *TNT* model contains residues 10-350 of the *C* subunit, all 20 residues of the peptide inhibitor, a single detergent molecule modeled as *n*-octane, and 207 solvent atoms, for a total of 3075 atoms.

Results. *Corrections to the* 2.7 Å *model.* The 2.0 Å model has the same basic backbone trace and placement of invariant protein-kinase residues as the 2.7 Å model. During the refinement, however, electron density and residue stereochemistry indicated that the initial 2.7 Å model had incorrect register for residues 55-64 and 309-339 (Fig. 1). The model was corrected without altering the connectivity of the main chain by using an omit map to rebuild Gly55, displacing the side chains of residues 56-64 one position toward the C terminus, and reinterpreting the turn at residues 64-65. The longer correction, residues 309-339, was along a surface strand which is not part of the conserved core and was also rebuilt into omit maps. The corrected structure refined smoothly with good stereochemistry.

The 55-64 correction changes part of the nucleotide binding site to place the highly conserved Val57 adjacent to the position of the ATP ribose and adenine moieties in the ternary complex, rather than Met58. The side chain of Met58, in fact, points outward from the nucleotide site (Zheng *et al.*, 1993). The 309-339 correction invalidates the interaction of Glu 331 with the $P-3$ Arg of PKI(5-24) proposed from the initial model (Knighton, Zheng, Ten Eyck, Xuong *et al.*, 1991). The side chain replacing Glu331, Tyr330 (Fig. 2*a*), is still in proximity to the $P-3$ Arg and may form a hydrogen bond with it under some circumstances. In this binary *C*:PKI(5-24) complex structure, the $P-3$ Arg side chain has two conformations (refined occupancy 2:1). In the major conformation the guanidinium forms two hydrogen bonds with the Glu127 side chain and a single hydrogen bond to the acyl O atom of Thr51 and is unfavorably disposed for interaction with Tyr330. In the minor conformation only a single hydrogen bond to Glu127 is seen, with the guanidinium also approaching the side chain of Ser130 and Asp328 to form weaker hydrogen-bond interactions. In this minor conformation, Tyr330 forms a hydrogen bond to the N^ϵ proton. Asp329 and Glu331 are orientated away from the $P-3$ Arg, and the side chain of Asp329 comes close to the side chain of Lys47.

Peptide interactions and structure. In most other respects, however, the PKI(5-24)-enzyme interactions reported in the 2.7 Å model have been confirmed. Besides the noted alternate conformation of the $P-3$

Arg, however, additional interactions can be described (Fig. 2*a*). First, the guanidinium of Arg133 is seen to form a hydrogen bond to the acyl O atom of the $P-5$ Thr and forms water-bridged interactions with the acyl O atoms of the $P-4$ Gly and the $P-6$ Arg. Second, besides two hydrogen bonds to the $P-2$ Arg guanidinium, the side chain of Glu170 also forms a hydrogen bond to the $P-2$ Arg amide proton. Third, in an interaction of the peptide inhibitor with itself, the $P-6$ Arg guanidinium interacts through a bridging water molecule with the acyl O atom of the $P-1$ Asn, in addition to its interaction with Glu203. The single good backbone-backbone hydrogen-bond between the acyl O atom of Gly200 and the amide proton of $P+1$ Ile is confirmed. Finally, the two C-terminal ($P+2$, $P+3$) inhibitor residues are still disordered. The present model attempts to place these two residues in their best position, but the *B* factors are very high, and no matter how these residues are placed, there is positive density in $F_o - F_c$ maps, indicating probable multiple conformations.

Ser338 phosphorylation site. Correction of Ser338, a site of phosphorylation on the *C* subunit, reveals that the phosphate moiety forms a hydrogen bond with the amide proton of Ile339 and is near the side-chain tips of Asn340 and Lys342, and perhaps of Arg336 as well. The side chain of Asn340 is approximately 3.7 Å away from the phosphate. Lys342 extends toward the phosphate but has only weak electron density at its tip.

Detergent and N*-terminal extension.* The 14 N-terminal residues were absent in the electron density of the *C* subunit crystallized without detergent (Knighton, Zheng, Ten Eyck, Ashford *et al.*, 1991). In contrast, the *C* subunit crystallized in the presence of MEGA-8 lacks electron density only for residues 1-9. A rod of density was observed extending into the interior of the protein between the N-terminal helix and its interface with the remainder of the enzyme (Fig. 2*b*). The environment of the rod was almost exclusively hydrophobic, and the density could be modeled as the aliphatic portion of a MEGA-8 detergent molecule, the polar sugar moiety apparently being disordered. Residues 10-14, which were not visible in previous work, could be modeled near the detergent binding site as an additional turn of the N-terminal helix. The proximity to the protein N terminus and the hydrophobicity of the site are consistent with this site being part of the normal myristic acid binding site of the mammalian *C* subunit. Ser10, a site of autophosphorylation in the recombinant *C* subunit (Yonemoto, McGlone & Taylor, 1993*b*) does not have clear phosphate density.

Discussion. Crystallization of a single isoform of Ser139Ala catalytic subunit together with MEGA-8 detergent has allowed extension of the resolution of the *C*:PKI(5-24) complex structure to 2.0 Å from the initial 2.7 Å model of the mixed phosphoform complex, providing a more reliable basis for modeling the *C*:PKI(5-24) complex. New structural features seen in this corrected structure and at this higher resolution explain several earlier observations about the *C* subunit and its PKI(5-24) interactions. The protection of the Ser338 phosphate from phosphatase treatment (Shoji, Titani, Demaille & Fischer, 1979) and the protection of Lys342 from modification by acetic anhydride treatment (Buechler, Vedvick & Taylor, 1989) are likely due in part at least to the proximity of the Lys342 side chain to the phosphate group. In addition, the stretch of acidic residues from 328 to 334 displayed residue-specific changes in 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC)-labeling behavior in the presence and absence of MgATP and PKI(5-24) (Buechler & Taylor, 1990), and both crys-

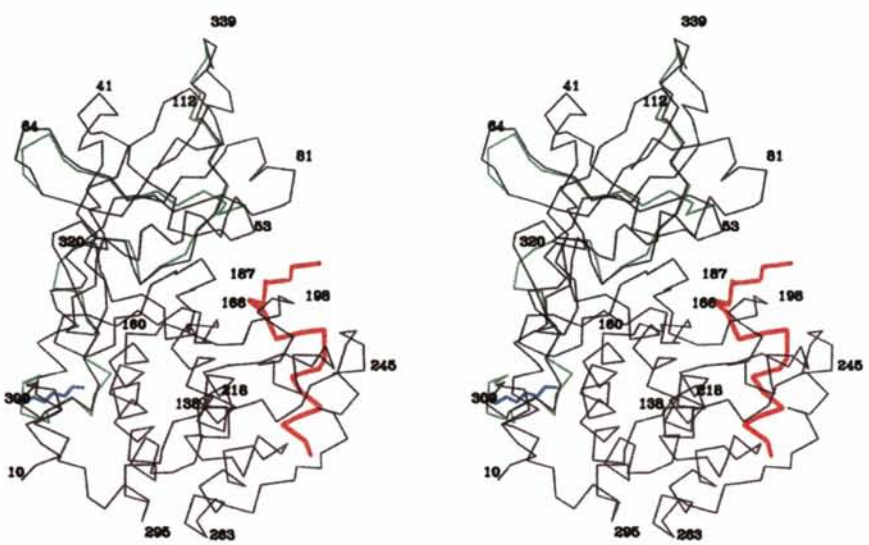

Fig. 1. *C*:PKI(5-24) C^{α} backbone trace. The PKI(5-24) peptide inhibitor is shown in red. The MEGA-8 detergent, modeled as *n*-octane, is shown in blue in the lower left. In green is the superimposed C^{α} trace of the superseded 2.7 Å 1CPK model for residues 54-67 and 307-341.

tallographic locations of the $P-3$ Arg side chain are close to Asp328, which is protected in the ternary complex. This 2.0 Å structure also highlights the role of Arg133 noted earlier (Gibbs, Knighton, Sowadski, Taylor & Zoller, 1992) in maintaining affinity of a yeast *C* subunit for its regulatory subunit while not greatly affecting its interaction with a peptide substrate beginning at the $P-4$ residue. The 2.0 Å structure confirms that the preponderance of Arg133 interaction with the peptide is with the $P-5$ and $P-6$ residues beyond the end of the peptide substrate.

A novel feature of this structure is the presence of a detergent binding site near the amino terminus of the visible electron density. The mammalian *C* subunit is *N*-myristylated (Carr, Biemann, Shuji, Parmalee & Titani, 1982), a modification that stabilizes the protein (Yonemoto, McGlone & Taylor, 1993*a*) rather than localizing it to membranes. Co-crystallization of recombinant *C* subunit with MEGA-8 detergent here conferred additional stability to residues 10–14 such that they had crystallographic order, in contrast to their invisibility in the 2.7 Å density with no detergent. The detergent is adjacent to Val15, the first visible residue in the structure without detergent. Enhanced Ser10 phosphorylation in this crystal

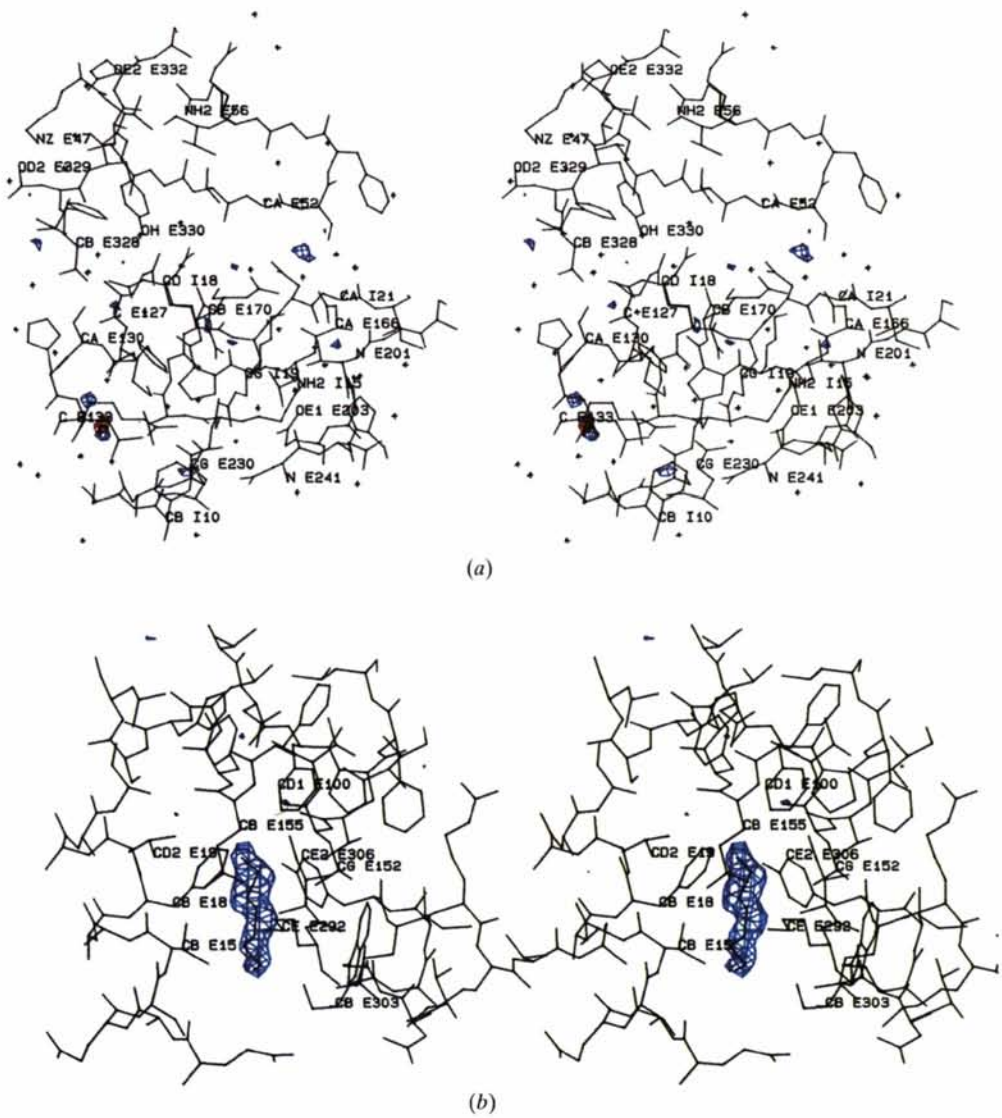

Fig. 2. Peptide, inhibitor and detergent interactions. (*a*) PKI residues *I*15–*I*22 and their interactions with nearby enzyme (*E*) residues and solvent (drawn as stars) are shown superimposed on $\pm 3.5\sigma$ $mF_o - DF_c$ difference electron-density contours (blue positive, red negative). The major conformation of the $P-3$ Arg(*I*18) points toward Thr*E*51 and Glu*E*127, and of Arg*E*56 toward Glu*E*332. (*b*) The *n*-octane model of the co-crystallized MEGA-8 detergent molecule in 3.5σ $mF_o - DF_c$ difference electron density (blue) is shown plugging a hydrophobic hole between the N-terminal helix and adjacent enzyme surface. Model structure factors in the 10–2.0 Å range were used after 120 cycles of *X-PLOR* conjugate-gradient positional refinement of the model without detergent. Solvent atoms are not displayed.

over the mixed phosphoform protein used formerly does not seem to be responsible for the additional local order, since ordered phosphate density is not evident. The consistency of this observed detergent structural effect with myristic acid function, the accessibility of this location to the enzyme amino terminus, and the hydrophobic character of the binding site, together suggest that this site binds myristic acid in the mammalian enzyme. Crystallographic work with the mammalian *C*-subunit structure has confirmed this prediction (Zheng, 1993).

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The 2.7 Å coordinates of the corrected mixed-phosphoform recombinant *C* subunit are available from the Brookhaven Protein Data Bank as entry 2CPK. The 2.0 Å coordinates and structure factors for the Ser139Ala mutant crystal have been deposited with the Protein Data Bank.*

*Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1APM, R1APMSF). Free copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Supplementary Publication No. SUP 37077). At the request of the authors, the structure factors will remain privileged until 1 April 1994. A list of deposited data is given at the end of this issue.

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