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Crystallization studies of the catalytic subunit of cAMP-dependent protein kinase: crystals of murine recombinant catalytic subunit and a mutant, Cys 343→Ser, diffract to 2.7 Å resolution

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Abstract. The recombinant mouse catalytic subunit of cAMP-dependent protein kinase, expressed and purified from *E. coli*, has been successfully cocrystallized as a binary complex with an inhibitor peptide and as a ternary complex with an inhibitor peptide and MgATP. In contrast to the catalytic subunit obtained from porcine heart, the recombinant catalytic subunit lacks a myristoyl group at the amino terminus and differs in sequence at nine positions out of 350 amino acids. The catalytic activities of the two enzymes, however, are nearly identical. Both enzymes cocrystallized with a 20-amino-acid inhibitor and MgATP; however, the porcine-heart enzyme crystallized in a hexagonal space group ($P6_122$) while the recombinant murine catalytic subunit crystallized in an orthorhombic space group ($P2_12_12_1$, a = 73.70, b = 76.26, c = 80.74 Å). The orthorhombic crystals of the recombinant catalytic subunit exhibit the best diffraction characteristics of all catalytic subunit crystals obtained so far: 2.7 Å resolution. Unlike the mammalian porcine-heart enzyme, no crystals of the recombinant apo-enzyme were obtained under the same crystallization conditions. These results are consistent with earlier conclusions that the catalytic subunit exists in at least two distinct conformational states and furthermore suggests that the inhibitor peptide alone is sufficient to induce the major conformational changes that distinguish the two states. A mutant form of the catalytic subunit where Cys343 was replaced with Ser was also cocrystallized with the 20-amino-acid peptide inhibitor and MgATP, and resulted in an orthorhombic crystal isomorphous to crystals of the unmutated enzyme with a similar diffraction of 2.7 Å.

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Successful crystallization of proteins and obtaining good heavy-atom derivatives remain among the most difficult and elusive tasks in X-ray crystallography. Recent advances in molecular biology have provided the crystallographer, however, with an array of new techniques that may facilitate both of these tasks. First, both eukaryotic and prokaryotic expression systems offer the possibility of obtaining large quantities of pure protein for extensive crystallization trials (Marston, 1987). Second, the use of prokaryotic expression systems eliminates post-translational modifications, which, in some cases, tend to complicate crystallization experiments. Third, the potential for generating mutant forms of the protein offers greater possibilities for both obtaining high-quality crystals and better understanding of the interaction between structure and function. Protein kinases, for example, represent a large and very diverse family of related enzymes that play major regulatory roles in eukaryotic cells (Hanks, Quinn & Hunter, 1988). Some, such as the catalytic subunit of the cAMP-dependent protein kinase (cAPK), can be purified from mammalian tissues in a homogeneous form in reasonable quantities (Nelson & Taylor, 1981). Others, however, such as protein kinase C, often represent a mixture of different isozyme forms (Coussens, Parker, Rhee, Yang-Feng, Chen, Waterfield, Francke & Ullrich, 1986). Finally, many of the protein kinases, particularly those catalyzing

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Table 1. *Summary of reflection intensities (Y) and R factors by resolution shells*

A total of 26 017 observations of 11 778 reflections were made. Overall R = 4.09%. $R = \sum(I - Y)^2/\sum Y^2$, where Y is the average intensity and I is an individual intensity observation.

Shell lower limit (Å)	Average reflection intensity	Average $Y/\sigma(Y)$	Number of reflections with $Y/\sigma(Y)$ in given range							R factor (%)
			<2	<5	<10	<20	<40	<60	>60	
4.62	955.69	36.563	74	121	176	322	726	656	419	3.23
3.66	931.54	23.935	110	223	321	522	793	401	79	3.41
3.20	495.04	12.065	336	422	595	611	411	65	5	5.43
2.91	260.56	6.351	599	666	664	400	90	0	0	8.40
2.70	176.07	3.997	702	659	405	134	9	0	0	7.07

the phosphorylation of tyrosine residues, are typically expressed in very low quantities. Thus, the development of expression systems is an important first step towards the goal of solving the crystal structure. Unfortunately, many eukaryotic protein kinases can be overexpressed in *E. coli*, but typically in an insoluble and inactive form (Gilmer & Erikson, 1981). The catalytic subunit of cAPK is an exception, since large quantities can be overexpressed in *E. coli* (Slice & Taylor, 1989). The recombinant enzyme is catalytically active although it lacks a myristoyl group at the amino terminus (Slice & Taylor, 1989). This system not only provides a ready source of protein, but also has the potential for generating mutant forms of the enzyme that may, among other things, facilitate solving the three-dimensional structure.

Since eventually one hopes to compare the three-dimensional structure of the free enzyme with the structure containing bound substrate, considerable effort has been devoted in initial studies with the mammalian enzyme to obtaining crystals both of the free enzyme and of the ternary complex containing MgATP and a 20-residue inhibitory peptide, PKI(5–24) (Fig. 1) (Sowadski, Xuong, Anderson & Taylor, 1985; Knighton, Xuong, Taylor & Sowadski, 1991). These crystallization conditions were repeated here using the recombinant catalytic subunit. In addition to lacking a myristoyl group, the recombinant mouse catalytic subunit differs in sequence at nine out of 350 positions (Uhler, Carmichael, Lee, Chrivia, Krebs & McKnight, 1986). Crystals of a ternary complex containing the recombinant catalytic subunit, MgATP, and the PKI(5–24) inhibitor were obtained; however, those crystals, obtained under conditions identical to the mammalian enzyme, were orthorhombic (Zheng, Knighton, Parello, Taylor & Sowadski, 1991). The purified recombinant catalytic subunit was concentrated to 10 mg ml^{-1} and dialyzed at 277 K against 50 m*M* Bicine* buffer (pH = 8.0–8.3), 150 m*M* ammonium acetate and 10 m*M* 2-mercaptoethanol. Crystallization was carried out at 277 K using the standard hanging-drop technique, with the drop consisting of equal volumes of protein solution, reservoir solution and 10 m*M* DTT* with MgATP and PKI(5–24) to yield a molar ratio of protein:inhibitor:ATP:Mg^{2+} equal to 1:1:20:5 and 1:3:20:5. The reservoir contained 10 m*M* DTT and 8% (*w*/*v*) Dow polyethylene glycol 400 (Knighton *et al.*, 1991). Before sealing the cover slip, methanol was added to the reservoir solution to a concentration of 15% (*v*/*v*).

The resulting crystals of the recombinant catalytic subunit complexed with MgATP and PKI(5–24) were in the orthorhombic space group, $P2_12_12_1$. The crystals were examined with a multiwire area detector (Xuong, Nielsen, Hamlin & Anderson, 1985). The unit-cell dimensions of the crystals are a = 73.70, b = 76.26, c = 80.74 Å. The crystals* diffract to 2.7 Å resolution. Assuming 2.7 $Å^3$ $dalton^{-1}$ (Matthews, 1968), there is one molecule per asymmetric unit. Table 1 summarizes the data of the ternary complex crystals.

A ternary complex of the mutant catalytic subunit Cys343→Ser has also been cocrystallized under identical crystallization conditions. Both the space group and the unit-cell dimensions of the resultant crystals are identical to crystals of the unmutated recombinant catalytic subunit.

As Table 1 shows, crystals of the ternary complex of the murine catalytic subunit with PKI(5–24) peptide inhibitor and MgATP diffract the best in comparison with all crystal forms of the catalytic subunit reported so far (Sowadski *et al.*, 1985; Knighton *et al.*, 1991). Its superiority may result from the differences between the murine recombinant catalytic subunit and the porcine-heart catalytic subunit. One of these is that the murine catalytic subunit expressed in *E. coli* is devoid of the hydrophobic myristic acid at the amino terminus. Another difference is that the murine recombinant enzyme differs by nine amino acids from the porcine-heart enzyme. Most changes happened in the small lobe on the surface. None of these amino acids occur in the regions that are conserved in the entire family of over 150 protein kinases (Hanks *et al.*, 1988). Why changes in these positions, as well as the lack of myristic acid, would have caused the cocrystallization of the catalytic subunit in a different space group

* Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] (Aldrich); DTT, Cleland's reagent (*threo*-1,4-dimercapto-2,3-butanediol, dithiothreitol) (Aldrich).

* It has been observed that the crystals of the ternary and binary complexes exhibit variability in unit-cell dimensions depending on enzyme preparation. These changes in unit-cell dimensions exceeded 0.5%. Analysis of both purification protocols and unit-cell dimensions of the crystals indicates that it is most likely that changes in the eluting buffers occurring from preparation to preparation are the primary reasons for this apparent 'non-isomorphism' of the native crystals of the ternary complex.

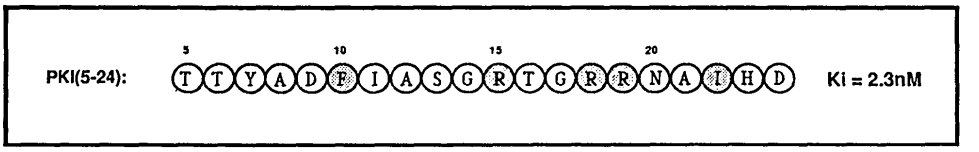

Fig. 1. The amino-acid sequence of the specific peptide inhibitor PKI(5–24) of the catalytic subunit derived from a potent thermostable naturally occurring protein kinase (PKI) inhibitor described by Walsh and his colleagues (Cheng, Kemp, Pearson, Smith, Misconi, Van Patten & Walsh, 1986). The shaded circles represent the residues identified as important residues for peptide binding.

is not immediately apparent at this time. The enzymatic activity of both porcine-heart and recombinant murine catalytic subunits is nearly identical. However, the enzymes differ in thermostability with the murine catalytic subunit having a 5 K lower temperature transition (T_m) than the porcine-heart enzyme (Yonemoto, McGlone, Slice & Taylor, 1992).

Preliminary results at this time using small-angle neutron scattering (SANS) to measure the radius of gyration (R_g) demonstrate that the free recombinant catalytic subunit and the ternary complex represent distinct conformational states that differ by approximately 1.0 Å in R_g. The results showed, furthermore, that PKI(5–24) alone was sufficient to induce this change (Parello, Timmins, Sowadski & Taylor, 1992). On the basis of these results the recombinant catalytic subunit was crystallized as a binary complex using the same identical conditions described above, but omitting MgATP. The binary complex crystallized in a form that was nearly isomorphous to the ternary complex; space group $P2_12_12_1$, a = 73.62, b = 76.52, c = 80.14 Å.

Since all four crystal forms reported so far have been obtained under identical crystallization conditions, except for the monoclinic crystal form of porcine-heart free catalytic subunit, it is possible to correlate more extensively the crystallographic results with those obtained through SANS studies.

The present crystallization studies show that both the ternary and the binary complex of murine recombinant catalytic subunit crystallize in the same space group. Since the crystallization conditions for both these complexes were identical, it is likely that the enzyme exists in the same major conformational state with or without MgATP. Consequently, since the R_g values for both of these complexes were identical, it is likely that the enzyme exists in the same major conformational state with or without MgATP. Therefore, the binding of PKI(5–24) alone is responsible for identical crystal packing. This is in agreement with the SANS experiments recently reported by Parello *et al.* (1992) showing that both the ternary and binary complexes of the murine recombinant catalytic subunit display the same value for their radii of gyration. Both results strongly suggest similarities between the conformational states of the enzyme in the ternary and binary complexes.

Furthermore, the SANS studies have shown that in the absence of PKI(5–24) and MgATP, the enzyme exhibits a somewhat more elongated shape than that adopted by the enzyme in both ternary and binary complexes. The decrease in R_g by 0.9 from 20.0 Å upon binding of PKI(5–24) very likely represents a significant conformational change of the enzyme molecule (Parello *et al.*, 1992). Although the crystals of the apo-enzyme recombinant catalytic subunit have not been obtained, previous studies have shown (Knighton *et al.*, 1991) that under identical crystallization conditions, addition of the PKI(5–24) and MgATP into the bovine-heart catalytic subunit results in the changing of the space group from cubic $P4_132$, representing the apo-enzyme, to hexagonal $P6_122$, representing the ternary complex. Thus, both crystallographic results, the one previously reported and the one presented now, would suggest, along with the SANS studies, a strong possibility that under identical crystallization conditions the catalytic subunit exists in at least two distinct conformational states and that the inhibitor peptide alone is sufficient to induce the major conformational changes that distinguish the two states.

In the case of hexokinase, a decrease of 0.9 Å in R_g was found as a consequence of glucose binding. This change was well documented by comparing two crystal structures of hexokinase – the free enzyme and the binary complex containing bound glucose (Fletterick, Bates & Steitz, 1975; Bennett & Steitz, 1978; Anderson, Zucker & Steitz, 1979; McDonald, Steitz & Engelman, 1979). This glucose-induced conformational change represents the closing of a cleft between two lobes and this type of substrate-induced conformational change is predicted to occur in a number of kinases that act on small molecules. In a similar way, a decrease in R_g of 0.9 Å upon binding of the PKI(5–24) peptide inhibitor might well represent a major conformational change of the enzyme which undergoes a transition from an open form (free enzyme) to a more compact (closed) form in the presence of PKI(5–24). Although the magnitude of the SANS changes seen with the catalytic subunit are analogous to those observed for hexokinase, it should be emphasized that the substrate here is very large (20 amino acids) compared to glucose, so that the actual changes in the protein may be even more pronounced than those seen for hexokinase.

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References

ANDERSON, C. M., ZUCKER, F. M. & STEITZ, T. A. (1979). *Science*, **204**, 375–380.

BENNETT, W. S. & STEITZ, T. A. (1978). *Proc. Natl Acad. Sci. USA*, **75**, 4848–4852.

CHENG, H.-C., KEMP, B. E., PEARSON, R. B., SMITH, A. J., MISCONI, L., VAN PATTEN, S. M. & WALSH, D. A. (1986). *J. Biol. Chem.* **261**, 989–992.

COUSSENS, L., PARKER, P. J., RHEE, L., YANG-FENG, T. L., CHEN, E., WATERFIELD, M. D., FRANCKE, U. & ULLRICH, A. (1986). *Science*, **233**, 859–866.

FLETTERICK, R. J., BATES, D. J. & STEITZ, T. A. (1975). *Proc. Natl Acad. Sci. USA*, **72**, 38–42.

GILMER, T. M. & ERIKSON, R. L. (1981). *Nature (London)*, **294**, 771–773.

HANKS, S. K., QUINN, A. M. & HUNTER, T. (1988). *Science*, **241**, 42–52.

KNIGHTON, D. R., XUONG, N. H., TAYLOR, S. S. & SOWADSKI, J. M. (1991). *J. Mol. Biol.* **220**, 217–220.

MCDONALD, R. C., STEITZ, T. A. & ENGELMAN, D. M. (1979). *Biochemistry*, **18**, 338–342.

MARSTON, F. A. O. (1987). *DNA Cloning: a Practical Approach*, Vol. 3, edited by D. M. GLOVER, p. 59. Oxford: IRL Press.

MATTHEWS, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

NELSON, N. C. & TAYLOR, S. S. (1981). *J. Biol. Chem.* **256**, 3743–3750.

PARELLO, J., TIMMINS, P. A., SOWADSKI, J. M. & TAYLOR, S. S. (1992). *J. Mol. Biol.* In the press.

SLICE, L. W. & TAYLOR, S. S. (1989). *J. Biol. Chem.* **264**, 20940–20946.

SOWADSKI, J. M., XUONG, N. H., ANDERSON, D. & TAYLOR, S. S. (1985). *J. Mol. Biol.* **182**, 617–620.

UHLER, M. D., CARMICHAEL, D. F., LEE, D. C., CHRIVIA, J. C., KREBS, E. G. & MCKNIGHT, G. S. (1986). *Proc. Natl Acad. Sci. USA*, **83**, 1300–1304.

XUONG, N. H., NIELSEN, C., HAMLIN, R. & ANDERSON, D. (1985). *J. Appl. Cryst.* **18**, 342–350.

YONEMOTO, W., MCGLONE, M. M., SLICE, L. W. & TAYLOR, S. S. (1992). *Methods Enzymol.* In the press.

ZHENG, J., KNIGHTON, D. R., PARELLO, J., TAYLOR, S. S. & SOWADSKI, J. M. (1991). *Methods Enzymol.* **200**, 508–521.