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Solution Structure of the cAMP-Dependent Protein Kinase

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Abstract

This is the final report of a three-year, Laboratory Directed Research and Development (LDRD) project at Los Alamos National Laboratory (LANL). Protein phosphorylation is well established as one of the most important mechanisms of signal transduction and cellular regulation. Two of the key enzymes that catalyze these phosphorylation reactions are the cAMP- (PKA) and cGMP- (PKG) dependent protein kinases. PKA has served as the prototypic model of this class of enzymes that now comprises in excess of 300 phylogenetically related proteins. A large number of these protein kinases are critical for the regulation of cell function and a full analysis of their similarities and differences is essential to understand their diverse physiological roles. The cAMP-dependent protein kinase has the subunit structure R2C2, in which C and R refer to the catalytic and regulatory subunits, respectively. The cGMP-dependent protein kinase (PKG) is highly homologous to PKA but is distinguished from it by having the regulatory and catalytic domains on a contiguous polypeptide. The studies described here use small-angle scattering and Fourier Transform InfraRed (FTIR) spectroscopy to study domain movements and conformational changes in these enzymes in different functional states in order to elucidate the molecular bases for the regulation of their activities.

Background and Research Objectives

Understanding the molecular basis for biochemical regulation is a fundamental problem in human medicine. A multitude of events must be switched on, switched off, accelerated or decelerated in response to a myriad of signals that have to be correctly processed and responded to for normal healthy function. Unregulated or uncoordinated activities generally lead to pathological conditions and, if not corrected, to uncontrolled proliferation or cell death. This project focused on studies of the cyclic adenylic acid (cAMP)-dependent protein kinase, which has served as the central model for modulation of cell function by protein phosphorylation, one of the most important mechanisms of cell

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regulation. Protein phosphorylation by the cAMP-dependent protein kinase controls a broad diversity of cellular activities that includes cell proliferation and the cell cycle, energy availability and its distribution, transcription and translation of macromolecules, muscle contraction, cell movement and aggregation, secretion of macromolecules, ion transport into and out of subcellular compartments and cells, and the ionic milieu with which the cell is surrounded. Understanding the molecular (structural) mechanisms underlying the activity of this protein kinase is central to understanding how it regulates cell function.

Since its discovery twenty five years ago by one of the principal investigators of this project, the cAMP-dependent protein kinase, a key enzyme that catalyzes many phosphorylation reactions, has served as the model enzyme of a class that now comprises in excess of 350 phylogenetically related proteins.[1] cAMP is a cyclic adenosine nucleotide, and its activation of the cAMP-dependent protein kinase is the mode whereby it acts as a "second messenger" of protein hormones. The cAMP-dependent protein kinase has the subunit structure R_2C_2, in which C and R refer to the catalytic and regulatory subunits, respectively. The R_2C_2 holoenzyme is catalytically inactive, whereas C subunit alone is fully catalytically competent. The three-dimensional structure of C in combination with a pseudosubstrate molecule PKI (protein kinase inhibitor) has been solved by x-ray crystallography[2] and PKI has also served to determine the nature of interaction between protein substrates and the catalytic subunit.[3,4] The dimeric R subunit contains two binding sites for cAMP (i.e., 4 per R_2 dimer) and at least two distinct sites for interaction with the catalytic subunit, one of which is pseudosubstrate in nature.[5] cAMP binding causes a conformational change in the R subunit and the holoenzyme that leads to enzyme activation. In the presence of cAMP even relatively mild physical methods such as gel filtration and ion exchange chromatography lead to separation of the subunits, from which has come the proposal that cAMP-dependent activation of the protein kinase is a consequence of dissociation of the subunits according to the equation

$$R_2C_2 + 4\,cAMP \rightleftarrows R_2\,cAMP_2 + 2\,C \ .$$

The long-term objectives of this collaborative project were to use molecular biology and biochemistry with advanced physical techniques such as small-angle x-ray and neutron scattering with Fourier Transform Infrared (FTIR) spectroscopy to determine the solution structures of the cAMP-dependent protein kinase and its subunits, and to understand in detail how the subunit structures are modulated to achieve their function.

Importance to LANL's Science and Technology Base and National R&D Needs

The studies presented depend upon a unique combination of biotechnology and biochemistry at the University of California at Davis (UCD), and advanced physical techniques that are available at Los Alamos National Laboratory (LANL). The project addresses the underlying molecular principles of biochemical regulation, which is critical to healthy function. The project strengthens our capabilities in structural biology (scattering, spectroscopy and isotope chemistry) and hence strengthens the Laboratory's core competency of bioscience and biotechnology. The project also contributes to the Neutron Laboratory tactical goal of the Laboratory by highlighting our neutron scattering capabilities in relation to an important biomedical application.

Scientific Approach and Accomplishments

Our approach was to use FTIR spectroscopy and small-angle scattering to study the cAMP-dependent protein kinase holoenzyme and its subunits. In order to make the large amounts of pure material needed for these measurements, recombinant technology was developed to produce pure samples of R and C subunits. Small-angle scattering gives information on the conformational shapes of proteins in solution, essentially defining molecular boundaries, while FTIR spectroscopy gives information on the secondary structural element within those molecular boundaries. The parameters that can be determined by small-angle scattering include the distance distribution function P(r) (which is simply the frequency of interatomic distances within the scattering particle), the value of the maximum linear dimension (d_{max}), the radius of gyration (R_g), and the molecular weight of the scattering species. By combining the scattering data with data from other techniques such as FTIR, crystallography and/or NMR, one can develop models of structures for testing. This approach has been used successfully by us with proteins such as phosphorylase kinase,[6] calmodulin,[7,8] and troponin C.[9,10]

Small-angle scattering can be done using neutrons or x-rays. Neutron scattering has the unique capability of distinguishing between different components of a complex using isotope labeling. Unlike x-rays, neutrons are scattered by atomic nuclei, and as a result different isotopes of the same element can have very different neutron scattering properties. This is particularly so for hydrogen and deuterium. Substituting deuterium for hydrogen can dramatically alter the scattering density of a molecule. Neutron scattering signal

strength from a protein in solution depends upon the magnitude of the difference between the neutron scattering density of the protein and the solvent (i.e., the contrast). Thus by manipulating the deuterium content of a protein or of the solvent ("contrast variation") one can vary the neutron scattering signal strength. Solvent matching refers to the case when the neutron scattering density of the dissolved particle equals that of the solvent, thus making the particle "invisible" in the neutron experiments. Neutron scattering from a complex formed between deuterated and non-deuterated components, and dissolved in solvents containing different D_20:H_2O ratios, gives information on the structures of the individual deuterated and non-deuterated components. In addition, the contrast dependence of R_g gives information on the relative dispositions of the components. Examples of these types of studies are described in Trewhella et al.[7-10] To do these experiments for the cAMP-dependent protein kinase, both R and C must be expressed in E. coli ingrown on deuterated algal hydrolysate and D_20 as we have described previously for the preparation of deuterated calmodulin and troponin C.[6,7,9] Using the protein obtained by these procedures we can reconstitute the holoenzyme with one or the other of the subunits deuterated.

The combination of FTIR spectroscopy, x-ray and neutron scattering will provide evidence of the nature of the conformational changes induced by cAMP in the overall complex and its subunits, and will give direct information on the dissociation mechanism underlying the function of this protein kinase.

X-ray and neutron scattering and FTIR spectroscopic investigations have allowed a determination of the conformational change of the catalytic subunit that occurs upon the binding of the pseudosubstrate PKI.[11] The three-dimensional crystal structure of the C subunit shows that its catalytic core consists of two lobes held together by one strand of polypeptide.[2] Our FTIR studies confirmed that the secondary structure of C was unchanged by the binding of PKI, whereas both x-ray and neutron scattering showed a significant reduction in both the d_{max} and R_g of C. We were able to show the binding of PKI thus causes closure of the cleft between the two lobes around the protein kinase substrate site as a consequence of movement about a hinge at a specific glycine residue. This provides a model for the molecular mechanism of C-subunit activation that can now be tested. Sequence analysis of all known protein kinases showed that this glycine was absolutely conserved in a number of branches on the kinase phylogenetic tree thus providing additional insight into a fundamental underlying mechanism common to many kinases related through evolution.

We also used FTIR spectroscopy and small-angle x-ray scattering to investigate the structural determinants in the sequence of the PKI pseudosubstrate peptide.[12] We were able to show that the PKI peptide in solution preserved the helix and turn structures that are

seen when it binds the C-subunit. By preparing PKI peptides in which specific amino acid residues were substituted for residues with similar, opposite or neutral properties, we were able to shed light on which amino acids are key to allowing the PKI peptide to fold into its bound conformation and are critical to the high affinity interaction with the C-subunit.

A key long-term objective of this work is to use neutron scattering to determine the structures of the R- and C-subunits in the complex and to understand how to modulate their conformation to achieve function. These experiments depend upon molecular biology and biochemistry development that allows reconstitution of the holoenzyme from purified components in which individual subunits are deuterated. Thus a major accomplishment of this project has been the development of this technology and the preparation of proteins. For the catalytic subunit, the full length cDNA (Slice and Taylor[13]) was inserted into the pRSET plasmid and expressed in E. coli BL21 under control of the T7 promoter. For the regulatory subunit, the full length cDNA for the RII subunit (Scott et al.[5]) was inserted into pETII-d plasmid and expressed in E. coli BL21 (DE3). Both of these proteins have been obtained in milligram quantities and purified to near homogeneity. In the case of the R-subunit, we have also succeeded in producing it in deuterated form. From the R- and C-subunits produced using these expression systems, we reconstituted the holoenzyme and purified to homogeneity by a sequence of association-dissociation steps. We have measured x-ray scattering data that show the reconstituted kinase is well behaved in solution and not aggregated. These data show the holoenzyme is a highly asymmetric structure, with an almost disk-like shape. We have neutron beam time in the December '95–March '96 time frame to measure the neutron scattering data from samples of reconstituted holoenzyme with the R-subunit deuterated, both with and without cAMP bound.

Expression of the monomeric regulatory subunit has also been initiated so that we can prepare samples with a single C- and R-subunit, with the R-subunit deuterated to provide further detail on the nature of the R- and C-subunit interactions from neutron scattering measurements. The cDNA/pETII construct designed for expression of the RII was mutated using a PCR strategy to eliminate the first 45 nucleotides of the coding sequence that should yield (des15)-R, which should be unable to dimerize. The modified plasmid has been transformed into HMS174 bacterial hosts for initial screening. Upon determination of recombinants, the DNA construct will be retransformed into BL21 (DE3) lysogens for protein expression. With expression of (des15)R we will then be able to construct monomeric holoenzyme [(des15)R-C], which will remain monomeric because it lacks the amino terminal regulatory subunit dimerization domain.

Conclusions

It is expected that the work done on this project will lead to an elucidation of the solution structure of the cAMP-dependent protein kinase and of topographical interactions of the R- and C-subunits, and thus will provide important insights into the molecular basis of its biochemical function. This is of central importance to an understanding of the mode whereby hormonal actions are mediated by cAMP. The shorter term accomplishments reported here give us a model for the activation of the catalytic (C-)subunit of cAMP-dependent protein kinase, and the capability to prepare large scale amounts of purified subunits deuterated and/or non-deuterated for reconstitution into functional holoenzyme position us to succeed in the longer term goals of elucidating the shapes and dispositions of the catalytic (C-) and regulatory (R-)subunits in the holoenzyme and how their conformations are modified to facilitate function. On the basis of the work done with this project, we have submitted a joint Los Alamos/UC Davis NIH R01 proposal ("The Solution Structures of the cAMP- and cGMP-Dependent Protein Kinases") that addresses these longer term goals, not only for the cAMP-dependent protein kinase but for the structurally and functionally related cGMP-dependent protein kinase.

Publications

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