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ARTICLE

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Structural themes and variations in protein kinase A as seen by small-angle scattering and neutron contrast variation

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Abstract Using small-angle solution scattering and neutron contrast variation, we have studied the structure of the multi-subunit protein kinase A. We have gained insights into how nature can take a set of common structural domains (or themes) and modulate their interactions via sequence variations and second messenger mediated signaling to affect enzyme activity and receptor binding important for targeting this multi-function enzyme to specific sub-cellular locations. These studies demonstrate the power of neutron contrast variation to expand our knowledge of the dynamic supra-molecular structures that carry out biological function.

Keywords Protein structure · Protein kinase A · Isoforms · Neutron contrast variation · Small-angle scattering

Introduction

The essential processes of life are accomplished via the interactions of biological macromolecules as they carry out highly regulated and coordinated functions. The resultant dynamic interactions are not easily studied by traditional crystallography, NMR or electron microscopy. Small-angle solution scattering with neutron contrast variation can contribute unique structural data on supra-molecular structure, and in cases where there are structural data available on the individual components, fill critical gaps in our knowledge.

Protein phosphorylation is the most wide-spread protein modification used for modulating function. The phosphorylation step involves the transfer of a phosphate group from nature's principal carrier of bio-chemical energy, ATP, to a target protein. This step is catalyzed by protein kinases. Protein kinase A (PKA) is a multi-subunit kinase that is ubiquitous in mammalian cells. Phosphorylation of its targets is known to be critical for regulating a many cellular processes including metabolism, gene transcription, ion flux, growth, cell death (Walsh et al. 1992) as well as multi-cellular processes such as learning and memory (Kandel 2001). PKA activity is regulated by the second messenger cAMP. The so-called "second messengers" inside cells are small, diffusible molecules or ions that are released as a result of extra-cellular signals and elicit the needed cellular response by binding to effector proteins such as the regulatory subunit of PKA.

In its inactive form, PKA exists as a dimer of dimers (R_2C_2). The catalytic (C) subunits are responsible for catalyzing the phosphoryl transfer reaction while the regulatory (R) subunits confer cAMP dependence and localize the holoenzyme to specific locations within the cell (Tasken and Aandahl 2004). There are several functional domains in the R subunits; a dimerization domain that is also the site for binding to A Kinase Anchoring Proteins (AKAPs) is at the N-terminus while the C-terminus has the two tandem cAMP-binding domains. Between these domains is a linker region that includes an inhibitor sequence that binds to the active site of C and inhibits substrate binding and hence catalysis. There are a number of isoforms of PKA that localize PKA to different parts of the cell. They differ in their regulatory subunits; designated RIα, RIβ, RIIα and RIIβ. The cAMP-binding domains among these R isoforms are highly conserved (Canaves and Taylor 2002), while the linker regions show considerable variation (Vigil et al. 2004a). Despite a conserved domain organization and many similar biochemical properties, the R subunit isoforms have quite distinct biological roles as evidence by gene knock out studies (Amieux and McKnight 2002, Cummings et al. 1996, Rao et al. 2004).

High-resolution structures of C subunit (Knighton et al. 1991), isolated cAMP-binding domains of RIα

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(Su et al. 1995) and RIIβ (Diller et al. 2001), and isolated dimerization domains of RIIα (Newlon et al. 1999) and RIα (Banky et al. 2003) have provided important molecular insights into the phosphoryl transfer reaction, cAMP binding, and tethering to AKAPs, respectively. There is also a crystal structure for a complex between the C subunit and a deletion mutant of RIα that includes the inhibitor sequence and one cAMP-binding domain (Kim et al. 2005). However, there are currently no high-resolution structures of full-length R subunit homodimers or holoenzyme complexes; it has been speculated that the inability to crystallize these molecules is due to the inherent flexibility of the linker regions. Small-angle X-ray and neutron scattering with contrast variation have enabled us to expand our knowledge of the structures this complex enzyme adopts in solution and thus gain insights into the structural features that facilitate its activation and targeting.

Experimental methods

Small angle- scattering

Small-angle scattering occurs when radiation passes through a sample containing discrete particles, such as proteins in a solution, whose dimensions are large in comparison to the wavelength of the incident radiation and whose scattering density differs from that of their solvent (i.e., there is "contrast") (for reviews see Trewhella 1997 and Wall et al. 2000). The angular dependence of the small-angle scattering gives information about the particle's shape and size. For a dilute solution of mono-disperse, identical particles, the scattered intensity $I(Q)$ can be expressed in the form of a Fourier transform:

$$I(Q) = 4\pi \int P(r) \frac{\sin Qr}{Qr} \mathrm{d}r \qquad (1)$$

where $Q = (4\pi \sin\theta)/\lambda$ is the magnitude of the scattering vector (also known as the momentum transfer), θ is half the scattering angle, and λ the wavelength of the incident radiation. $P(r)$ is the frequency distribution of all interatomic distances within the scattering particle weighted by the product of the scattering powers of each respective pair of atoms and goes to zero at the maximum linear dimension, $d_{\max}$. $P(r)$ contains real space information about the general shape of the scattering particle and the scattering density distribution within its boundaries and is calculated as the inverse Fourier transform of $I(Q)$. The zeroth moment of $P(r)$ gives the forward scatter, $I(0)$, which is related to the mass of the scattering particle, and hence gives information on the association state of the molecules in a solution. The second moment of $P(r)$ gives R_g which is the root-mean-square of all elemental scattering volumes from their center-of-mass and is a simple but convenient measure of the relative asymmetry of the scattering particle. One can also obtain an estimate of the molecular volume from $I(Q)$.

Contrast variation

X-rays are electromagnetic radiation and as such are scattered principally by the electrons in a sample. Hence electron scattering power is proportional to the number of electrons in an atom, and increases steadily with increasing atomic number. Neutrons are neutral particles that interact with the atomic nuclei, and neutron scattering lengths depend on the properties of the compound nucleus formed when the neutron and nucleus interact. Neutron scattering lengths thus vary randomly with atomic number and different isotopes of the same element can have very different neutron scattering properties. With the exception of hydrogen, all the neutron scattering lengths for atoms typically found in proteins are positive and similar in magnitude. The scattering length for hydrogen is negative resulting from a resonance effect that gives rise to a 180° phase change upon scattering from hydrogen. Neutron scattering density is calculated by adding the scattering lengths of each atom in a volume element and dividing by that volume. Hence by substitution of hydrogen for deuterium one can dramatically change the neutron scattering length density for a specific component in a system, i.e., you can vary its contrast and hence its relative contribution to the small-angle scattering signal. In a complex of two or more proteins, one can manipulate the relative scattering density of protein components by replacing hydrogen with deuterium. Assuming internal scattering density fluctuations are negligible, the total scattering from a complex of a deuterated (D) and non-deuterated protein (H) in any solvent can be written as:

$$I(Q, \Delta\rho_{\mathrm{D}}, \Delta\rho_{\mathrm{H}}) = \Delta\rho_D^2 I_{\mathrm{D}}(Q) + \Delta\rho_{\mathrm{D}}\Delta\rho_{\mathrm{H}} I_{\mathrm{DH}}(Q) + \Delta\rho_H^2 I_{\mathrm{H}}(Q) \qquad (2)$$

$\Delta\rho_{\mathrm{D}}$ is the contrast for the deuterated component and equals $\rho_{\mathrm{D}} - \rho_{\mathrm{S}}$ where ρ_{D} is the mean scattering density for the deuterated protein and ρ_{S} is that for the solvent. A similar definition holds for $\Delta\rho_{\mathrm{H}}$. The three basic scattering functions in eq. 2 are $I_{\mathrm{D}}(Q)$ and $I_{\mathrm{H}}(Q)$, representing the scattering due to the individual components in the complex, and $I_{\mathrm{DH}}(Q)$ which is the cross-term. A contrast variation study is done by measuring neutron scattering data from a complex containing a deuterated components in solutions with different ratios of D_2O:H_2O. Each data set gives an equation of the form of eq. 1 which can then be solved for the basic scattering functions from which structural parameters [R_g, $d_{\max}$, and $P(r)$] for the overall complex and for the deuterated and non-deuterated components can be calculated. The cross term I_{DH}(Q) is gives information on the distance between the deuterated and non-deuterated components.

The interpretation of scattering data in terms of structural parameters for an individual molecule

depends upon being able to prepare samples of monodisperse, identical molecules. Even small amounts of aggregate or large molecular weight contaminants can significantly bias the data. In addition, one needs to be able to approximate the conditions of dilute solution. If there is conformational heterogeneity on the samples, the scattering data will yield the time and ensemble average of the conformations present. If the rigorous sample requirements for small-angle scattering are met, and the interpretation of the scattering data in terms of useful three-dimensional models is approached with great care, the precise structural parameters and general shape information derived from small-angle X-ray and neutron scattering are very powerful constraints for structural modeling. If there are crystal or NMR structures available for individual domains or subunits of supramolecular complexes, then models can be built, tested, and optimized against the scattering data.

Results

Neutron contrast variation studies of PKA, in combination with X-ray scattering data, have revealed important details of the enzymes regulatory and catalytic subunit interactions and the remarkable structural diversity in the different isoforms of PKA, in spite of its having high sequence and structural homology as well as a common domain organization within its sequences. The figure summarizes some of what we have learned so far of how the different isoforms of PKA organize this common set of domains into different global structures. Among the type 1α, IIα, and IIβ R_2 homodimers only the type Iα homodimer has a well-defined structure in solution (top Fig. 1) (Vigil et al. 2004a). Both of the type II homodimers show evidence of structural flexibility in the form of expanded apparent volumes calculated from the scattering data. The type Iα homodimer has a distinctive lobster shape, with the body formed by the dimerization domains and the claws by the cAMP-binding domains. Using neutron contrast variation from type Iα PKA holoenzyme in which the R subunit was deuterated, we showed that the R_2 homodimer undergoes a conformational change upon C subunit binding such that the cAMP-binding domains move apart and one C subunit binds to each in such a manner that the C subunits do not interact (second from top Fig. 1) (Heller et al. 2004). Additional neutron contrast variation (Zhao et al. 1998) and X-ray solution scattering studies (Vigil et al. 2006) of the type-IIα and type-IIβ holoenzymes have shown that C-subunit binding to the R_2 homodimers for these isoforms reduces their conformational flexibility in each case, but the resulting spatial arrangements of their respective domains results in dramatically different global shapes. The type IIα holoenzyme is highly extended (third from top Fig. 1), while the type IIβ is much more compact (bottom Fig. 1). The compact molecular shape observed in the scattering data for the RIIβ isoform can only be achieved if there are contacts between the R subunit linker sequences and cAMP and dimerization domains of R as well as the C subunit. In contrast the more open RIα subunit can only form one of two of these contacts given its more open and extended shape and there is evidence that this isoform is conformationally flexible.

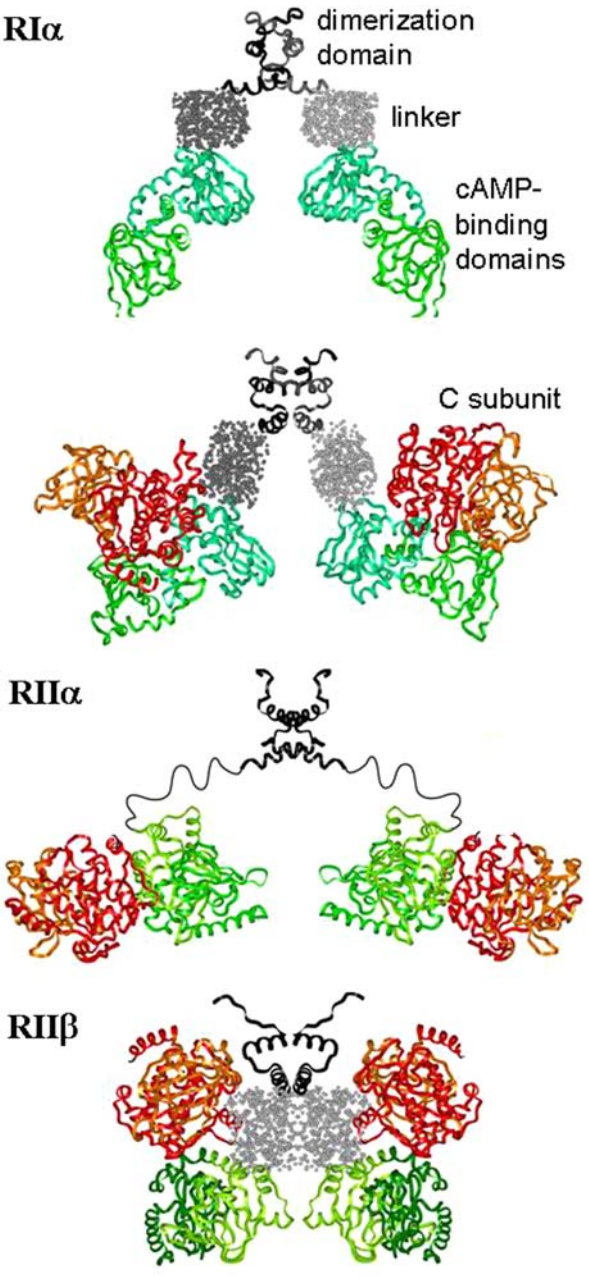

Fig. 1 Structures of three isoforms of PKA. *Top panel* homodimer and holoenzyme structures for RIα, *middle and lower panels* RIIα and RIIβ holoenzyme structures. Relative scaling of models is approximate. In all models, the catalytic cleft of the C subunit is between its small mostly β-structure lobe, shown in *orange*, and large mostly α-helical lobe, shown in *red*. The dimerization domain of the R subunit is *black* and the linker is represented by *gray spheres* that fill the volume of the representative cylinders. The cAMP-binding domain A is *light green*, and domain B is *dark green*

These dramatic differences in global structures among the homodimers and holoenzymes of PKA are attributed to sequence variations in the R subunits in the linker regions between the more highly conserved cAMP binding domains and the dimerization domain that facilitate different arrangements of the domains and result in very different overall shapes. The different shapes give rise to differences in binding surfaces and conformational dynamics that presumably optimize their interactions as needed for sub-cellular targeting via interactions with their respective AKAPs.

Neutron and X-ray scattering studies of R deletion mutants missing their dimerization domains (ΔR) complexed with C subunit have shown that there is yet another layer of structural complexity in R-C interactions (Zhao et al. 1998; Tung et al. 2002; Vigil et al. 2005). Studies of the heterodimeric ΔRIIβC and ΔRIαC complexes have shown that the RIα in its complex with C is significantly more extended than it is as a free subunit and also compared to RIIβ in its complex. Based on the crystal structures of RIα (Knighton et al. 1991) and the complex of the cAMP binding domain A of RIα with C (Kim et al. 2005), we have been able to interpret this difference in terms of a large conformational transition in the helix that connects the two cAMP binding domains upon binding C that results in one cAMP binding domain being pushed away from the C subunit such that it is almost fully solvent exposed. In contrast the RIIβ isoform when complexed with C forms a very compact structure in which both cAMP binding domains have extensive contacts with C.

When two cAMP molecules bind to each R subunit of PKA, the R-subunit's pseudosubstrate is released by C and substrate binding and modification can proceed. Using small-angle scattering and neutron contrast variation on the type IIα holoenzyme and its subunits (Olah et al. 1993; Zhao et al. 1998) we showed that the pseudosubstrate release results in an opening of the C subunit catalytic cleft that would facilitate substrate binding. The pseudosubstrate release upon binding of cAMP weakens the R-C interaction. The early dogma of PKA activation said that the C subunits fully dissociated from the R_2 homodimer. In a set of small-angle X-ray scattering experiments designed to address the question of PKA dissociation (Vigil et al. 2004b), we monitored the zero–angle scattering from solutions of PKA holoenzyme to which increasing amounts of cAMP and substrate were added. In this manner we were able to quantify the extent of PKA dissociation for different isoforms. For the type-Iα isoform, a 1000-fold excess of cAMP leaves about 40% holoenzyme and it is only upon addition of cAMP plus the peptide substrate that the holoenzyme almost fully dissociates. In the case of the type-IIβ isoform, 1000-fold excess cAMP and peptide substrate still leaves about 20% of the holoenzyme intact. These measurements, done under equilibrium conditions and without protein modifications, showed unequivocally that cAMP alone is not sufficient to cause PKA to fully dissociate, in agreement with a body of fluorescence, biotin labeling, and FPLC data that have been developing (Johnson et al. 2000, Kopperud et al. 2002, Yang et al. 1995). Further, the equilibrium between R-C association and dissociation in the presence of cAMP and substrate varies among the isoforms. These results thus contributed to the overturning of the early dogma and added new information about the complexity of PKA regulation.

Discussion

The information content in a solution scattering experiment is inherently low resolution, but the structural parameters are both accurate and precise. They are therefore very valuable constraints for structural modeling of complexes and for assessing molecular associations. In general, the most valuable small-angle scattering experiments address systems for which high resolution structural data are available, either on components of complexes, or on the system in one state such that questions can be asked about structural changes induced by a change in state. In these cases, computational modeling, combined where possible with additional structural constraints (e.g., from mutagenesis, cross-linking, fluorescence data, etc) can be very powerful and lead to detailed molecular models that provide a basis for understanding and predicting supramolecular function.

Neutron contrast variation in combination with X-ray scattering studies of the multi-functional protein kinase A (PKA) have revealed details of its regulatory catalytic subunit interactions and the remarkable structural diversity in the different isoforms of this multi-subunit enzyme achieves, in spite of its having high sequence homology and a common domain organization within its sequences. Scattering data from three of the four PKA isoforms show how sequence variations in the sequences linking functional domains in the R subunit facilitate dramatically different spatial arrangements and contacts among the domains and subunits of the holoenzyme. The structural variety seen in the R-C interactions, in the R homodimers, and in the holoenzymes of PKA results in differences in binding surfaces and accessibility that will confer differences in binding affinities, kinetics, and conformational dynamics that are key to understanding the structural basis for the PKA isoforms accomplishing their specific roles in different parts of the cell to which they are targeted via their interactions with the AKAPs. One of the frontiers in structural molecular biology is to understand how multidomain, multisubunit signaling proteins are organized and how they integrate various inputs. Small-angle scattering with neutron contrast variation, in combination with complementary data such as high-resolution structures, sequence analysis, and mutagenesis results will become increasingly important in this regard, especially as most multidomain proteins are notoriously difficult to crystallize.

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