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Detergent Binding to Unmyristylated Protein Kinase A—Structural Implications for the Role of Myristate

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Myristylation often governs the targeting of protein kinases to the plasma membrane. It is now known that a key member of the src family of protein tyrosine kinases, pp60$^{v\text{-}src}$, binds to the lipid bilayer of the plasma membrane via a myristylated amino terminal sequence. The mechanism of this interaction is not known; however, myristic acid (Myristic acid may also be referred to as Myristate) and residues 2 through 14 are also absolutely required (Resh and Ling, 1990). This review presents an analysis of crystal structures of detergent-modified recombinant and myristylated mammalian catalytic subunit of protein kinase A. Crystals of unmyristylated recombinant catalytic subunit of protein kinase A are grown in the presence of Mega 8, a glucamide-type of detergent, and only this detergent binds, which results in a resolution extension (Knighton *et al.*, 1991a). Comparisons of these two structures reveal that the detergent association with the recombinant enzyme binds in exactly the same hydrophobic pocket of the protein occupied by myristic acid in the mammalian protein (Karlsson *et al.*, 1993; Zheng *et al.*, 1993a). Removal of the detergent through soaking results in the local unwinding of the first helix, helix A, and disorder of the canonical recognition sequence of the phosphorylation site, Ser 10 (Zheng *et al.*, 1993b). These results suggest that anchoring the myristic acid inside the protein results in formation of a stable structural template, which includes the myristylated amino terminal sequence important for the recognition by protein kinases. This "inside out" motif might provide a structural paradigm for the recognition of myristylated proteins, including pp60$^{v\text{-}src}$.

KEY WORDS: Protein kinase A; protein phosphorylation; myristylated amino terminal; detergent interactions.

INTRODUCTION

Protein kinases play a key role in cellular signal transduction. These proteins, the largest family of enzymes, consist of a highly conserved catalytic core and a myriad of regulatory N- and C-terminal regions differentiating the modes of regulation of these enzymes (Hanks and Quinn, 1991). Specificity of these enzymes is often governed by their localization within the cell. Most protein kinases are also phosphoproteins, hence a reversible modification like phosphorylation is important for protein kinase activity. Equally as important are irreversible modifications, which define the destination of individual protein kinases. Among the irreversible modifications such as glycosylation and myristylation, the latter has been a focus of research concerning the src-subfamily. Myristylation is catalyzed by the enzyme N-myristyl transferase, which is specific for an N-terminal glycine. There are several important tyrosine kinases in the src subfamily and many of them are believed to be myristylated. The most extensively studied member has been pp60$^{v\text{-}src}$ and the association of this oncogenic protein kinase with the membrane is essential for its transforming

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ability (Cross *et al.*, 1984). Mutation of Gly 1, which results in the elimination of myristic acid, leads to the release of $pp60^{v\text{-}src}$ into the cytoplasm with severe physiological consequences (Kamps *et al.*, 1985). Through the study of the src family it is clear that there is no homology with regard to the N-terminal segments consisting of myristic acid. The sequence consists of only one common amino acid for all myristylated proteins including the src subfamily:Gly 1. Chimeric proteins with a sequence encompassing the myristylation site in src were shown to be incorporated into the membrane only if a specific segment of the protein adjacent to the Gly 1 was present (Brooks-Wilson *et al.*, 1989). Replacement of those adjacent sequences by importing sequences from other myristylated proteins resulted in a src which did not bind to the membrane, despite the presence of myristic acid. It is known from the work of a number of laboratories that myristic acid, Gly 1, and amino acids 2 through 14 constitute a membrane-binding region (Resh, 1990). Although the structure of src is not available, there is no reason to assume *a priori* that myristic acid must be embedded in the lipid bilayer since this fatty acid has also been found to be an integral part of the polio virus' three-dimensional structure (Chow *et al.*, 1987). Thus, while the hydrophobic chain length of myristic acid is clearly important for membrane interactions, the exact mechanism of the interaction must still be determined.

This review presents a discussion on a member of the protein kinase family, protein kinase A, in the context of extensive crystallographic studies carried out with this protein. Protein kinase A is the first kinase whose structure became known and provided the framework for the largest family of enzymes which encompasses hundreds of members. There are now nine crystallographic structures of protein kinase A in two major conformations (Karlsson *et al.*, 1993; Knighton *et al.*, 1991a, b, 1993; Zheng *et al.*, 1993a, b, c; Madhusudan *et al.*, 1994; Bossemeyer *et al.*, 1993). All crystal structures obtained from mouse recombinant catalytic subunit represent the "closed" conformation of the upper lobe of the enzyme in the presence of a pseudo-substrate. In the myristylated porcine heart catalytic subunit, three-dimensional structures of both open and closed conformations have been observed; the "open" conformation is characterized by a rotation of the upper lobe by 15° with regard to the lower lobe (Karlsson *et al.*, 1993; Zheng *et al.*, 1993a). This motion results in the opening of the cleft and loss of several hydrogen bonds between the upper domain and the inhibitor peptide (Karlsson *et al.*, 1994). The architecture of protein kinase A is common as well to other protein kinases—ERK 2 (Zhang *et al.*, 1994), cdk 2 (De Bondt *et al.*, 1993), twitchin protein kinase (Hu *et al.*, 1994), human insulin receptor (Hubbard *et al.*, 1994), casein kinase-1 (Xu *et al.*, 1995), and the cyclinA-CDK2 complex (Jeffrey *et al.*, 1995). Yet there are unique features of protein kinase A, one of which is the presence of helix A The N-terminal of helix A is myristylated, and this review also describes the structural motif formed by the presence of this posttranslational modification. The comparison the crystal structure of the myristylated porcine C-subunit with that of mouse recombinant unmyristylated catalytic subunit will show that myristic acid is essential for the formation of the structural motif encompassing the phosphorylation site.

2.9 Å STRUCTURE OF MYRISTYLATED CATALYTIC SUBUNIT OF PROTEIN KINASE A

The structure of myristic acid has been determined through the crystallography of the mammalian catalytic subunit. Gly 1 and myristic acid are well ordered in the electron density map and the myristic acid moiety inserts itself into a deep, hydrophobic pocket of the enzyme consisting of the residues of the N-terminus and the conserved catalytic core formed within the large lobe (Karlsson *et al.*, 1993; Zheng *et al.*, 1993a). The following residues are in the vicinity of the myristic moiety: Ala 4, Lys 7, Ser 10, Glu 11, Ser 14, Val 15, Phe 18, Leu 19, Phe 100, Leu 152, Glu 155, Ilu 303, and Tyr 306, presented in Fig. 1.

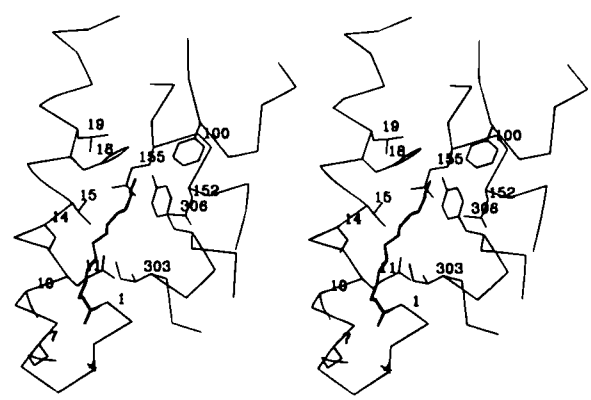


Fig. 1. Stereo view of the N-terminal of helix A of myristylated mammalian protein kinase A with residues interacting with myristic acid highlighted. Myristic acid is drawn in thick line. The coordinates are taken from Karlsson *et al.* (1993) and Zheng *et al.* (1993a).

Although a few hydrophilic residues come close to the myristic acid, it is the hydophobic part of these polar and charged residues which interacts with the myristic acid. A unique feature of helix A is the phosphorylation site, Ser 10, which is one of four phosphorylation sites of the recombinant catalytic subunit. Whether or not Ser 10, in the case of mammalian catalytic subunit (porcine heart), is phosphorylated by another protein kinase or by itself is not known, but it is known that the sites of the reversible posttranslation modification, phosphorylation, and the irreversible one, myristylation, are in close proximity of each other. The most revealing discovery is that myristic acid is not on the surface of the protein but embedded inside the hydrophobic pocket.

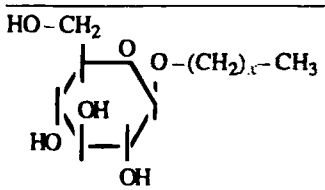
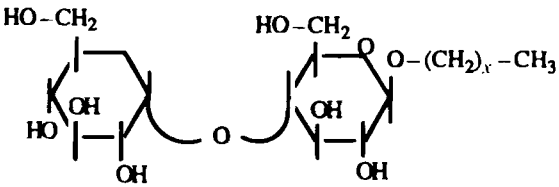
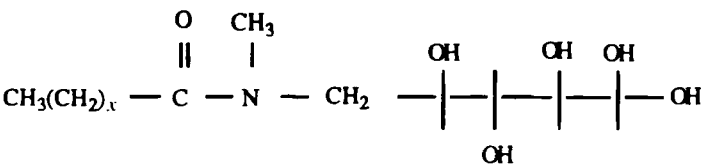
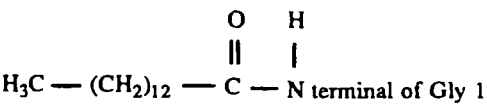
1.95 Å STRUCTURE OF UNMYRISTYLATED CATALYTIC SUBUNIT OF PROTEIN KINASE A WITH BOUND DETERGENT

The parallel crystallographic work on protein kinase A concerns the recombinant mouse catalytic subunit expressed in *E. coli* which is devoid of myristic acid. The first three-dimensional fold of the protein kinase family was obtained using this protein. X-ray crystallography of recombinant catalytic subunit initially started at a 2.7 Å resolution (Knighton *et al.*, 1991a, b) and further extended to 1.95 Å (Knighton *et al.*, 1993). The two important factors contributing to the extension of the resolution of the crystals are: (1) the use of detergents in crystallization and (2) the purification (Herberg *et al.*, 1993) and crystallization of isoelectrically pure isozymes of the catalytic subunit. It is difficult to determine the exact contribution of each factor in extending the resolution. The detergents used in the cocrystallization experiments are listed in Table I. In crystallization trials, the concentration of the detergent was 10 times less than that of the critical micelle concentration (CMC). The only detergent which resulted in successful crystallization was Mega 8. The crystals obtained with and without detergent were essentially isomorphous (Knighton *et al.*, 1991a, b, 1993). The structure solution revealed that the detergent was located in exactly the same hydrophobic pocket occupied by myristic acid in mammalian (porcine heart) catalytic subunit. The detergent inserts itself into this hydrophobic pocket consisting of residues Val 15, Phe 18, Leu 19, Phe 100, Leu 152, Glu 155, Ilu 303, and Tyr 306. The number of residues which interact with the detergent is smaller than those interacting with myristic acid, and the main reason is that the first nine residues are disorderd and not seen in the electron density map. In addition, the detergent has been modeled as N-octane. The interactions of detergent with the residues in the pocket are presented in Fig. 2 and in the context of the entire molecule in Fig. 3. The N-terminal helix A contains Ser 10, visible in both structures. Only the hydrophobic part of the Mega 8 is ordered; the remaining hydrophilic portion (sugar moiety) is disordered because, unlike in mammalian catalytic subunit, the detergent is not covalently linked with the N-terminus.

DETERGENT BINDING IS REVERSIBLE AND ENHANCES THE STRUCTURAL STABILITY OF HELIX A

The structure of the mouse recombinant catalytic subunit without detergent was examined to evaluate the extent of conformational changes. The quality of diffraction is comparable between crystals grown in the presence of detergent and crystals soaked in the absence of detergent. Analysis of the electron density map confirms that detergent is absent in the crystal, and the refinement of the structure has shown that the segment of helix A including Ser 10 is not visible in the electron density map (in fact, the first 14 residues are disordered in the structure) (Zheng *et al.*, 1993b). These results might also provide a function for the myristic acid. The catalytic subunit of protein kinase A is not known for its association with the cellular membrane, unlike the src subfamily of protein kinases. Translocation of the catalytic subunit into the nucleus was established, but so far there is no evidence concerning the cellular membrane association; however, the function of the insertion of the hydrophobic tail of myristic acid into the protein might be related to the structural stability of helix A. Helix A consists of the phosphorylation (P) site, Ser 10, which is preceded by a canonical sequence with either lysines or arginines in P-2 and P-3 sites. The structure of fully phosphorylated catalytic subunit has recently been solved and all four phosphorylation sites consist of a common structural motif encompassing the phosphorylation site and canonical sequence mentioned above (Madhusudan *et al.*, personal communication). Furthermore, this motif is also present in the structure of substrate peptide bound to the enzyme. Hence, structural requirements for recognition of phosphorylation sites, including Ser 10, have been established; this common motif cannot

Table I. Chemical Structure and Critical Micelle Concentration (CMC) of Detergents[a]

Chemical structure of detergent		Percentage critical micelle concentration %(w/v)
	Nonly-β-D-glucopyranoside $X = 8$ Octyl-β-D-glucopyranoside $X = 7$	0.2 0.6–0.7
	Dodecyl-β-D-maltoside $X = 11$	0.01–0.03
	Mega 8 $X = 8$ Mega 9 $X = 9$ Mega 10 $X = 10$	1.5 0.6 0.18
	Myristic acid	

[a] The concentration of detergents used for the crystallization experiment was ten times less than that of the CMC.

be seen in the electron density map of structure without detergent. Since the detergent locates itself in the same pocket as myristic acid, the local structural rearrangement of helix A in the absence of detergent would also occur in the absence of myristic acid in the hydrophobic pocket. Therefore, the structural stability that myristic acid exerts, results in the formation of a recognition motif for phosphorylation site, Ser 10. The binding of detergent results in the ordering of one and a half turns of N-terminal helix A.

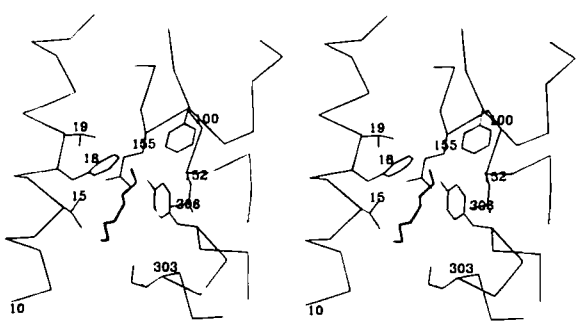


Fig. 2. Stereo view of the N-terminal helix A of unmyristylated recombinant protein kinase A with residues interacting with Mega 8 highlighted. Detergent is drawn in thick line. Coordinates are taken from Knighton *et al.* (1993).

JUXTAPOSITION OF THE REVERSIBLE AND IRREVERSIBLE POSTTRANSLATIONAL MODIFICATION

Juxtaposition of phosphorylation site Ser 10 and myristic acid, which is inserted into the protein, addresses one issue: Is it myristic acid itself, or the motif defined by myristic acid, that is important for recognition of the membrane-bound proteins? This question is very important in cellular signaling in regard to the large and functionally very important group of protein kinases, the src subfamily. This subfamily has been a continuous focus of research with

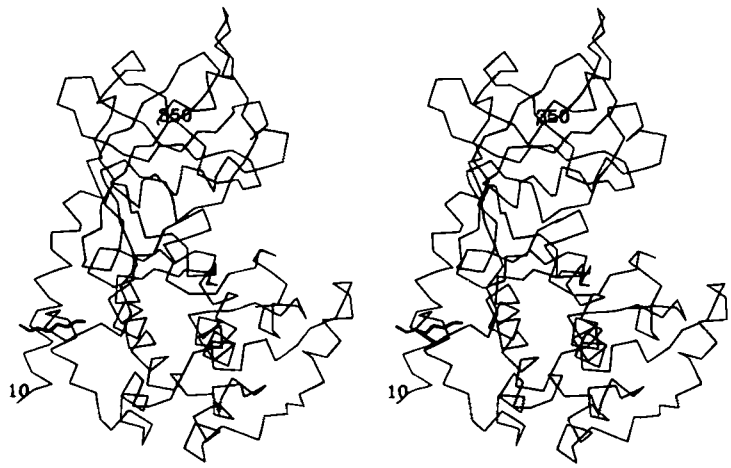


Fig. 3. Stereo view of recombinant catalytic subunit protein kinase A with bound detergent. Detergent is drawn in thick line. N- and C-terminals are highlighted. Coordinates have been taken from Knighton *et al.* (1993).

regard to their membrane association (Resh, 1990). There is no homology among sequences adjacent to the myristic acid. The only homologous feature is the presence of myristic acid and Gly 1, indicating that perhaps myristylation in this family is not caused by single N-myristyl transferase but by families of N-myristyl transferases. Src is tightly associated with the membrane and this association appears to resemble a stable transmembrane protein. The lack of myristic acid (by mutating Gly 1) releases src into the cytoplasm. However, the same results of releasing src can be obtained in the presence of myristic acid and Gly 1 but in the absence of the adjacent sequence of residues 2 through 14. Therefore, it has been proposed that myristic acid is not sufficient for stable association with the membrane and that the adjacent sequence is critically important to provide a structural template for high-affinity binding to the lipid bilayer (Sigal *et al.*, 1994). The importance of the structural template due to the presence of myristic acid has been further demonstrated in work done by McLaughlin and Aderem (1995). Through the use of cell radii and binding energy calculations, McLaughlin determines that there is an electrostatic interaction of a cluster of basic residues, present in some myristylated proteins, with acidic lipids. Phosphorylation of serine residues within these clusters of basic residues reduces their electrostatic interactions with acidic lipid, providing an "electrostatic switch" mechanism for the reversible binding of the myristylated protein. Myristate has been shown to provide barely an adequate amount of energy to anchor a myristylated protein to the plasma membrane (Sigal *et al.*, 1994). Therefore, it must be the template formed by the presence of myristate and not myristate alone that anchors the protein. Furthermore, the importance of the phosphorylation site can be seen as it is the "electrostatic switch" that controls the myristylated protein. The crystallographic studies of the catalytic subunit define the structure of the motif, which is formed by the presence of myristic acid. This motif in the catalytic subunit would furnish a stable phosphorylation site which could be recognized by other cellular components of the cytoplasm. While there is no evidence to suggest that the catalytic subunit interacts with the cellular membrane, these studies provide a plausible structural explanation for the template resulting from the presence of myristic acid. This motif, in which myristic acid is inserted into the proteins, can be a subject of reversible posttranslational modification, i.e., phosphorylation, whose function remains to be established in the case of the catalytic subunit. The formation of this structural template by the presence of myristic acid supports the proposition that it is not the myristic acid alone, but the template formed by the presence of myristic acid, that is important for interaction with other proteins.

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