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Single-step purification of myristoylated and nonmyristoylated recoverin and substrate dependence of myristoylation level

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Abstract

Recoverin is cotranslationally modified by the covalent linkage of a myristoyl group to its N terminus. It is a member of a family of Ca^{2+}-myristoyl switch proteins. Recombinant myristoylated revoverin is currently produced by the cotransformation of bacteria with recoverin and an enzyme that allows N-myristoylation and by supplementing the culture medium with myristic acid. A large variation in the myristoylation level of recoverin and in the amount of myristic acid supplied to the culture medium can be found in the literature. Moreover, although it is known to strongly affect bacterial growth, the amount of ethanol used to solubilize myristic acid is only scarcely mentioned. To improve our understanding of the parameters responsible for recombinant recoverin myristoylation, the effects of myristic acid and ethanol on recoverin myristoylation and expression levels have been systematically studied. In addition, a single-step purification procedure to produce purified myristoylated and nonmyristoylated recombinant recoverin has also been devised. Finally, sodium myristate has been used as an efficient alternative substrate to achieve high myristoylation and expression levels of recoverin. Given that a large number of proteins are myristoylated, these procedures could be applied to several other proteins in addition to recoverin.

Keywords: Myristoylation; Purification; Recoverin; Calcium-binding proteins; Sodium myristate; Myristic acid; Ethanol; *Escherichia coli*

A large number of proteins are fatty acylated to their N-terminal glycine or to internal cysteine residues (for a recent review see [1]). N-myristoylation, which consists of the covalent attachment of a myristoyl group (C14:0) to an N-terminal glycine residue of a protein via an amide linkage, is the most described fatty acylation of proteins (for reviews see [2–4]). In fact, a new computer-based predictor of N-terminal myristoylation indicates that 0.5% of all proteins encoded in the human genome are apparent substrates of the *N*-myristoyl transferase (NMT)[1] [5]. NMT is the enzyme responsible for this cotranslational modification of proteins containing the proper recognition sequence motif at their N terminus [6,7]. Indeed, It has been shown that the consensus sequence Met-Gly-X-X-X-Ser/Thr is the common feature of myristoylated proteins (for a review see [3]). However, Maurer-Stroh et al. [8] have refined this sequence, suggesting the implication of two other motifs at positions 7–10 and 11–17 of the N terminus of myristoylated proteins. This hydrophobic modification plays a key role in protein targeting, protein–protein interaction, and/or binding to membranes. Interestingly, in some cases, ligands such as GTP, phosphate, or Ca^{2+} are involved in the modulation of membrane binding by controlling the orientation of the myristoyl moiety relative to the protein (for reviews see [9,10]). In these cases, myristoyl groups and ligands constitute the so-called molecular switch and, so far, recoverin is the most studied Ca^{2+}-myristoyl switch.

Recoverin is an EF-hand calcium-binding protein and a member of the neuronal calcium-sensors (NCS) family (for

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[1] *Abbreviations used:* NMT, *N*-myristoyl transferase; NCS, neuronal calcium-sensors; ROS, retinal rod outer segments; PMSF, phenylmethylsulfonyl fluoride; RP, reverse phase; TFA, trifluoroacetic acid; CMC, critical micelle concentration.

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a review see [11]). These proteins bind Ca^{2+} via the EF-hand motif, a helix–loop–helix of 29 residues arranged to coordinate Ca^{2+} with a pentagonal bipyramidal symmetry (for reviews see [12,13]). This structural feature was called EF-hand because this motif was originally described for the helices five (E) and six (F) of the carp parvalbumin [14]. Like 11 other members of the NCS [11], recoverin contains an amino-terminal myristoyl group [15,16]. Recoverin was originally purified from retinal rod outer segments (ROS) of vertebrates [17] where it was shown to prevent rhodopsin phosphorylation by inhibiting rhodopsin kinase at high concentration of Ca^{2+} [18–22]. Indeed, in the dark, the binding of two Ca^{2+} ions to recoverin induces the extrusion of its myristoyl group (calcium-myristoyl switch) which increases its affinity for ROS membranes [10,23,24] or membrane models [25,26]. In contrast, light induces lowering of intracellular Ca^{2+} which results in a conformational change of recoverin and sequestration of its myristoyl group in a hydrophobic cleft [27–29]. Moreover, several NCS are also calcium-myristoyl switch proteins or undergo important conformational changes upon calcium binding [11].

Considering the importance of myristoylated proteins in several biological pathways, the availability of an efficient method to produce homogeneous recombinant proteins bearing a myristoyl moiety is of utmost importance for structural and biochemical studies. In this regard, Duronio et al. [30] have developed a coexpression system in *Escherichia coli* consisting of the simultaneous expression of a target protein and the *Saccharomyces cerevisiae* NMT to circumvent the lack of endogenous NMT activity in this bacterial strain. In addition, the culture medium must be supplied with myristic acid as a substrate for NMT to achieve N-myristoylation [30]. Such an expression system is very useful compared to eukaryotic systems since it allows the overexpression of myristoylated and nonmyristoylated proteins and acylation with other substrates using the same expression system. This method has been used for myristoylation of many proteins such as recoverin [24,27,31–36], *S*-modulin [37], GCAP [38], ARF [39], NCS-1 (frequenin) [40,41], MARKS [42], p22 protein [43], and annexin [44]. However, partial myristoylation and/or variations of the myristoylation level have been observed for recoverin [24,26,32,45], ARF [39], and NCS-1 [41]. Furthermore, the amount of myristic acid used to supply the culture medium varied drastically from 1.25 to 1000 mg/250 ml of culture medium [27,32,33,41], indicating a lack of systematic assays to optimize protein myristoylation. Furthermore, a two-step procedure consisting of a phenyl Sepharose chromatography followed by an anion-exchange chromatography is currently used for recombinant recoverin purification [24,27,31,33–36,46].

Herein, we report that the detailed characterization of the eluted fractions from low-substituted phenyl Sepharose chromatography allows purification of myristoylated and nonmyristoylated recoverin in a reliable single-step procedure. In addition, we show that the ethanol used to solubilize myristic acid in the culture medium reduces the amount of purified recoverin. As an alternative, we demonstrate that the water-soluble sodium myristate can be used to achieve a more efficient overexpression and N-myristoylation of recoverin than the widely used myristic acid.

Materials and methods

Materials

Ultrapure NaCl (99.9%) was purchased from J.T. Baker (Phillipsburg, NJ, USA). *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes) (99.5%), β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA), and sodium myristate (99%) were from Sigma (St. Louis, MO, USA). $CaCl_2$ and myristic acid (GC grade) were purchased from Fluka (Neu-Ulm, Germany).

Preparation and cloning of recoverin cDNA

RNA from freshly dissected bovine (Bos Taurus) retina was isolated by the Tri-reagent method (Sigma) and used for reverse transcription (RT) reaction (RevertAid H minus First Strand cDNA Synthesis Kit; Fermentas, Burlington, ON, Canada). Then, first-strand cDNA was used as a template for PCR using primers designed to amplify the coding sequence of the bovine recoverin gene (M95858) and to introduce *Nde*I and *Bam*HI restriction sites. The full-length bovine cDNA was ligated into the pET11a plasmid (Novagen) to generate the pET11a-recoverin expression vector (pET11a-Rec).

Expression and purification of myristoylated recombinant recoverin

Myristoylated and nonmyristoylated recoverin were expressed essentially as reported by Ray et al. [33] with some modifications. Briefly, recoverin was expressed in *E. coli* strain BL21 (DE3) pLysS (Novagen) containing plasmids encoding for recoverin (pET11a-Rec) and *N*-myristoyl transferase (pBB131) (kindly provided by James B. Hurley). The culture was grown in 250 ml of LB (Luria–Bertani) medium at 37 °C containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). At an $A_{600nm} = 0.3$, protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside and cells were incubated for an additional 3 h at room temperature. To allow N-myristoylation of recoverin, myristic acid [30] or sodium myristate was added 20 min before the induction (see the following section). Then, cells were harvested by centrifugation and resuspended in 10 ml of buffer A (50 mM Hepes (pH 7.5) 100 mM NaCl, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 0.1 mM PMSF). After sonication and centrifugation (20,000*g* for 30 min at 4 °C), the cleared lysate was loaded at a flow rate of 0.4 ml/min to a column (10 mm id × 10 cm long equipped with a flow adaptor; Econo-column, Bio-