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Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitylated

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Covalent coupling of bovine rhodopsin to CPG-thiol glass was used for separation of CNBr peptides. It is shown that cysteine residues 322 and 323 in the C-terminal cytoplasmic fragment of rhodopsin are modified with palmitic acid.

Rhodopsin; Carrier; Covalent coupling; C-terminal fragment; Palmitylation

1. INTRODUCTION

Since the pioneering paper of Folch and Lees on the discovery of proteolipid [1], a series of covalent protein-lipid complexes extending from viral membrane glycoproteins [2] to proteins with the tumorogenic transformation activity [3] and a number of cellular proteins as diverse as ankyrin (a peripheral red cell membrane protein) [4], receptor of transferrin [5], calcium binding subunit of calcineurin [6] have been reported (review [7]).

Bovine rhodopsin is one of the best characterized membrane proteins. Its amino acid sequence is known both from protein and DNA sequencing [8,9]. Along with the most common post-translational glycosylation, rhodopsin has been shown to be modified by fatty acids [10–12].

In this paper, we show that Cys-322 and -323 in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitylated.

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2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

The carrier used was porous CPG-thiol glass (Pierce, USA), bead size 125–177 μm and pore diameter 500 Å. Chemicals specifically purchased were 2,2'-dipyridyl disulfide (Aldrich, USA), SDS (Serva, FRG), β-mercaptoethanol and DTT (Sigma, USA). For HPLC separation, Nucleosil C_8 and C_{18} (particle diameter 7.5 μm) were obtained from Macherey-Nagel (FRG). Palmitic acid and a standard mixture of fatty acids were from Sigma (USA). All other reagents were of analytical grade.

2.2. *Methods*

Activation of the carrier and determination of its capacity were according to [13].

Photoreceptor membranes from rod outer segments (ROS) of bovine retina were prepared essentially as described [14] except that no thiol reagent was used at any stage of disk membrane preparation. The amount of rhodopsin in each preparation was determined spectrophotometrically and the spectral criteria of purity (A_{280nm}/A_{500nm}) were found to be about 1.8–2.0.

For reduction of rhodopsin, membranes were solubilized in 100 mM Tris-HCl, pH 8.6, containing 3 mM EDTA·Na_2, 4% SDS, 20-fold excess of DTT per mol of cysteine and the resulting solution was incubated at 20°C for 12 h. Unreacted DTT was removed on a Bio-Gel P-2 column (1.5 × 45 cm) equilibrated in 100 mM sodium acetate, pH 4.0, with 3 mM EDTA·Na_2 and 1% SDS.

For coupling of rhodopsin to the activated carrier, if no prior reduction was required, photoreceptor membranes were solubilized in 100 mM sodium acetate, pH 4.0, containing 3 mM EDTA·Na_2 and 4% SDS and incubated for 2 h. In the

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case of the reduced rhodopsin, it was necessary to carry out immobilization immediately following reduction and passage through the Bio-Gel P-2 column. For this purpose the protein fraction from the column was directly collected into a reaction vial. Dried activated carrier was added to each preparation (250 nmol of protein per 1 g of carrier) and the resulting slurry was gently rotated for 12 h.

After immobilization the lipids, detergent and noncovalently bound protein were removed by washing the carrier sequentially with 10 vols, each 100 mM, sodium acetate, pH 4.0; 4% SDS:50% methanol; 100% methanol; chloroform/methanol (2:1, v/v) and 99.7% formic acid. The conjugate was thoroughly washed with water and methanol, then dried.

The amount of the immobilized protein was determined by the amino acid analysis. A 10-fold excess of 5.7 N HCl was added to the conjugate, the ampoule was then evacuated, sealed and incubated at 105°C for 24 h. After hydrolysis, the carrier was washed with a 5-fold excess of 5.7 N HCl and the soluble extract chromatographed on a Durrum D-500 amino acid analyzer (Durrum, USA).

The CNBr cleavage was performed as follows: the immobilized protein was washed several times with 80% formic acid. After the last washing 2–4 vols of 75% formic acid were added to the conjugate. Solid cyanogen bromide was added in a 500-fold excess per mol of methionine, and the reaction was carried out at 20°C for 20 h in the dark. The conjugate was washed with 10 vols of formic acid, water and methanol, and then dried.

Peptides were detached from the glass by incubation in 50 mM Tris-HCl, pH 8.6, containing 3 mM EDTA·Na_2 and a 20-fold excess of DTT or β-mercaptoethanol per mol of cysteine for 2 h at 20°C. The peptides soluble in the buffer solution were combined with those soluble in formic acid and then subjected to gel filtration on Bio-Gel P-2 in 80% formic acid.

The peptides were separated on a 1.5 × 100 cm Bio-Gel P-30 column equilibrated with 80% formic acid. For HPLC of peptides the instrument used was an Altex 322 system (Altex, USA) with a flow cell spectrophotometer. The effluent was monitored at 206 nm. The samples were injected in 80% formic acid.

The covalently bound fatty acids were released from the purified C-terminal CNBr peptide (80 nmol) in 1.0 N KOH for 12 h at 37°C. The reaction mixture was acidified with HCl and fatty acids were extracted several times with hexane. The extracts were dried under a stream of N_2 at 60°C and subjected to methanolysis at 80°C for 4 h using HCl in methanol. Fatty acid methyl esters were separated and quantified on a Chrom-5 gas chromatograph with a flame ionization detector (FID). The samples were also analyzed by mass spectrometry.

3. RESULTS AND DISCUSSION

Covalent coupling of rhodopsin to the carrier (CPG-thiol) through the thiol-disulfide exchange reaction proved to be extremely useful both for exhaustive cleavage and facile separation of hydrophobic peptides [13]. In brief, the essence of this approach included: coupling of rhodopsin solubilized in SDS to the carrier; removal of detergent and lipids by washing with organic solvents; cleavage of the conjugate with CNBr in 70% formic acid; extraction of free peptides into 80% formic acid; release of bound peptides by treatment with β-mercaptoethanol and subsequent 80% formic acid extraction. Thus already at this stage peptides were efficiently separated according to their cysteine content that provides further facile fractionation of each fraction on Bio-Gel P-30 and HPLC, if necessary.

However, we found that depending on whether the solubilized rhodopsin was subjected to β-mercaptoethanol treatment before coupling to the support or not, the C-terminal peptide Val-Thr-Thr-Leu-Cys-Cys-Gly-Lys-Asn-Pro-Leu-Gly-Asp-Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala was covalently bound or remained in the supernatant, respectively. Moreover, this peptide found in the supernatant can be easily coupled to the carrier after β-mercaptoethanol treatment. For the simplicity of further discussion the C-terminal peptides derived from CNBr cleavage of rhodopsin coupled to the carrier with and without β-mercaptoethanol treatment are designated 'C-r' and 'C-n.r', respectively.

The unusual behavior of this peptide led to alternative suggestions: either cysteines 322 and 323 form an intramolecular disulfide bond or they are modified with some β-mercaptoethanol-sensitive group. If we imply that the cysteine residues 322 and 323 in C-n.r, corresponding to residues 5 and 6, form a disulfide bond, the Edman degradation using hot and cold phenylisothiocyanate (PITC) at these steps, respectively, would show the appearance of radioactivity at the sixth step, the previous one being nonradioactive and blank. Such analysis revealed not only the absence of radioactivity at the sixth step but also the complete blocking of the sequence beginning from the fifth one. On the contrary, the sequence of 'C-r' including the two cysteine residues either with free or iodo-[^{14}C]acetamide modified groups is easily determined.

It should be noted that unlike C-r or C-r modified with iodo-[^{14}C]acetamide, C-n.r is irreversibly absorbed on a reverse-phase HPLC column and cannot be eluted either with CH_3OH or CH_3CN at concentrations as high as 90%. That was unexpected since even the more hydrophobic peptides of rhodopsin including those with free thiol groups can be readily recovered from HPLC

columns with high yields. Oxidation with performic acid or reduction with β-mercaptoethanol drastically changes the properties of C-n.r and results in its high recovery from the HPLC column.

Data presented, on the one hand, exclude the possibility of a disulfide bond between the two adjacent (322–323) cysteine residues in rhodopsin, and, on the other, indicate that these residues are modified with some groups conferring on the peptide unusually high hydrophobic features.

Highly purified samples of bovine rhodopsin were shown to contain 1 or 2 mol covalently bound palmitates per mol protein [10]. Incorporation of [^{3}H]palmitates was demonstrated using both the whole retina or crude rod outer segments (ROS). The covalent nature of palmitate attachment was proven on the basis of stability of the lipoprotein complex in detergent solutions and organic solvent commonly used for extraction of lipids from membrane proteins.

Furthermore, taking into consideration the lability of the complex toward reducing agents, hydroxylamine and mild alkaline treatment, it was suggested that the fatty acid is bound to rhodopsin via thioester linkage [12]. The results of these studies taken together with our data on the C-terminal fragment of rhodopsin imply that the two adjacent cysteines in this peptide are most probably acylated with palmitates.

In order to demonstrate that C-terminal fragment contains covalently bound fatty acids, peptide-bound lipids were released from 80 nM C-n.r by alkali hydrolysis and subsequently subjected to acid methanolysis. The exact quantity of palmitic acid was determined using the GC method with the marganic acid methyl ester (C17:0) as an internal standard. The initial quantity of the standard was 50 mg. After transesterification with HCl/CH_3OH and extraction with hexane the sample was analyzed in described conditions (see fig.1). The coefficient for palmitic acid equal to 0.94 was calculated by the triangular method. The total quantity of C16:0 in the initial sample was found to be 47 mg, which corresponds exactly to 2 mol palmitic acid per mol of the C-terminal CNBr peptide.

Palmitic acid was further identified by mass spectrometry (fig.1). The mass spectra for palmitic acid methyl ester released from the C-terminal peptide matched exactly that of the standard sample of C16:0 as far as the molecular ion and other characteristic ions are concerned [14].

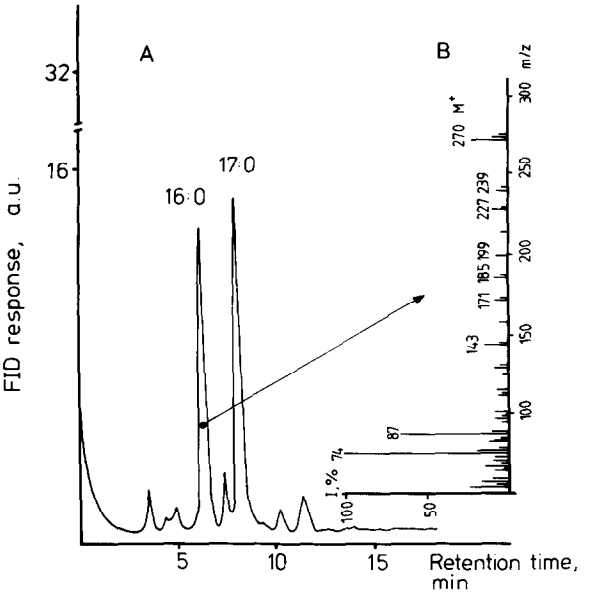

Fig.1. Covalently bound fatty acids in bovine rhodopsin. (A) The separation of fatty acid methyl esters on a gas chromatograph; column: 5% silar 10C on chromosorb W-AW (100–120 mesh), 2500 × 4 mm; GC conditions: 150°C, initial t; t inj, 200°C; t det, 245°C. (B) Mass spectrum of 16 carbon methyl ester (17:0 – internal standard).

In the light of these results the unusual properties of C-n.r and the cease of the sequence at the fifth step may now be easily explained. First, two fatty acid molecules confer hydrophobic properties on this peptide preventing its elution from a HPLC column. Second, as mentioned above, the sequential degradation of C-n.r ceased at the fifth step corresponding exactly to the first cysteine residue in the peptide. Interaction of the thioester carbonyl function with a newly exposed amino group of cysteine 322 may result in water elimination with subsequent cyclization [15] and blocking of the sequence.

The physiological importance of rhodopsin acylation is still unclear. The absorption of a photon of light by rhodopsin results in the *cis* to *trans* isomerization of retinal. This configurational change in the chromophore is expressed in a series of conformational changes in the protein. According to a number of biochemical data the most

obvious of these changes occur in the C-terminal cytoplasmic domain of rhodopsin. It is conceivable that the structural changes in this fragment expose the binding sites for transducin either on its own or on other cytoplasmic regions. The conformational alterations followed by light absorption not only provide transducin binding sites but also drive phosphorylation of up to nine threonine and serine residues in the C-terminal fragment by opsin kinase. Then phosphorylation induces the subsequent binding of a protein known as '48 kDa' protein leading to rhodopsin inactivation. Thus the C-terminal cytoplasmic fragment of photoexcited rhodopsin acquires a substrate status for at least three soluble proteins [16].

Increased sensitivity of this fragment to proteolysis after illumination provides important additional evidence of its conformational change upon photoactivation of rhodopsin. In fact, the accessibility of 12 C-terminal amino acids to thermolysin, although reversible in a time-dependent manner is significantly increased at the stage of metharhodopsin II [17]. Interestingly enough, this cleavage always precedes the exposure of additional protease sensitive sites on the second and third cytoplasmic loops connecting the 3–4 and 5–6 transmembrane segments. Furthermore the C-terminal peptide of about 40 amino acid residues is shown in all proposed models [16] as being exposed (fig.2). It contains a number of sites commonly sensitive to proteases of high specificity, such as trypsin, *S. aureus* V8 protease, however, neither these nor the protease of such a wide specificity as papain result in exhaustive cleavage of this fragment. Limited cleavage at the peptide bond Leu–Cys-321–322 by papain is achieved when using an enzyme substrate ratio as high as 1:10 and for at least 5–6 h at room temperature. On the contrary, exhaustive cleavage of this fragment is observed on apomembranes obtained by treatment of rhodopsin with hydroxylamine in the light [18].

It is tempting to suggest that fatty acylation of rhodopsin may promote a proper functional anchorage of the C-terminal fragment to the photoreceptor membrane thus preventing it from non-physiological exposure and protease action.

Recent sequence analysis demonstrated that the C-terminal fragments of various rhodopsins contain from one to three cysteines [16]. Careful

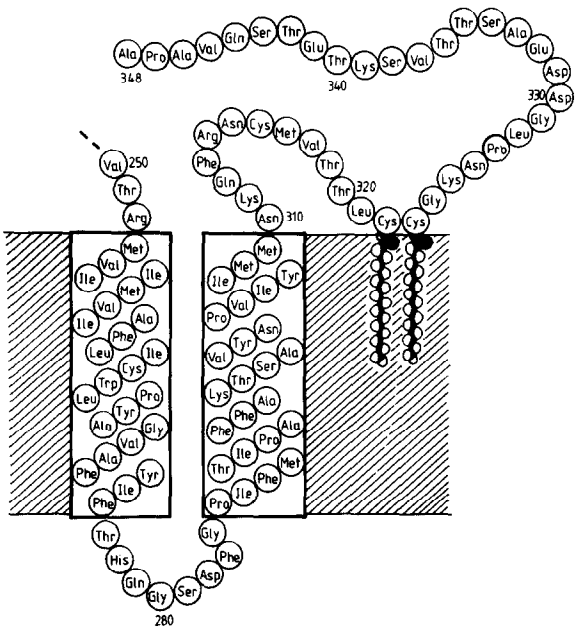

Fig.2. Sixth and seventh transmembrane rods and cytoplasmic fragment of the rhodopsin molecule. Lys residue responsible for retinal binding is in segment VII. Residues Cys-322 and Cys-323 are palmitylated.

analysis of possible fatty acid acylation of both vertebrate and invertebrate proteins may further clarify the functional importance of this modification. In any case bovine rhodopsin palmitylation raises questions related to cleavage and reformation of the thioester bond and involvement of some well known or still unknown proteins of ROS in this process.

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