Palmitylation of a G-Protein Coupled Receptor
DIRECT ANALYSIS BY TANDEM MASS SPECTROMETRY*

(Received for publication, April 9, 1992)

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Bovine rhodopsin has been reported to be S-palmitoylated at cysteines 322 and 323 (Ovchinnikov, Y. A., Abdulayev, N. G., and Bogachuk, A. S. (1988) FEBs Lett. 230, 1–5). Using a combination of enzymatic and chemical cleavage techniques in conjunction with tandem mass spectrometry, the sites of incorporation of the palmityl groups are shown. Bovine rhodopsin in disc membranes was digested with thermolysin to generate the C-terminal fragment (241–327), which was subsequently cleaved with cyanogen bromide to generate the peptide Val-Thr-Thr-Leu-Cys-Cys-Gly-Lys-Asn-Pro (318–327). A bis-S-palmitylated synthetic peptide cleaved the two thioester-linked palmitoyl groups to produce reduction products of the same approximately decreased molecular weight (MH+ 1511.7) by liquid secondary ion mass spectrometry. Dithiothreitol reduction of both the isolated and the synthetic peptide cleaved the two thioester-linked palmitoyl groups to produce reduction products of the same appropriately decreased molecular weight (MH+ 1035.5). Tandem mass spectrometry of the isolated and the synthetic peptide identified the sites of attachment of the palmitoyl groups on cysteines 322 and 323. These results prove the modification of cysteines 322 and 323 with palmitic acid in bovine rhodopsin, and illustrate the utility of mass spectrometry to characterize the post-translational modifications in G-protein-coupled receptors.

Translation of messenger RNA into protein does not usually result in a final product since numerous post-translational modifications can occur after protein synthesis. One recently discovered type of post-translational modification is fatty acylation usually with either myristic or palmitic acid (1). The functional significance of this type of modification is presently unclear, although some evidence supports the involvement of protein acylation in membrane targeting and other G-protein-coupled receptors indicates that the reported site of palmitylation is conserved within the C terminus of most of the G-protein-coupled receptors (7). This conservation of cysteine residues suggests that palmitylation of G-protein-coupled receptors may represent a common structural motif important in receptor structure and function.

Interest in the palmitylation of rhodopsin began in 1984 when [3H]palmitic acid was shown to be incorporated into bovine rhodopsin through an alkali labile linkage, suggestive of an ester bond (6). Subsequently cysteines 322 and 323 were shown not to be susceptible to labeling with iodo-[2-14C]acetic acid, leading Al-sa'leh et al. (8) to conclude that cysteines 322 and 323 were involved in a disulfide bridge. O’Brien et al. (9) concluded that the bond between palmitic acid and bovine rhodopsin was a chioester since the bond was labile to β-mercaptoethanol. In 1988, Ovchinnikov et al. (10) reported that the peptide containing residues 318–348 from bovine rhodopsin was the site of palmitic acid attachment. From the results of earlier studies, and their own experiments, Ovchinnikov et al. surmised that cysteines 322 and 323 must be palmitoylated. This conclusion was based upon indirect evidence as Ovchinnikov et al. could not isolate the peptide fragment with the palmitic acid groups attached because the peptide was adsorbed irreversibly to their C-18 high performance liquid chromatography (HPLC) column. The same year, Karciauskas et al. (11), using site-directed mutagenesis, concluded that the presence of palmitoyl groups on cysteines 322 and 323 is not essential for transducin activation. The lack of certainty in the site and nature of the palmitic acid bonds prompted us to use mass spectrometry to directly identify the sites of palmitylation in bovine rhodopsin. Since incorporation of palmitic acid was reported to be nonenzymatic (9), we also investigated the possibility of acylation by other fatty acids such as stearic or oleic acid (12). In this paper we describe the successful isolation of a palmitoylated peptide fragment from bovine rhodopsin and its characterization by mass spectrometry.

*This research was supported by Grants EY06239 and EY04939 from the National Institutes of Health, National Science Foundation Grant DIR 8804502, and an unrestricted grant from Research to Prevent Blindness, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: G-protein, guanyl nucleotide-binding regulatory protein; HRPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Boc, t-butyloxycarbonyl; Fmoc, N-(9-fluorenylmethyl)oxycarbonyl; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; LSIMS, liquid secondary ion mass spectrometry; Pal, palmitoyl.
diluted 10-fold with H2O and lyophilized. The sample was monitored at 280 nm, and aliquots of the fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to verify separation of F1 and F2.

Cyanogen Bromide Cleavage of F2—The fractions containing F2 were pooled and dried in vacuo. The F2 fragment was dissolved in 0.7 ml of 99% formic acid and adjusted to 90% formic acid with ethanol to a total volume of 5 ml. The sample was filtered with a Millex-LS 5.0-μm filter unit (Millipore, Bedford, MA) and then applied to a Sephadex LH-60 column (2.5 × 90 cm) equilibrated with 30% formic acid in ethanol (16). The two fragments, F1 and F2, were separated at a flow rate of 1.5 ml/h with 15-ml fractions collected into silanized glass tubes. The eluent was monitored at 280 nm, and aliquots of the fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to verify separation of F1 and F2.

Mass Spectrometry—Mass spectra were recorded on a JEOL HX110/HX110 4-sector tandem mass spectrometer (JEOL, Tokyo, Japan). Samples were dissolved in 30 μl of 0.1% trifluoroacetic acid, acetonitrile/1-propanol (2:1) (1:1), applied to the probe in a matrix of glycerol/m-nitrobenzyl alcohol (1:1) and ionized with 18 kV cesium ions. Single stage analysis was performed with an accelerating voltage of 10 kV and a resolution of 1500. The tandem mass spectrometry (MS/MS) experiments were performed with the collision cell operated at 3 kV filled with helium sufficient to attenuate the ion current of the C-12 monoisotopic peak by 70%.

RESULTS AND DISCUSSION

Our goal was to identify the site of palmitic acid attachment in bovine rhodopsin. Determining the site of palmitylation in bovine rhodopsin required cleaving the protein into pieces which were small enough (M < 2000) to be easily sequenced by tandem mass spectrometry. Under the assumption that Ovchinnikov et al. (10) had identified the correct site of palmitylation of rhodopsin, a strategy was devised to generate a peptide containing cysteines 322 and 323 which would have a molecular weight less than 2000. Rhodopsin was first cleaved while still in the disc membrane with thermolysin, since the sites of cleavage were well documented (20, 21). Thermolysin cleaves rhodopsin into two large pieces termed F1, the N-terminal fragment containing residues 1–240, and F2 containing residues 241–327 (see Fig. 1). Thermolysin cleavage also generated by cleavage of rhodopsin while in the membrane. CNBr indicates one of the sites of palmitoylation of rhodopsin, a strategy was devise...
generates three other smaller fragments from the C-terminal end which are released into the supernatant and are easily removed (Fig. 1). Cleavage with thermolysin provides two advantages: first, cleavage generates a new C terminus only 4 residues away from cysteine 323 and, second, cleavage reduces the problems of aggregation which occur when intact rhodopsin is digested with cyanogen bromide. Separation of F2 from F1 was accomplished using an established gel permeation chromatography (Sephadex LH-60) method for hydrophobic proteins (16). Monitoring the eluent at 280 nm, a chromatographic profile as reported by Hargrave et al. (21) was observed. The separation of F1 and F2 was verified by analyzing the individual fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The pattern observed on the gel was similar to that reported by Hargrave and Fong (22). The purified F2 fragment was then subjected to cyanogen bromide digestion to produce 7 peptides, one of which would contain both cysteines 322 and 323. This peptide (residues 318-327) shown in Fig. 1B if palmitylated at both cysteines would have a mass of 1511.9 Da for the protonated molecular ion (MH⁺) when analyzed by liquid secondary ion mass spectrometry (LSIMS).

Although all the peptides generated from cyanogen bromide digestion of F2 were expected to have unique masses, separation of the peptides by HPLC was necessary to avoid problems associated with competition for ionization from the glycerol/m-nitrobenzyl alcohol matrix. This competition would result in preferential ionization of the more surface active peptides (23, 24). Separation was achieved with the HPLC conditions described under “Experimental Procedures.” It was anticipated that nonstandard HPLC conditions would be required to separate and recover the palmitylated peptide, since Ovchinnikov et al. (10) had reported that they could not isolate the putative palmitylated peptide by reversed-phase HPLC even with acetonitrile concentrations as high as 90%. Other investigators, seeking to identify the sites of palmitylation in the myelin proteolipid protein, found that removal of the fatty acids was necessary because of poor recovery of the fatty acylated peptides from HPLC columns (12). A modification of a method first described by Tarr and Crabb (25) which incorporated a cyanopropyl column with an organic mobile phase of acetonitrile/1-propanol (2:1), was used to separate the CNBr digest of F2. They had shown that this ratio of the organic solvents produced the best recoveries for the hydrophobic protein cytochrome P-450. Fig. 2A shows a typical chromatogram obtained for the cyanogen bromide digest of F2. Fractions collected from the HPLC were analyzed by LSIMS. A peptide with MH⁺ = 1511.7 Da was observed in a fraction which eluted at 67.8 min. The mass of 1511.7 Da corresponds to the predicted protonated molecular ion mass for the peptide Val-Thr-Thr-Leu-Cys(Pal)-Cys(Pal)-Gly-Lys-Asn-Pro if two palmitoyl groups are incorporated into the peptide.

Although a peptide was identified with the expected unique molecular weight for the palmitylated digest of F2, an authentic standard was synthesized for reference in regards to chromatographic retention time and MS/MS fragmentation behavior. The synthesis of the peptide Val-Thr-Thr-Leu-Cys-Gly-Lys-Asn-Pro was achieved on the “super acid-sensitive resin,” Sasrin, which allows the removal of the protected peptide from the solid support by treatment with 1% trifluoroacetic acid in methylene chloride leaving all of the other protecting groups intact. The thio-S-t-butyl protection for the 2 cysteine side chains was chosen to allow selective cleavage with the reducing agent tributylphosphine. Once the cysteines were deprotected they could be exclusively acylated with palmityl chloride. In a final step all the remaining protecting groups were removed with neat trifluoroacetic acid, and after separation by HPLC the product Val-Thr-Thr-Leu-Cys(Pal)-Cys(Pal)-Gly-Lys-Asn-Pro was obtained. The synthesis of the initial protected peptide was verified by amino acid analysis and mass spectrometry. Each step of the synthesis to incorporate the palmitoyl groups was monitored by mass spectrometry. The identity of the final product was verified by LSIMS based upon the expected MH⁺ mass of 1511.9 Da (Fig. 3). The synthetic peptide was treated with dithiothreitol to reductively cleave the two palmitic acid groups generating the unmodified peptide (Val-Thr-Thr-Leu-Cys-Gly-Lys-Asn-Pro) with a predicted MH⁺ of 1035.5. When the reduced material was analyzed by LSIMS an MH⁺ of 1035.4 was observed providing confirmation of the identity of the synthetic material (data not shown).

The chromatographic behavior of the synthetic peptide (Fig. 2B) was identical to that of the peptide isolated from rhodopsin (Fig. 2A), with each having HPLC retention times of 67.8 min. Examination of Fig. 3 reveals that both the isolated material and the reference material exhibited similar mass spectra when analyzed by LSIMS. Both spectra exhibit intact molecular ions (MH⁺) of m/z 1511.7 and doubly charged species (M + 2H)⁺ of m/z 756.1. LSIMS is a soft ionization process which characteristically produces few fragmentation products. Fragmentation of the thioester bond produced a
significant peak at m/z of 1273.4 which is apparent in both spectra, suggesting that the thioester bond is very susceptible to cleavage. The peak at m/z 1273.4 is not due to the monopalmitoylated peptide since the mono- and dipalmitoylated products do not coelute on the HPLC. The peaks at 515 and 621 are due to chemical background. A peak at m/z 1539.7 (MH⁺ + 28) is observed in the spectrum from the isolated peptide. This increase in mass of 28 Da could result from addition of one formyl group, one ethyl group, or two methylene groups. Formylation of the peptide could occur during the cyanogen bromide cleavage process, while formation of the C-terminal ethyl ester could occur during the Sephadex chromatography. An increase of two methylene groups could also suggest the replacement of one of the palmitic acid groups with a stearic acid group. In some spectra (data not presented), a peak of m/z 1567.7 (MH⁺ + 56) is observed which could correspond to the addition of either two formyl groups or four methylene groups indicative of incorporation of two stearic acid groups.

Comparison of the retention time and the mass spectrum of the synthetic peptide with the isolated peptide supports the identification of the isolated peptide as the predicted peptide Val-Thr-Thr-Leu-Cys(Pal)-Cys(Pal)-Gly-Lys-Asn-Pro. Another way to verify the structure was to reduce the isolated material with dithiothreitol and examine the mass spectrum for a mass shift corresponding to a decrease in mass of 476.45 Da (loss of 2 palmitoyl groups). A peak with m/z 1035.5 was indeed observed in the mass spectrum from the reduced isolated peptide. A smaller peak at m/z 1063.5 was also apparent in the spectrum and was most likely a result of either formylation or esterification of the isolated peptide, since reduction with dithiothreitol would not cleave the ester bond. The peptide contains two threonine residues which are the likely sites of formylation, and would account for the appearance of both MH⁺ + 28 and MH⁺ + 56 (26). One carboxyl group is also present which could be esterified to form the ethyl ester with a resulting MH⁺ + 28. If the increases of 28 and 56 Da were the result of incorporation of one and two stearic acid groups, respectively, reduction of the peptide would only produce an ion at m/z 1035.4.

Shown in Fig. 4, A and B, are the tandem mass spectra recorded by collision induced dissociation of the m/z 1511.9 MH⁺ ions from both the isolated and synthetic peptide, respectively. The MS/MS spectra of the isolated and synthetic peptides are essentially identical except for differences in the relative intensities of the peaks. These differences are most likely due to the inability to generate the same ion current between samples and differences in the collision cell gas pressure between sample runs. The match between the two spectra is sufficient to conclude that the isolated and the synthetic peptides are identical. Interpretation of the fragment ions allowed conclusive localization of the sites of palmitic acid attachment. The identity of the 15 labeled peaks in Fig. 4 are listed in Table I. The ions which gave the most information regarding the site of the palmitic acid attachment are peaks labeled 6 and 8 which are two w ions, w6 (m/z 469.0) and w8 (m/z 810.1), respectively. The difference of 341 Da between these two ions indicates the loss of a palmitolysine

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2 Peptide fragment ion nomenclature from K. Biemann (33).
residue. Although no complete ion series was observed, the y1-7 series was present and was sufficient to discern the location of the two palmityl groups (these lower intensity ions are not labeled in Fig. 4). The mass differences of 341 Da between the y3 ion m/z 755.1 and the y4 ion m/z 415.0 and between the y6 ion m/z 1097.6 and the y7 ion correspond to the sequential loss of two palmitylcsine residues. The presence of the two b ions, b2 and b3, with m/z of 301.0 (Fig. 4, peaks 4) and 301.9, respectively, indicate that the 2 threonine residues present are unmodified. The presence of these ions, plus the susceptibility of the peptide to reductive cleavage of the palmityl groups with dithiothreitol, rules out the possibility of the palmityl groups being bound through ester linkages to the 2 threonines. Additional support for the palmitate being bound through a thioester linkage to the 2 cysteines comes from the appearance of an ion at m/z 314.2 which corresponds to a palmitylcysteine immonium ion. All of the fragment ions with intensities greater than 0.5% relative to the MH⁺ could be accounted for based upon the proposed sequence. Most of the ions were C-terminal ions, probably due to the sequential loss of two threonine residues through thioester linkages producing losses characteristic of the corresponding fatty acyl group as the major cleavage products (27). The major ion in the spectra, m/z 1272.5, corresponds to cleavage between a sulfur atom on a cysteine residue and the carbonyl carbon of the fatty acid, releasing the palmityl group. Cleavage at the two fatty acid groups apparently predominate to such an extent that fragmentation at the other sites does not occur readily, thereby not generating a complete fragment ion series which would make sequence interpretation easy. Further confirmation of the proposed structure was the presence of several immonium ions indicative of the amino acid content of the peptide. The observed ions were m/z 72, 74, and 86 indicative of valine, threonine, and leucine, respectively.

To facilitate interpretation of the MS/MS spectra of the palmitylated peptide, various derivatives of the peptide were prepared. The methyl ester was prepared (28) and yielded an MH⁺ 1525.9 ion by LSIIMS, indicating that one methyl group was incorporated as expected (the only carbonyl group is at the C terminus of the peptide). MS/MS of the methyl ester derivative allowed easy identification of the C-terminal ions (x, y, z, v, w) from the derivatized peptide since methyl esterification shifts the mass of all the C-terminal ions up by 14 Da. In an effort to obtain a complete ion series, the trimethylammonium acetyl derivative of the palmitylated peptide was prepared according to Vath and Biemann (29). Placing a fixed positive charge on the N terminus was expected to produce a clear series of N-terminal ions. Additions of one MH⁺ 1610.9 Da and two MH⁺ 1709.9 Da trimethylammonium acetyl groups were observed corresponding to incorporation of trimethylammonium groups at the two primary amines in the peptide: the N terminus and the lysine residue. Unfortunately MS/MS of the peak at MH⁺ 1610.9 did not produce a strong N-terminal series, however, additional N-terminal ions were observed which further confirmed the identity of the palmitylated peptide. To better understand the fragmentation process observed for the palmitylated peptide, another analog of the peptide was synthesized in which the palmityl groups were replaced with myristyl groups. MS/MS of the myristylated synthetic peptide produced an analogous spectrum to that of the palmitylated peptide except that the ions containing either one or two myristyl groups differed by a fixed amount of the difference in mass between malonic and myristic acids (56 Da) respectively. These shifts aided in assigning the identity of peaks containing the fatty acyl groups. As with the palmitylated peptide, the major fragmentation occurred at the thioester bonds producing losses characteristic of the corresponding fatty acyl group as the major cleavage. This characteristic fragmentation behavior should be helpful in identification of thioester type fatty acylated peptides.

If the palmitylation of bovine rhodopsin is nonenzymatic as has been reported (9), we speculated that other fatty acids should also be incorporated into rhodopsin, since (i) another fatty acylated protein (myelin proteolipid protein) is acylated in a nonenzymatic manner (30) with palmitic, stearic, and oleic acid (31) and (ii) the lipid content of the disc membrane is relatively high in other fatty acids in addition to palmitic acid (32). Mass spectrometric analysis of all the fractions collected from the HPLC showed no evidence that fatty acids other than palmitic acid were incorporated into bovine rhodopsin. These findings were not totally unexpected since Ovchinnikov et al. (10) reported observing 2 mol of palmitate/mol of rhodopsin. The presence of other fatty acids on cysteines 322 and 323 in small percentage cannot totally be ruled
out since authentic standards were not prepared for all the possibilities to determine where these other fatty acylated analogues would elute on our HPLC system.

The function of the palmitoyl groups in rhodopsin remains unclear, although the most reasonable function yet proposed may simply involve incorporation of the fatty acid into the lipid membrane to form a “fourth” cytoplasmic loop (10). When Karnick et al. replaced cysteines 322 and 323 with serines, the protein exhibited the correct absorption spectra and activated transducin, suggesting that the palmitoyl groups do not affect wavelength regulation or transducin binding and activation (11). However, removal of the palmitate groups released in response to bleaching (10). When we analyzed bleached retina, the peak at 67.8 min was still observed, and no monopalmitoylated or unpalmitoylated peptide was found when all the HPLC fractions were analyzed by mass spectrometry. This suggests that the palmitate groups are not released in response to bleaching.

In summary we have developed a method with which to obtain from bovine rhodopsin, the peptide 318–327 with the two palmitoyl groups attached. From this peptide we have conclusively identified that cysteines 322 and 323 in bovine rhodopsin are palmitoylated by comparing the retention time on HPLC, the LSIMS spectrum, and the tandem mass spectra with those from a synthetic palmitoylated peptide. We obtained from bovine rhodopsin, the peptide with hydroxylamine apparently decreases activation of transducin with GTPγS (34).

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