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A Major G Protein α_O Isoform in Bovine Brain Is Deamidated at Asn346 and Asn347, Residues Involved in Receptor Coupling†

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ABSTRACT: The structural differences between two major forms of the α subunit of the heterotrimeric G protein G_O were found to be due to deamidation of either of two Asn residues near the C-terminus of the proteins, in a region involved in receptor recognition. G_O is the most abundant heterotrimeric G protein in mammalian brain. Two forms of the protein, G_{OA} and G_{OB}, are known to be generated by alternative splicing of a single $G_O\alpha$ gene. A third isoform, α_{OC}, represents about $^1/_3$ of the α_O protein in brain and is related to α_{OA}, from which it is thought to be generated by protein modification. Mass spectrometry and chemical derivatization of tryptic fragments of the proteins were used to localize the structural difference between α_{OA} and α_{OC} to a C-terminal peptide. Sequence analysis of a C-terminal chymotryptic fragment both by ion trap mass spectrometry and by Edman degradation identified Asn346 and Asn347 of α_{OA} as alternative deamidation sites in α_{OC}. These structural differences have immediate implications for G protein function, as they occur in a conformationally sensitive part of the protein involved in receptor recognition and activation. Since Asn347 is a conserved residue present in most G protein α subunits outside the α_s family, these observations may have general significance for many G proteins. Deamidation may be a component of a novel process for modifying or adapting cellular responses mediated by G proteins.

Heterotrimeric G proteins are integral parts of a major mechanism used by cells to respond to their extracellular environment (*1*−*5*). These proteins mediate the effects of possibly hundreds of receptors, allowing them to have a primary role in integrating cellular responses to multiple signals (*6*). Signal integration is one role of G proteins likely explaining the diversity of subunit isoforms making up these proteins (*7*, *8*). In addition to regulation by receptors, a number of established and potential mechanisms modulate signaling through G proteins. These include receptor desensitization (*9*), which may also initiate switching the G protein preference of receptors (*10*), and attenuation of G protein signaling through RGS[1] proteins (*11*, *12*). There are other less well understood but emerging processes as well,

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[1] Abbreviations: GAP, GTPase-activating protein; MAP kinase, Mitogen-activated protein kinase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; *m*/*z*, mass/charge ratio; RGS, Regulators of G protein signaling; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; urea/SDS−PAGE, SDS−PAGE in the presence of 6 M urea; TCEP, tris(2-carboxyethyl)phosphine.

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such as receptor-stimulated cycling of α subunit palmitoylation (*13*, *14*), and activation of G proteins by accessory proteins (*15*, *16*).

The primary G protein in brain is G_{OA}, which mediates the inhibition of calcium channels (*17*), the activation of MAP kinase (*18*), the development of neuronal growth cones (*19*, *20*), and the regulation of vesicle trafficking (*21*−*24*). The G_O proteins constitute from 0.2% to 1% of brain particulate protein (*25*, *26*). Related to G_{OA} by immunological reactivity (*27*, *28*) and by primary sequence (*27*) is another protein called G_{OC}. A similar protein has been observed by many laboratories (*27*−*34*). The α subunit of this G protein, α_{OC}, is purified as a heterotrimer containing a population of $\beta\gamma$ dimers distinct from those associated with α_{OA} (*28*). Recently, we found that this protein accounts for as much as a third of all α_O protein in brain (McIntire, Dingus, Wilcox, and Hildebrandt, unpublished experiments). Although α_{OC} and α_{OA} are related to one another, the α_{OC} isoform is structurally distinct from α_{OA} (*30*, *32*, *35*). It is thought to differ from α_{OA} by some covalent modification (*28*), although previous efforts to identify this difference were not successful (*31*).

The α_{OA} and α_{OC} proteins have different mobilities by urea/SDS−PAGE (*30*, *32*). Despite this difference, the proteins were found to have little or no (<2 Da) difference in their molecular weights (*35*). Many arguments suggested that these proteins would differ at their N-termini, including differential processing of the related α_t protein (*36*, *37*) and a recently described novel modification of the N-terminus of the α_S protein (*38*). Surprisingly, the N-termini of α_{OA} and α_{OC} are identical; the difference between α_{OA} and α_{OC} was localized to the C-terminus (*35*).

Here, we report the location and identity of the difference between α_{OA} and α_{OC}. The α_{OC} protein was found to be a mixture of two isoforms containing Asp substitutions for either Asn346 or Asn347 of α_{OA}. These substitutions are in a conformationally sensitive part of the C-terminus (*39*−*41*) that responds to activated receptor and initiates the G protein activation process (*39*). One of these residues, Asn347, is found in most G protein α subunits outside the $G_s\alpha$ family and is crucial for receptor activation (*42*). Since the work here shows that α_{OC} is a deamidated form of α_{OA}, deamidation may be part of a G protein regulatory mechanism involved in attenuation or modulation of receptor responses.

MATERIALS AND METHODS

Purification of G Protein Isoforms. G proteins were purified from bovine brain using a modification (*43*, *44*) of the method of Sternweis and Robishaw (*25*). Isoforms of G proteins were purified using a MonoQ anion exchange column with a 0−300 mM NaCl gradient (*28*). G protein subunits were separated in the presence of aluminum, magnesium, and fluoride as described previously (*43*, *44*).

Proteolytic Digestion of G Protein α *Subunits*. For digestion with trypsin, 3 nmol of purified α_{OA} and α_{OC} were boiled for 10 min, and then precipitated with nine volumes of ice-cold acetone. The precipitates were resuspended in 150 μL of 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.01% of the nonionic detergent Thesit (polyoxyethylene 9 lauryl ether, Sigma) containing TPCK-treated trypsin (Sigma, 1:50 w/w) and digested at 32 °C overnight. For digestion with chymotrypsin, 1.5 nmol of isolated α_{OA} and α_{OC} was precipitated in nine volumes of ice-cold acetone. Precipitates were resuspended in 40 μL of 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 mM $CaCl_2$, and 0.01% Thesit containing sequencing grade chymotrypsin (Boehringer Mannheim, 1:50 w/w, protease/protein) and digested for 30 min at 25 °C.

HPLC Separation of Digested Proteins. Proteolytic fragments of α_{OA} and α_{OC} were separated on a 4.6 × 30 mm Aquapore C8 RP300 column (Brownlee). Buffer A was 10% acetonitrile, 0.1% TFA in water, and buffer B was 75% acetonitrile, 25% 2-propanol, 0.095% TFA. A linear gradient of 2%−80% B over 60 min (trypsin) or 2%−60% over 30 min (chymotrypsin) at 0.5 mL/min was used, and absorbance at 214 nm was measured during the gradient. Five percent of the eluate was analyzed by a Finnigan LCQ ion trap mass spectrometer with an electrospray ionization (ESI) source while the other 95% was collected as fractions at 30 or 60 s intervals.

Methyl Esterification of Proteolytic Fragments and MALDI Mass Spectrometry. HPLC fractions containing peptides were lyophilized and reduced with 1.5 μL of 100 mM aqueous tris(2-carboxyethyl)phosphine (TCEP) for 20 min, and then lyophilized again. Peptides were esterified with 2 N methanolic HCl, prepared according to the method of Knapp (*45*), in which 150 μL of acetyl chloride was added dropwise to 1 mL of ice-cold methanol, while stirring. Approximately 1.5 μL of 2 N methanolic HCl was added to the dried samples, which were incubated at room temperature for various times, and then lyophilized. MALDI (matrix-assisted laser desorption ionization) mass analysis using a Voyager-DE MALDI mass spectrometer (PerSeptive Biosystems) was performed on samples with the matrix α-cyano-4-hydroxycinnamic acid (Aldrich), in 70% acetonitrile, 0.1% TFA. Samples were spotted on a sample plate and allowed to crystallize at room temperature. Masses were internally calibrated with lys-bradykinin, 1188.2, and insulin, 5734.5. Typically 256 laser shots were averaged to produce a mass spectrum.

Electrospray Ionization Mass Spectrometry (*ESI-MS*). C-terminal peptides from α_{OA} and α_{OC} were analyzed by a Finnigan LCQ ion trap mass spectrometer with a standard ESI source either directly during HPLC separation or by concentrating HPLC fractions and performing nanospray ESI-MS. The nanospray source was constructed in house and used a pulled glass capillary, situated approximately 1 mm from the heated metal capillary, with an applied voltage of 1500 V. The capillary was loaded with 1−2 μL of sample solubilized in 47% water/47% methanol/6% acetic acid, delivered at a flow rate of nL/min. Tandem mass spectrometry was achieved by selection and fragmentation of the precursor ion of interest with a window of 2 *m*/*z* units. Mass spectra were then collected for the fragment ions. Predicted *m*/*z* values were generated with MacBioSpec software v. 1.0.1 (PE SCIEX Instruments, 1992, Thornhill Ontario, Canada).

Edman Sequencing. Aliquots of HPLC fractions from the separation of chymotryptic digests of α_O subunit isoforms were sequenced by Edman Degradation on an ABI 494 Protein Sequencer in the MUSC Protein Chemistry Facility.

RESULTS

The α_{OA} and α_{OC} proteins have different mobilities by urea/SDS−PAGE (*30*, *32*) but have little or no (<2 Da) difference in their molecular weights (*35*). On the basis of proteolytic mapping studies, and by using this difference in electrophoretic mobility, we were able to localize the site of the difference between α_{OA} and α_{OC} to a 17 kDa C-terminal fragment (*35*). To determine the nature of the difference between these α subunits, 17 kDa C-terminal fragments from α_{OA} and α_{OC} were further fragmented and characterized by mass spectrometry and by Edman degradation. After digestion of either protein with trypsin, five major peptides from the 17 kDa C-terminal fragment could be found by monitoring the HPLC effluent by ESI-MS (Figure 1). Selected ion chromatograms, obtained by specifying the mass (±0.5 Da) of the predicted peptide fragments, located the distribution of each peptide during the separations (Figure 1B,C). The identities of these fragments were established by their intact masses and by their partial MS/MS fragmentation patterns (data not shown). From the selected ion chromatograms there were no obvious differences in mass or retention time of the five peptides from α_{OA} and α_{OC} (Figure 1). Fractions containing these peptides were then used for more detailed characterizations to determine sites of structural differences in the proteins.

Methyl Esterification of Peptides from C-terminal Fragments of α_{OA} and α_{OC}. The difference between α_{OA} and α_{OC} was previously shown to be 2 Da or less for the intact proteins, and for their 17 kDa C-terminal fragments (*35*). One covalent modification consistent with this small mass difference is deamidation, a structural change which converts Asn and Gln to Asp and Glu, respectively. This modification generates an additional carboxyl group in proteins, yet changes the mass by a single mass unit. To evaluate this hypothesis, and to identify a possible peptide fragment containing a deamidation site, HPLC fractions containing the tryptic peptides from the 17 kDa region of α_{OA} and α_{OC} were analyzed by MALDI-MS after methyl esterification of the available carboxyl groups in the peptides (Figure 2). Methyl esterification of a carboxyl group causes a 14 Da shift in the mass of the peptide, a mass change readily detectable by MALDI-MS.

The five major tryptic fragments (Figure 1, P1−P5) cover all but 8 amino acids in the 17 kDa fragment and contain all of the Asn and Gln residues (which are the potential deamidation sites) in this region. Sites predicted to be available for methyl esterification, including Asp and Glu residues and carboxy termini generated from tryptic digestion, are designated with asterisks (Figure 1). MALDI mass spectra before and after methyl esterification, for both α_{OA} and α_{OC} digests, are shown for P1 in Figure 2A, for P3 in Figure 2B, and for P2, P4, and P5, which coelute in the same fraction, in Figure 2C. The reaction times for methyl esterification were chosen so as to count the number of free carboxyl groups in each peptide. For P1, P2, and P3, α_{OA} and α_{OC} digests had equivalent methyl esterification patterns. For P1 and P3, these were entirely consistent with the predicted number of free carboxyl groups in the peptides (Figure 2A,B). For P2, the parent mass had a second peak 16 Da larger than predicted and consistent with the presence of an oxidized residue. This peak was the same in α_{OA} and α_{OC} digests and corresponds to an analogous peak 16 Da larger than P2 before methylation. Therefore, the seventh peak in the methyl esterification spectra for peptide P2

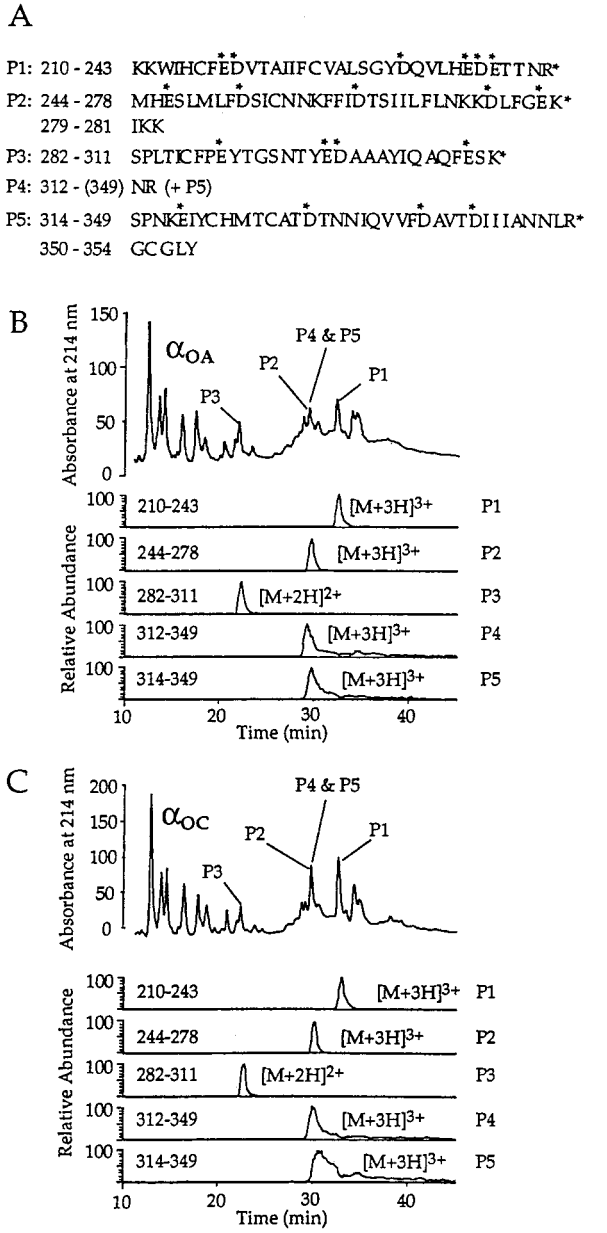


FIGURE 1: Separation of tryptic peptides generated from α_{OA} and α_{OC} and identification of fractions containing fragments from the 17 kDa C-terminal domain. (A) Sequence of α_{OA} broken into major peptides generated by tryptic cleavage and labeled P1, P2, P3, P4, and P5. Two short sequences (279−281 and 350−354) are not analyzed here. P4 and P5 differ only in the first two residues in P4 that are missing in P5. Asterisks indicate the sites of carboxyl groups that would be available for methyl esterification. (B) α_{OA}. (C) α_{OC}. Digests were generated and separated by HPLC as described under Experimental Procedures. Separations were monitored by absorbance at 214 nm and by in ESI-MS on a Finnigan LCQ ion trap mass spectrometer. For each separation the 214 nm absorbance profile is shown along with the selected ion chromatograms for five major peptides generated from the 17 kDa C-terminal fragment, showing the abundance of the respective *m/z* ions during the elution. Predicted *m/z* values used for selected ion chromatograms are the following: 282−311 $[M + 2H]^{2+} = 1673.9$, range = 1673.4−1674.4; 244−278 $[M + 3H]^{3+} = 1385.3$, range = 1384.8−1385.8; 312−349 $[M + 3H]^{3+} = 1437.3$, range = 1436.8−1437.8; 314−349 $[M + 3H]^{3+} = 1347.2$, range = 1346.7−1347.7; and 210−243 $[M + 3H]^{3+} = 1328.5$, range = 1328.0−1329.0.

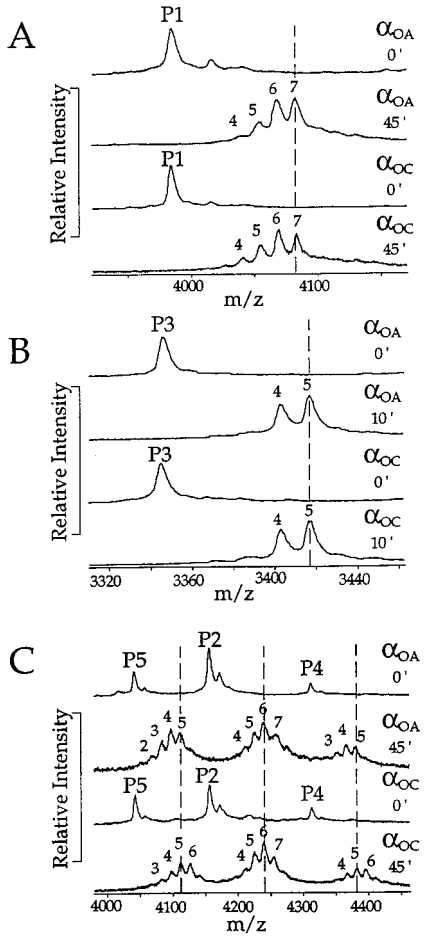


FIGURE 2: Methyl esterification of tryptic peptides generated from the 17 kDa fragments of α_{OA} and α_{OC}. (A) MALDI mass spectra of P1 peptides from α_{OA} and α_{OC} before and after (45 min) methyl esterification. Numbers above peaks indicate the number of methyl groups that were incorporated into the each peptide. (B) Same as in A but for P3. (C) Same as in A but for P2, P4, and P5, which were all recovered in the same fraction. Dotted lines align the methylated peptides with the highest common derivatization between α_{OA} and α_{OC}.

(Figure 2C) was most likely the oxidized form of P2.

Peptides P4 and P5 are related fragments resulting from tryptic cleavage after Lys311 and Arg313 in the α_O sequence and extending to Arg349. Both peptides were recovered in the same fraction (Figure 2C). The methyl esterification patterns of these two peptides were clearly different in digests from α_{OA} and α_{OC} (Figure 2C). Both the P4 and P5 peptides from the α_{OC} digest had one more methyl esterification site than did the peptides from α_{OA}. Whereas 5 sites are predicted, and were found in the α_{OA} fragments, 6 sites were found in P4 and P5 from α_{OC}. This suggests there is one additional carboxyl group available for methyl esterification in residues 314−349 of α_{OC}, compared to α_{OA}; this would be the case if one of the five Asn or one Gln residues in this peptide was deamidated in α_{OC}.

Chymotryptic Digestion and Mass Spectrometric Analysis of the C-terminus of α_{OA} and α_{OC}. Differential recognition of α_{OC} and α_{OA} by a polyclonal antisera made to a decapeptide homologous to the C-terminus of α_O, and subtle differences in MS/MS data acquired from the 314−349 peptide (data not shown), focused our efforts on the extreme C-terminus of α_{OC}. To obtain additional support for a

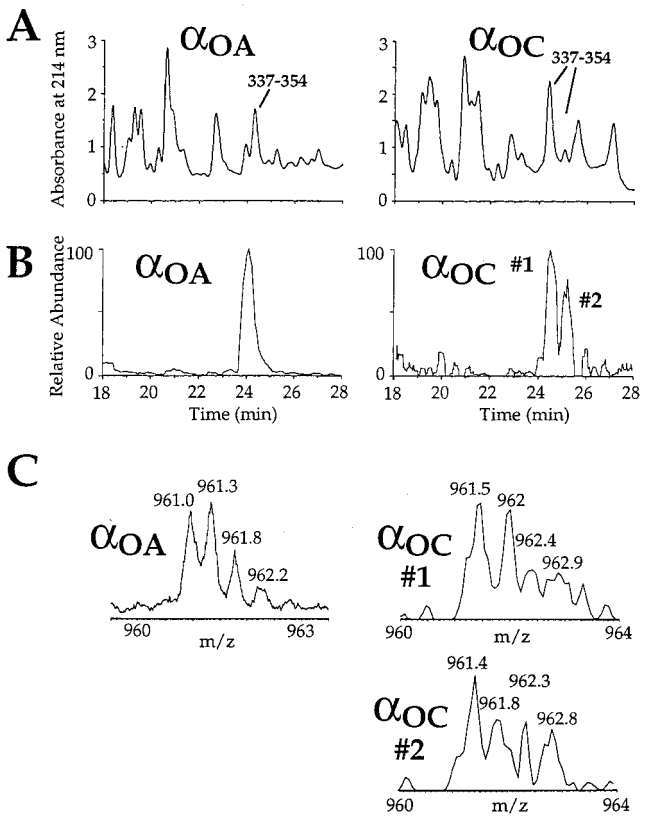


FIGURE 3: Analysis of the C-terminal peptides obtained from the chymotrypsin digest of α_{OA} and α_{OC}. Analysis of the C-terminus of α_{OA} and α_{OC} by HPLC and LCQ mass spectrometry. (A) Separation of chymotryptic digests of α_{OA} and α_{OC} showing the absorbance at 214 nm; indicated are peaks corresponding to the elution of the C-terminal peptide 337−354. (B) Selected ion chromatograms for the predicted monoisotopic $[M + 2H]^{2+}$ ion for 337−354 for α_{OA} (m/z = 961.0) and the $[M + 2H]^{2+}$ ion for 337−354 for α_{OC} (m/z = 961.5), considering the one deamidation site predicted from Figure 2. Note the resolution of the 337−354 peptide into two peaks, #1 and #2, for α_{OC}. (C) Isotopic cluster of the $[M + 2H]^{2+}$ ions for peaks in B: α_{OA} and α_{OC} #1 and α_{OC} #2. Note that the isotopic cluster is shifted 0.5 m/z units higher in α_{OC} for both peak #1 and peak #2, consistent with one deamidation site.

deamidated site in α_{OC} and to identify the site, a smaller C-terminal peptide was produced from the α_O isoforms by digesting them with chymotrypsin. Digests were separated by HPLC (Figure 3A) and analyzed by ESI-MS. Fractions containing peptides corresponding to 337−354 of α_{OA} and α_{OC} were identified in selected ion chromatograms for the doubly charged 337−354 fragment from α_{OA} (m/z 961.0) and for the comparable doubly charged fragment from α_{OC} with one deamidation site (m/z 961.5) (Figure 3B). While the selected ion chromatogram for α_{OA} described only one peak, the selected ion chromatogram for α_{OC} resolved the C-terminal peptide into two peaks, #1 and #2 (Figure 3B). The predicted monoisotopic m/z of the 337−354 peptide from α_{OA} in the absence of any deamidation and having two charges is 961.0, which corresponds to a mass of 1920 Da. Mass spectral data from α_{OA} are in perfect agreement with this predicted mass (Figure 3C). This spectrum shows the expanded region around the signal at 961.0 m/z, which has additional characteristic peaks at 0.5 m/z unit intervals corresponding to peptides containing 0, 1, 2, and 3 ^{13}C atoms according to the naturally occurring abundance of this isotope. In contrast, the m/z of the analogous charged

peptides from α_{OC} in both peaks #1 and #2 was 961.5 (Figure 3C), which corresponds to a mass of 1921, 1 Da higher than the predicted mass, and accounts for the one deamidation site predicted from the methyl esterification data (Figure 2C). These data identified the peptide 337−349 as the region of α_{OC} that differs from α_{OA}.

To characterize the exact location of the 1 Da difference, the chymotryptic C-terminal fragments from α_{OA} and α_{OC} were sequenced by tandem mass spectrometry on a Finnigan LCQ ion trap mass spectrometer. Sequence data from α_{OA} are consistent with the predicted sequence including two Asn residues located at 346 and 347 in the α_O sequence (Figure 4A). In contrast, sequence data from peaks #1 and #2 of the α_{OC} digest identified Asp residues at positions 346 and 347, respectively. Other than the change of Asn to Asp, no other difference from the C-terminal peptide from α_{OA} was found. This sequence difference is reflected in the 1 Da larger observed masses beginning with ions b_{10} and y_9 in the MS/MS data for the α_{OC} peptide #1 (Figure 4B), compared to the α_{OA} peptide (Figure 4A). These ions implicate Asn346 as the deamidation site in α_{OC} peptide #1. In the case of α_{OC} peptide #2 (Figure 4C), the MS/MS data show a b_{10} ion 1 Da lower than the b_{10} ion in α_{OC} peptide #1 (Figure 4B), suggesting that residue 346 in α_{OC} peptide #2 is the predicted Asn. However, ions beginning with b_{12} and y_9 in α_{OC} peptide #2 are 1 Da higher than the α_{OA} peptide, implicating residue 347 as the deamidation site in α_{OC} peptide #2. These data characterize the identity of α_{OC} as resulting from the conversion of either Asn346 or Asn347 in α_{OA} into Asp residues.

Edman Analysis of the C-Terminus of α_{OA} *and* α_{OC}. As a complementary technique to compare the sequence of α_{OA} and α_{OC}, HPLC fractions of the C-terminal peptide 337−354 from α_{OA} and of peaks #1 and #2 from α_{OC} were analyzed by Edman degradation. The sequence of α_{OA} was in agreement with the predicted sequence (Figure 5), as well as the mass spectrometric data (Figure 4A). Peak #1 from α_{OC} was in agreement with the analogous mass spectrometric data in Figure 4B, describing an Asp at position 346 instead of an Asn. Sequence analysis of peak #2 from α_{OC} also agreed with the mass spectrometric data in Figure 4C, identifying Asn347, instead of Asn346, as the residue converted to an Asp.

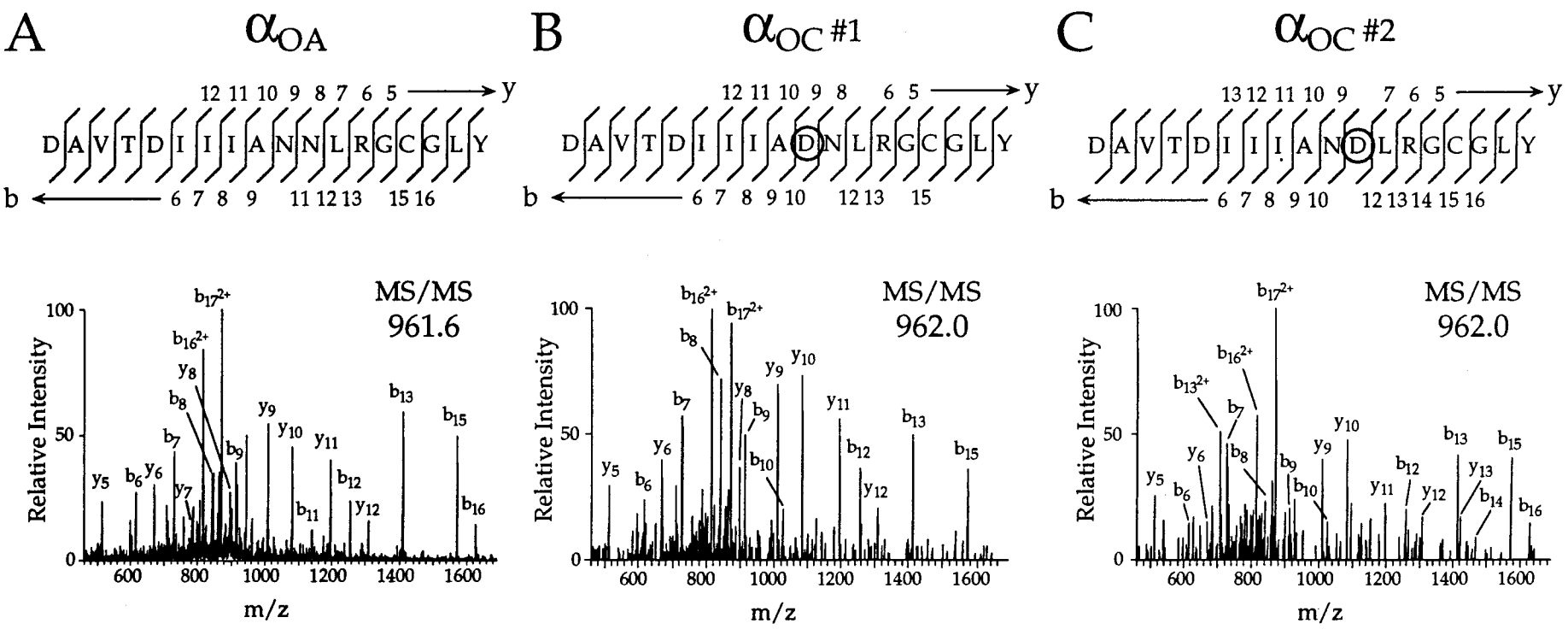
DISCUSSION

The α_{OC} variant of α_O has been observed by a large number of laboratories (*27*, *28*, *30*, *31*, *33*, *34*, *46*). It is present in brain of all mammalian species so far investigated (*30*). Recently, we have shown that this protein accounts for about a third of all of the G_O protein present in brain cortex, where it is expressed in a constant ratio to α_{OA} (McIntire, Dingus, Wilcox, and Hildebrandt, unpublished experiments). Interestingly, α_{OC} does not appear to be found in heart (*47*), which also expresses α_{OA}, so it may arise by some mechanism specific to brain. The G_{OA} and G_{OC} proteins are functionally different in that they are isolated as heterotrimers containing different $\beta\gamma$ populations (*28*).

Here we report that the α_{OC} isoform consists of deamidated forms of α_{OA} in which either Asn346 or Asn347 is converted to an Asp residue. The identification of this change as the structural difference between α_{OA} and α_{OC} is consistent with previous observations in the literature, as α_{OC} was noted to elute at higher salt concentrations by MonoQ anion-exchange chromatography (*28*) and was also reported to have a slightly more acidic pI than α_{OA} (*31*). Other additional structural differences between α_{OA} and α_{OC} are unlikely. One paradoxical observation about these proteins was their clear difference in electrophoretic mobility by urea/SDS−PAGE even though the proteins have a negligible difference in mass (*35*). This was true not only of the intact proteins but also of the 17 kDa fragments of the proteins characterized to contain the difference between them. The change from Asn to Asp at a single site explains these data since this induces only a 1 Da difference in the two proteins. Numerous arguments suggested that the difference between the two proteins was at their N-termini, where the proteins are multiply modified, but α_{OA} and α_{OC} have identical N-terminal peptides (*35*). Thus, extensive structural characterization of these proteins narrowed their site of difference down to a single region of the protein and data here explain the differences in this region.

There are several possible mechanisms by which Asn346 and Asn347 could be changed to Asp residues. They could arise by alternative splicing of mRNAs, as do α_{OA} and α_{OB}, by RNA editing, as recently described for the 5HT-2C receptor (*48*), or by deamidation of Asn to Asp. Alternative splicing and RNA editing seem less likely than deamidation since no specific cDNAs coding for Asp346 or Asp347 have been described although α_{OC} is nearly as abundant at the protein level (60%) as α_{OA} is (McIntire, Dingus, Wilcox, and Hildebrandt, unpublished experiments). In addition, explaining our results would necessitate postulating the existence of two additional mRNAs, one coding for Asp346 and the other for Asp347. Thus, although alternative spicing and RNA editing cannot be explicitly ruled out from our data, it seems much more likely that α_{OC} arises by deamidation of α_{OA}.

Deamidation as a post-translational modification can occur by several mechanisms. For example, in bacterial chemotaxis, the chemoreceptor proteins themselves contain Gln residues that are enzymatically deamidated during the signal transduction process (*49*, *50*). Further, it has recently been shown that dermonecrotizing factor from *Escherichia coli* can catalyze the deamidation Gln63 of the GTP binding protein Rho, causing constitutive activation of the protein (*51*−*53*). Alternatively, nonenzymatic deamidation has been observed in proteins as an age-related phenomenon in cells with little or no protein turnover, or in vitro under certain conditions (*54*, *55*). The β-aspartyl shift mechanism is one source of nonenzymatic deamidation in proteins, which proceeds via formation of a cyclic imide from an Asn residue, particularly when followed by Gly or Ser (*56*, *57*). The imide ring can open in two ways, generating either aspartic acid or isoaspartic acid. Thus, one hallmark of the β-aspartyl shift mechanism is the production of both isoaspartic acid and aspartic acid, often in a ratio of 3:1 (*58*, *59*). The sequence at the deamidation sites in α_{OA} is not favorable for this reaction, however, and we did not find a block to Edman sequencing at the deamidation site, which would be expected if the peptides contained large amounts of isoaspartate at that site. It will be important to determine the mechanism of this deamidation, as there are several conformationally determined autocatalytic mechanisms or enzy-



Ion	Predicted $[M+H]^{1+}$	Observed	Ion	Predicted $[M+H]^{1+}$	Observed
b_6	615.3	615.1	y_5	512.2	512.2
b_7	728.4	728.3	y_6	668.3	668.2
b_8	841.5	841.1	y_7	781.4	781.3
b_9	912.5	912.2	y_8	895.5	895.5
b_{10}	1026.6	n.d.	y_9	1009.5	1009.4
b_{11}	1140.6	1140.4	y_{10}	1080.5	1080.6
b_{12}	1253.7	1253.5	y_{11}	1193.6	1193.5
b_{13}	1409.8	1409.6	y_{12}	1306.7	1306.7
b_{14}	1466.8	n.d.	y_{13}	1419.8	n.d.
b_{15}	1569.8	1569.7			
b_{16}	1626.8	1627.0			

Ion	Predicted $[M+H]^{1+}$	Observed	Ion	Predicted $[M+H]^{1+}$	Observed
b_6	615.3	614.8	y_5	512.2	512.0
b_7	728.4	728.1	y_6	668.3	668.3
b_8	841.5	841.2	y_7	781.4	n.d.
b_9	912.5	912.3	y_8	895.5	895.3
b_{10}	1027.5	1027.1	y_9	1010.5	1010.5
b_{11}	1141.6	n.d.	y_{10}	1081.5	1081.2
b_{12}	1254.7	1254.4	y_{11}	1194.6	1194.4
b_{13}	1410.8	1410.4	y_{12}	1307.7	1307.7
b_{14}	1467.8	n.d.	y_{13}	1420.8	n.d.
b_{15}	1570.8	1570.6			
b_{16}	1627.8	n.d.			

Ion	Predicted $[M+H]^{1+}$	Observed	Ion	Predicted $[M+H]^{1+}$	Observed
b_6	615.3	615.0	y_5	512.2	511.9
b_7	728.4	728.1	y_6	668.3	668.4
b_8	841.5	841.0	y_7	781.4	781.2
b_9	912.5	912.2	y_8	896.4	n.d.
b_{10}	1026.6	1026.0	y_9	1010.5	1010.5
b_{11}	1141.6	n.d.	y_{10}	1081.5	1081.7
b_{12}	1254.7	1254.2	y_{11}	1194.6	1194.2
b_{13}	1410.8	1410.7	y_{12}	1307.7	1307.7
b_{14}	1467.8	1467.8	y_{13}	1420.8	1420.7
b_{15}	1570.8	1570.6			
b_{16}	1627.8	1627.8			

FIGURE 4: Sequencing by tandem mass spectrometry of the C-terminal peptides obtained from chymotrypsin digests of α_{OA} and α_{OC}. MS/MS analysis of the 337−354 peptide for α_{OA} and α_{OC}. (A) MS/MS spectra of the 337−354 peptide from α_{OA} described in Figure 3. (B) Same as A, but for peak #1 from α_{OC}. (C) Same as A, but for peak #2 from α_{OC}. Masses for the predicted and observed b and y ions (*61*) are illustrated for α_{OA}, α_{OC} peak #1, and α_{OC} peak #2. The observed b and y ions in α_{OC} peak #1 and α_{OC} peak #2 are compatible with deamidation at Asn 346 and Asn 347, circled in the sequences above. n.d. indicates not determined.

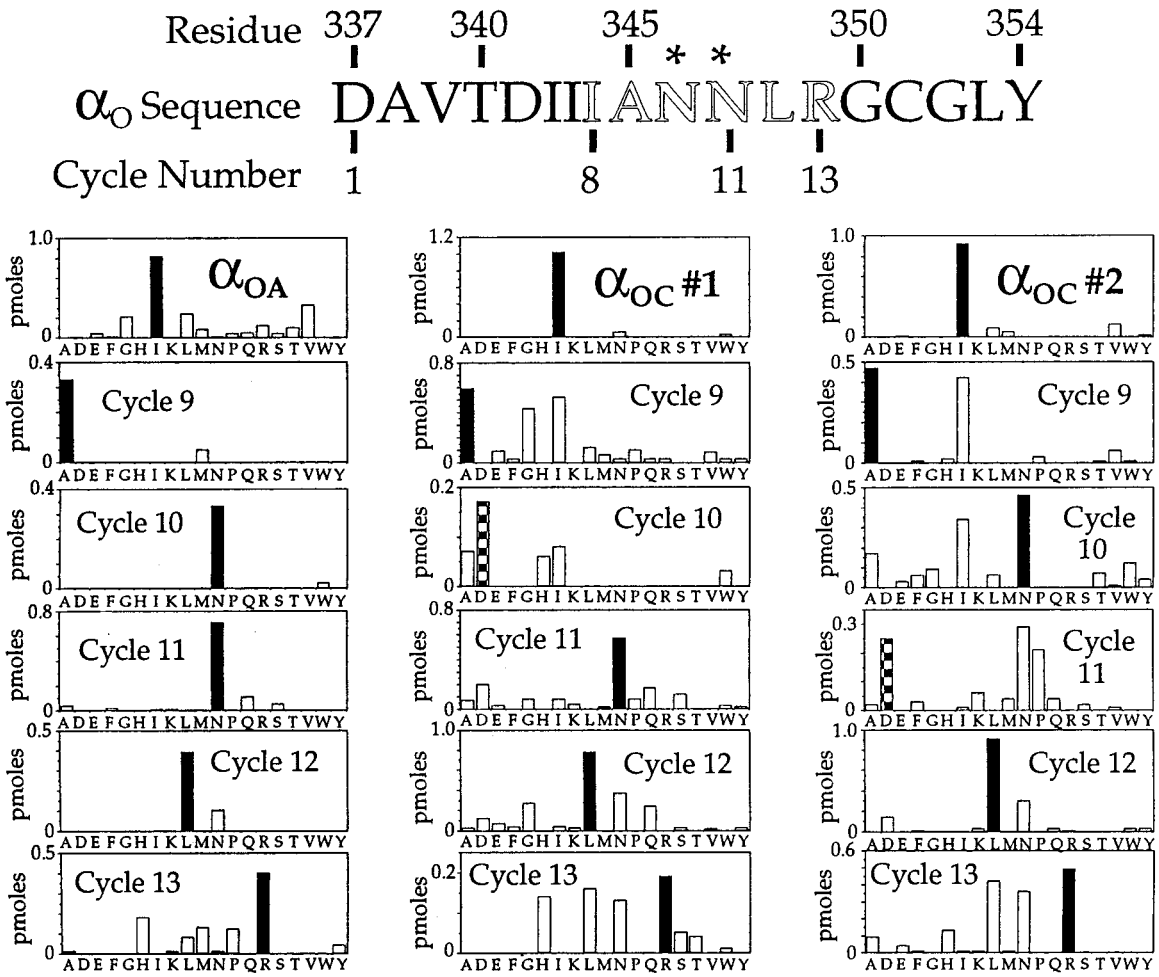


FIGURE 5: Sequencing by Edman degradation of the C-terminal peptides obtained from chymotrypsin digests of α_{OA} and α_{OC}. Across the top is the predicted sequence of α_{OA} for residues 337−354, corresponding to the chymotryptic peptide isolated in experiment in Figure 3. Fractions containing this peptide from α_{OA} or two peptides with the mass of deamidated analogues recovered from the digest of α_{OC} were sequenced by Edman degradation as described under Experimental Procedures. Shown in the bar graphs are the amino acid signals at each cycle corrected for background and individual signal intensity of the derivatized amino acids. Data for cycles 8−13 are shown in the figure; the corresponding residues in α_{OA} are denoted in the sequence at the top by outlined letters. Black bars show the corrected signal intensity for the expected residue from the α_{O1} sequence in each cycle. Hatched bars show the signals for Asp where an Asn was expected in the two α_{OC} peptides. Asterisks in the sequence above mark the sites of Asn variably identified in the sequencing records.

matic mechanisms that could mediate this reaction and would be of substantial biological significance.

The functional significance of the deamidation of α_{OA} reported here is suggested by the site of modification. Asn346 and Asn347 are at the C-terminus of the protein and in a region predicted to interact with receptors (*5*). We do not know the relative importance of the two different deamidation sites; however, it is interesting to note that we did not find an α_{OC} species deamidated at both Asn346 and Asn347. This suggests that deamidation at one site precludes deamidation at the other. An Asn at the site corresponding to Asn346 is found in relatively few G protein α subunits; α_{OA}, α_{i1}, α_{i2}, and α_z. An Asn corresponding to Asn347, however, is conserved in most G protein α subunits outside of the α_S family. The homologue of Asn347 in α_t is a predicted contact site with rhodopsin, and its mutation to an Ala decreases the ability of α_t to interact with or respond to rhodopsin (*42*). In the existing X-ray structures of G protein heterotrimers (*41*, *60*) the very C-terminus of the α subunit has an undefined structure but projects from a long α helix (helix α5) which terminates at Asn347 (*41*). On the basis of the NMR of a C-terminal peptide of α_t and its interaction with rhodopsin, the activated receptor induces formation of an extended helix projecting through Asn347 all the way to the C-terminus (*39*). This conformational change is proposed to induce the subsequent decrease in GDP affinity resulting in G protein activation. Deamidation of Asn347 (and possibly Asn346) is likely to have profound effects on the signaling properties of the proteins. In fact, α_{OC} has been reported to be less responsive to the muscarinic receptor than α_{OA} is (*31*). Deamidation of Asn residues at a critical site in the receptor-binding domain of G protein of α subunits is likely to be part of an important regulatory mechanism modulating G protein signaling.

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