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# Identification of Amine Components in a Glycolipid Membrane-Binding Domain at the C-Terminus of Human Erythrocyte Acetylcholinesterase†

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ABSTRACT: Purified human erythrocyte acetylcholinesterase was labeled by reductive radiomethylation with saturating amounts of [$^{14}$C]formaldehyde and sodium cyanoborohydride. Acid hydrolysis and automated amino acid analysis permitted both identification of radiomethylated components by their coelution with radiomethylated standards and quantitation of these components. The methylated N-terminal amino acids glutamate and arginine were observed at levels of 0.66 and 0.34 residues, respectively, per 70-kilodalton subunit, and lysine residues were methylated on their $\epsilon$-amino groups to a level of 7.40 residues per subunit [Haas, R., & Rosenberry, T. L. (1985) *Anal. Biochem. 148*, 154-162]. In addition, each subunit contained 1.35 residues of methylated ethanolamine and 0.98 residue of methylated glucosamine. Papain digestion cleaved the intact enzyme into two fragments, an enzymatically active hydrophilic fragment and a small hydrophobic fragment that represented the membrane-binding domain. The radiomethylated amino acids were quantitatively retained in the hydrophilic fragment, while the methylated ethanolamine and glucosamine were confined exclusively to the hydrophobic domain fragment. This fragment included the C-terminal dipeptide of the subunit. Peptide sequencing by manual Edman methods was combined with radiomethylation to demonstrate the sequence His-Gly-ethanolamine-Z for the hydrophobic domain fragment. The ethanolamine residue in this sequence is in amide linkage to the C-terminal Gly and is clearly distinct from the ethanolamine residues in Z which are susceptible to radiomethylation in the intact enzyme. Since Z also includes glucosamine and 2 mol of fatty acids [Roberts, W. L., & Rosenberry, T. L. (1985) *Biochem. Biophys. Res. Commun. 133*, 621-627], we conclude that the membrane-binding domain of human erythrocyte acetylcholinesterase is a covalently linked glycolipid at the C-termini of the subunits. Analogies to the membrane-binding domains of murine Thy-1 glycoprotein and trypanosome variant surface glycoproteins are discussed.

Several forms of acetylcholinesterase (AChE,[1] EC 3.1.1.7) are found in vertebrate tissues [see Massoulié & Bon (1982) and Rosenberry (1985)]. One major class of AChE forms corresponds to integral membrane proteins in brain and muscle, and this class is also represented by a dimeric ($G_2$) form (RBC AChE) that is the only AChE present in human erythrocyte membranes. We have purified RBC AChE to homogeneity (Rosenberry & Scoggin, 1984) and demonstrated that it is an amphipathic protein with a small hydrophobic domain that can be cleaved from the remaining enzymatically active hydrophilic fragment by papain (Dutta-Choudhury & Rosenberry, 1984). Papain cleavage dissaggregates detergent-free RBC AChE, abolishes the interaction of RBC AChE with Triton X-100 micelles, and releases RBC AChE from the membranes of reconstituted small liposomes (Dutta-Choudhury & Rosenberry, 1984; Kim & Rosenberry, 1985). In the preceding paper (Roberts & Rosenberry, 1986a), we showed that [$^{125}$I]TID is a selective radiolabel of the RBC AChE hydrophobic domain and that this label can be used to monitor the isolation of the hydrophobic domain fragment produced by papain. Amino acid compositions of the isolated fragment revealed only small amounts of amino acids inconsistent with a hydrophobic peptide domain (Roberts & Rosenberry, 1986a), but methanolysis of either intact RBC AChE or the isolated hydrophobic domain fragment released 2 mol of fatty acid esters per mole of domain (Roberts & Rosenberry, 1985).

Reductive radiomethylation, another useful procedure for labeling RBC AChE (Haas & Rosenberry, 1985), also provides important information about the hydrophobic domain

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[1] Abbreviations: AChE, acetylcholinesterase; RBC AChE, human erythrocyte acetylcholinesterase; $G_n$, a globular AChE form with $n$ catalytic subunits; [$^{125}$I]TID, 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)-diazirine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ATZ, 2-anilino-5-thiazolinone; PIPLC, phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*; mfVSG, membrane-bound form of trypanosome variant surface glycoprotein; kDa, kilodalton(s).

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that is the focus of this report. Reductive methylation with [$^3$H]- or [$^{14}$C]HCHO and $NaCNBH_3$ radiolabels the primary and secondary amines in RBC AChE. Both mono- and dimethylamine derivatives are produced that are stable to acid hydrolysis, and the hydrolyzed radiomethylated amino acids and amines can be identified on an amino acid analyzer. In this paper, we utilize reductive radiomethylation to localize the hydrophobic domain at the C-terminus of the catalytic subunits of RBC AChE and to identify ethanolamine and glucosamine as components of the hydrophobic domain.

## Materials and Methods

*Radiomethylation of RBC AChE.* RBC AChE was extracted from outdated erythrocytes with Triton X-100 and purified by affinity chromatography on acridinium resin (Rosenberry & Scoggin, 1984). Preparations to be radiomethylated at high specific activity were chromatographed on hydroxyapatite to remove Triton X-100 (Rosenberry & Scoggin, 1984), dialyzed against 5 mM sodium phosphate, pH 7, concentrated 5–30-fold in a Speedvac concentrator (Savant Instruments Inc.), reductively methylated with 10 mM [$^{14}$C]HCHO (ICN, 40 mCi/mmol) and 50 mM $NaCNBH_3$ (Haas & Rosenberry, 1985), and dialyzed extensively. Larger scale RBC AChE preparations were radiomethylated without Triton X-100 removal under identical conditions at a lower specific activity (0.5–3.0 mCi/mmol, by dilution with unlabeled HCHO) following elution from the affinity resin and dialysis and concentration as outlined above. The presence of 1–5% Triton X-100 had no effect on the extent of methylation or the distribution of the radiolabel in RBC AChE.

Dialyzed, radiomethylated RBC AChE was repurified by affinity chromatography on acridinium resin following additions of Triton X-100 (to 0.1%) and of a trace amount of [$^{125}$I]TID-labeled RBC AChE (Roberts & Rosenberry, 1986) to provide a marker for the hydrophobic domain. About 70–90% of the nondialyzable $^{14}$C label was typically retained on the affinity resin and eluted as enzymatically active RBC AChE with 10 mM decamethonium bromide and 20 mM sodium phosphate (pH 7) in 0.1% Triton X-100.

*Isolation of Fragments following Papain Digestion of RBC AChE in Triton X-100.* Repurified radiomethylated RBC AChE was dialyzed extensively and digested with activated papain resin (Dutta-Choudhury & Rosenberry, 1984), and the hydrophilic and hydrophobic fragments were isolated by affinity chromatography on acridinium resin and gel exclusion chromatography as outlined in Roberts and Rosenberry (1986a).

*Isolation of Fragments following Papain Digestion of Liposomal RBC AChE.* RBC AChE radiomethylated in the absence of Triton X-100 was further dialyzed against Tris buffer (10–20 mM Tris-HCl, pH 7.4) and reconstituted into small liposomes (RBC AChE, 420 units/mL; crude egg yolk phospholipid, 50 mg/mL; 31 mL) as outlined in Kim and Rosenberry (1985). The reconstituted small RBC AChE liposomes were purified by affinity chromatography on acridinium resin, dialyzed against Tris buffer, and digested at 25 °C with two cycles of activated soluble papain (RBC AChE, 9000 units; 0.7 mg of papain in each 1-h cycle; 25 mL) as described by Kim and Rosenberry (1985). The digest was subjected to affinity chromatography on acridinium resin and fractionated into a nonretained pool that passed directly through the column with little or no retardation and two retained pools. The first retained pool, eluted with Tris-buffered 5 mM decamethonium bromide following a Tris buffer wash, was enriched in residual intact RBC AChE liposomes (Kim & Rosenberry, 1985). The second retained pool was eluted with Tris-buffered 10 mM decamethonium bromide in 0.5 M NaCl following a wash with Tris-buffered 0.5 M NaCl and was enriched in the enzymatically active hydrophilic RBC AChE fragment released from the liposomes by papain. The retained pools were combined and dialyzed against Tris buffer for chromatography on Sepharose CL-4B.

*Radiomethylation of the RBC AChE Hydrophobic Domain Fragment.* Samples of hydrophobic domain fragment (0.4–3.2 nmol; 0.5–14 mL) isolated by chromatography on Sephadex LH-60 following papain digestion of $^{14}$C-radiomethylated RBC AChE were dried on 12 × 75 mm glass tubes in the Speedvac. Radiomethylation was conducted with 10 mM [$^3$H]HCHO (New England Nuclear, 75 mCi/mmol) and 50 mM $NaCNBH_3$ in sodium phosphate (20 mM, pH 7, 50 μL) for 15 min at 37 °C, and samples were dried for 2 h in the Speedvac prior to resuspension in 88% formic acid (1 mL) and repurification by Sephadex LH-60 chromatography.

*Radiomethylation of Amine Standards.* Samples (100 μL) of ethanolamine (Baker), glucosamine hydrochloride (Sigma), and galactosamine hydrochloride (Sigma) containing 1–20 mM amine, 1–2 mM [$^3$H]HCHO (84 mCi/mmol), and 50 mM $NaCNBH_3$ in sodium phosphate (20 mM, pH 7) were incubated for 15 min at 37 °C. Excess methylation reagents were either quenched by addition of a known amino acid (to 20 mM for 15 min at 37 °C) or removed by evacuation on the Speedvac. No correction was made for loss of radiolabel from these standards during acid hydrolysis (16 h). Comparison of analyzer recoveries of radiolabel from glucosamine before and after hydrolysis indicated a marginal loss of 11% ± 6% relative to radiomethylated quenching amino acids. The loss of $^{14}$C-methylated glutamate during hydrolysis was 5–7%, and this loss was included in the calculation of the methyl group specific activity (Haas & Rosenberry, 1985).

*Amino Acid and Radiomethylated Amine Analysis.* Acid hydrolysis, automated amino acid analysis, quantitation of radiolabeled components by single- or dual-isotope scintillation counting of the analyzer effluent, and determination of methyl group specific activity for undiluted [$^3$H]- or [$^{14}$C]HCHO stocks followed procedures in Haas and Rosenberry (1985). Methyl group specific activity in RBC AChE samples radiomethylated with low specific activity [$^{14}$C]HCHO and repurified by affinity chromatography was estimated by comparison of the observed ratio of $^{14}$C cpm incorporation to enzyme activity to the corresponding ratio for radiomethylation with undiluted [$^{14}$C]HCHO. The moles of RBC AChE and of the RBC AChE hydrophilic fragment produced by papain was calculated from the content of five selected amino acids (Haas & Rosenberry, 1985). The moles of the RBC AChE hydrophobic domain fragment was estimated from the content of radiomethylated ethanolamine and glucosamine (see Table I).

*Edman Sequencing.* RBC AChE hydrophobic domain fragment (1–10 nmol) isolated by chromatography on Sephadex LH-60 following papain digestion of $^{14}$C-methylated RBC AChE was dried on a 12 × 75 mm glass tube in the Speedvac. To the dried sample was added a 40-μL aliquot of pyridine (Baker)/triethylamine (Baker)/$H_2O$ (5:2:3) and 40 μL of 20% phenyl isothiocyanate (Pierce) in pyridine, and the mixture was incubated under $N_2$ for 5 min at 50 °C (Black & Coon, 1982). Following 0.5-mL additions of ethanol/88% formic acid (3:1) and 88% formic acid, the sample was applied to a 120-mL Sephadex LH-60 column (1.5 × 70 cm) equilibrated in ethanol/88% formic acid (3:1). The $^{14}$C-labeled peak was pooled, dried in the Speedvac, and incubated with 50 μL of trifluoroacetic acid (Pierce) under $N_2$ for 5 min at 50 °C to cleave the N-terminal amino acid as the ATZ derivative. The

mixture was dried in the Speedvac for 1 h prior either to radiomethylation or to another cycle of Edman degradation.

## RESULTS

*Isolation of the Hydrophilic and Hydrophobic Domain Fragments of RBC AChE following Reductive Methylation.* Radiomethylation of RBC AChE with 10 mM [$^{14}$C]HCHO under the conditions outlined under Materials and Methods is sufficient to convert nearly all amine groups in the enzyme to their labeled dimethylamine derivatives. Only a small fraction of the $\epsilon$-amino groups on lysine residues remain inaccessible [see Haas & Rosenberry (1985)]. To explore the distribution of the label, radiomethylated RBC AChE was reconstituted into liposomes and digested with papain. Digestion of liposomal RBC AChE had the advantage that subsequent fractionation by affinity and gel exclusion chromatography resulted in complete resolution of the hydrophilic and hydrophobic domain fragments from residual intact RBC AChE. The hydrophilic fragment remained fully active and, together with residual undigested RBC AChE liposomes, was retained on an acridinium affinity resin that bound to the enzyme active site. In contrast, the hydrophobic fragment remained embedded in the liposome membrane and passed directly through the affinity resin. The retained species were eluted with the competitive inhibitor decamethonium bromide and then separated by Sepharose CL-4B chromatography (Figure 1A). The earlier peak at 70 mL represented intact RBC AChE liposomes, as indicated by the correspondence both of its elution position and of its $^{14}$C cpm:enzyme activity ratio to those of nondigested RBC AChE liposomes [also see Kim & Rosenberry (1985)]. The later peak at 90 mL eluted at the position of the $G_2$ hydrophilic enzyme fragment (Dutta-Choudhury & Rosenberry, 1984; Kim & Rosenberry, 1985), and its $^{14}$C cpm:enzyme activity ratio was somewhat lower.

The hydrophobic domain fragment was isolated by the two-step procedure in Figure 1B,C. Fractionation of the nonretained pool on Sepharose CL-4B yielded a predominant $^{14}$C cpm peak at virtually the same elution volume as that of the RBC AChE liposomes in Figure 1A and a smaller $^{14}$C cpm peak near the column solvent volume. The smaller peak corresponded to low molecular weight radiolabeled peptide fragments and indicated the extent of nonspecific degradation of RBC AChE by papain under the digestion conditions. The small amount of enzyme activity in the nonretained pool was eluted some 6 mL earlier than the major $^{14}$C cpm peak in Figure 1B, indicating a minor population of slightly larger RBC AChE liposomes in which the enzyme was perhaps protected from papain-induced release by a bi- or multilamellar liposome structure. Confirmation that the major $^{14}$C cpm peak in Figure 1B corresponded primarily to liposomal hydrophobic domain fragment was obtained by rechromatography of the peak on Sephadex LH-60 in organic solvents (Figure 1C). The elution volume of 65 mL for the major $^{14}$C cpm peak in Figure 1C coincided with that for the major [$^{125}$I]TID-labeled hydrophobic domain fragment derived from a trace amount of [$^{125}$I]TID-labeled RBC AChE carried through the digestion, and this elution volume was identical with that previously observed for the [$^{125}$I]TID-labeled hydrophobic domain fragment obtained under a variety of digestion conditions (Roberts & Rosenberry, 1986a). The peak at 25 mL in Figure 1C represented the residual intact enzyme in the Figure 1B pool.

*Comparison of Radiomethylation Label Distribution in the Hydrophilic and Hydrophobic Domain Fragments.* Our previous studies indicated that the hydrophilic subunit fragment produced by papain cleavage of RBC AChE was only slightly smaller than the intact 70–75-kDa subunit, and we proposed that the hydrophobic domain must be localized to the N- or C-terminal segment of the intact subunit (Dutta-Choudhury & Rosenberry, 1984). To investigate this point, we compared the radiolabel distributions obtained by automated amino acid analysis of intact radiomethylated RBC AChE and of the hydrophilic and hydrophobic domain frag-

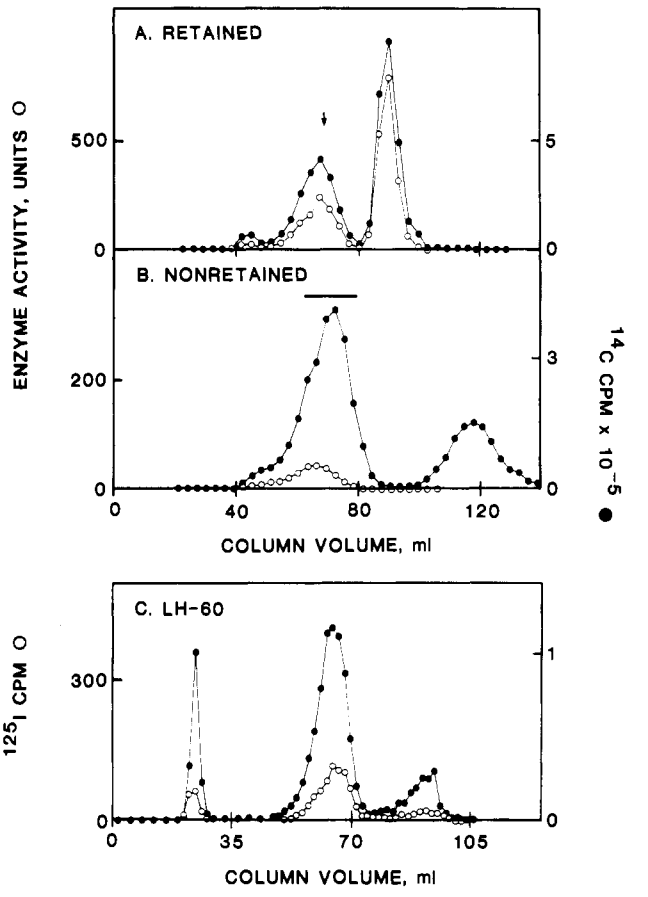


FIGURE 1: Cleavage of radiomethylated liposomal RBC AChE with papain and isolation of the released hydrophilic enzyme and the enzyme hydrophobic domain fragment. RBC AChE was radiomethylated with [$^{14}$C]HCHO and $NaBH_3$, mixed with a trace amount of [$^{125}$I]TID-labeled RBC AChE, reconstituted into small liposomes, digested with soluble papain, and fractionated into nonretained and retained pools by affinity chromatography on acridinium resin as outlined under Materials and Methods. (A) Chromatography of the retained pool containing residual intact RBC AChE liposomes and released hydrophilic RBC AChE on Sepharose CL-4B. The dialyzed retained pool was concentrated from 28 to 2.5 mL by vacuum dialysis and applied to a 120-mL Sepharose CL-4B column (1.5 × 70 cm) equilibrated at 4 °C in 20 mM Tris-HCl, pH 7.4. Elution of an added column solvent volume marker (saturated $K_2Cr_2O_7$ (10 $\mu$L), monitored by $A_{450nm}$] was normalized to 120 mL. Open symbols show enzyme activity (units), and closed symbols indicate $^{14}$C cpm. Recoveries of enzyme activity and $^{14}$C label were greater than 85%. The downward arrow indicates the elution position of nondigested liposomes [see Kim & Rosenberry (1985)]. (B) Chromatography of the nonretained pool containing cleaved liposomal hydrophobic domain fragment on Sepharose CL-4B. The nonretained pool was concentrated from 37 to 11 mL by vacuum dialysis and chromatographed as in panel A. Recoveries of enzyme activity and $^{14}$C label were greater than 90%. (C) Fractions indicated by the horizontal bar in panel B were pooled, dialyzed against several changes of sodium phosphate (pH 7) progressing from 15 to 5 mM, and dried on the Speedvac concentrator. The dried sample was resuspended in 88% formic acid (1 mL) and applied to a 120-mL Sephadex LH-60 column (1.5 × 70 cm) equilibrated in ethanol/88% formic acid (3:1). Open symbols denote $^{125}$I cpm, and closed symbols indicate $^{14}$C cpm. Overall recovery of $^{14}$C label in the major peak at about 65 mL from the fractions pooled in panel B was 39%.

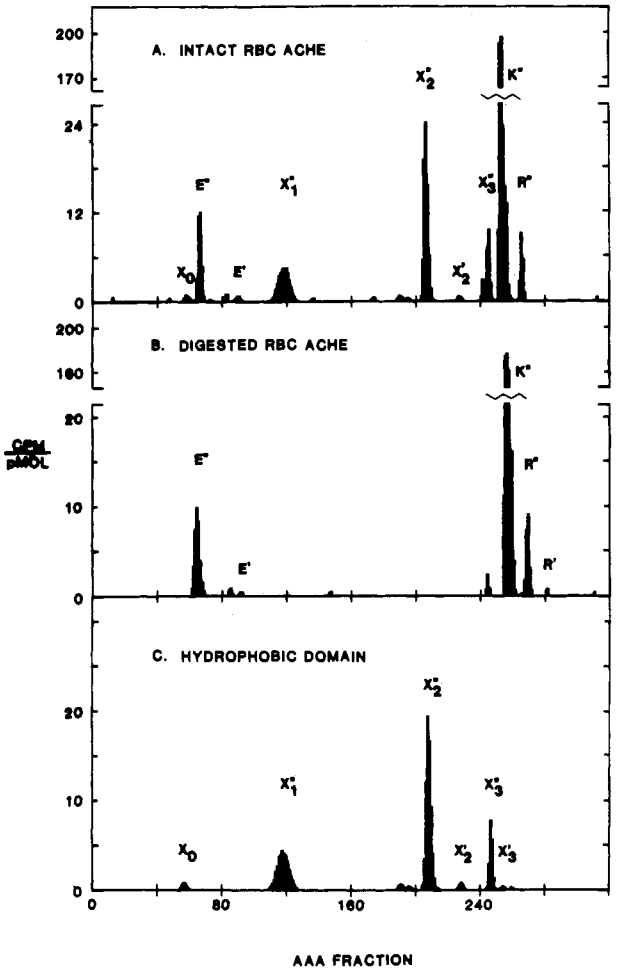


FIGURE 2: Distribution of radiomethylated components obtained by automated amino acid analysis of intact RBC AChE and of the hydrophilic and hydrophobic domain fragments isolated in Figure 1. Samples were hydrolyzed in 6 N HCl and subjected to automated amino acid analysis (AAA), and the analyzer effluent was collected for scintillation counting as outlined under Materials and Methods. (A) Radiomethylated and dialyzed intact RBC AChE (790 pmol); same stock as that reconstituted in Figure 1. (B) The hydrophilic fragment (310 pmol) isolated following Sepharose CL-4B chromatography in Figure 1A. (C) The hydrophobic domain fragment (230 pmol) isolated in the major peak at 65 mL from Sephadex LH-60 chromatography in Figure 1C. Picomole estimates for each component assumed that intact subunits contained 70 kDa of protein and were determined either from the nonlabeled amino acid content (panels A and B; see Materials and Methods) or from the recovered radioactivity (panel C) as described in Table I. Radioactive peaks were identified by comparison to radiomethylated amino acid standards (Haas & Rosenberry, 1985): E designates N-methylated Glu; K, $N^{\epsilon}$-methylated Lys; R, $N^{\alpha}$-methylated Arg; and X, non-amino acids identified in Figure 3. Primes and double primes designate mono- and dimethylated derivatives, respectively.

ments isolated as outlined in Figure 1. Several radiomethylated components were resolved from each of these species (Figure 2), and we drew a number of conclusions. First, some of the labeled peaks from the intact enzyme in Figure 2A corresponded in elution times to radiomethylated amino acid standards (Haas & Rosenberry, 1985). These included derivatives of Glu and Arg methylated on the $\alpha$-amino group and derivatives of Lys methylated on the $\epsilon$-amino group. The respective N-terminal stoichiometries of Glu and Arg were determined to be 0.66 and 0.34 per 70-kDa subunit. It is unclear at this point whether this finding implies either slight subunit heterogeneity or allelic variation in RBC AChE [see discussion in Haas & Rosenberry (1985)]. Of particular interest here, however, is the fact that all three of these radiolabeled amino acids were quantitatively retained in the hydrophilic enzyme fragment produced by papain digestion (Figure 2B, Table I). This observation demonstrated conclusively that the hydrophobic domain was not attached at the subunit N-termini.

Several of the radiomethylated peaks obtained by amino acid analysis of intact RBC AChE did not correspond to methylated amino acid standards, and these peaks are denoted X in Figure 2A. Additional evidence that these components were not $N^{\alpha}$-methylated amino acids was the [$^{14}$C]HCHO concentration dependence of their radiomethylation, which indicated a slower rate typical of alkylamines [see Haas & Rosenberry (1985) and Sherman et al. (1983)]. Significantly, the X components segregated exclusively in the hydrophobic domain fragment following papain cleavage (Figure 2C), and their relative amounts were identical in the intact enzyme and the isolated fragment (see Table I). Identical label distributions were observed for hydrophobic domain fragments isolated following papain digestion of radiomethylated RBC AChE after either reconstitution into liposomes or incorporation into Triton X-100 micelles.

*Identification of Radiomethylated Non-Amino Acid Components in the Hydrophobic Domain Fragment.* Possible identities for the methylated components in the RBC AChE hydrophobic domain were suggested in a report by Campbell et al. (1981) on a hydrophobic membrane-binding structure linked to the C-terminus of the Thy-1 glycoprotein of rat brain. Amino acid analysis of the C-terminal tryptic peptide of Thy-1 gave ninhydrin staining peaks that indicated glucosamine and galactosamine and suggested ethanolamine. $^{3}$H-Methylated standards of these amines were prepared at several concentrations of amine and [$^{3}$H]HCHO to permit identification of the mono- and dimethylamine derivatives [data not shown; see Haas & Rosenberry (1985)], and aliquots of $^{14}$C-labeled hydrophobic domain fragment from RBC AChE were mixed with each standard prior to acid hydrolysis and analysis on the amino acid analyzer. Methylation conditions exemplified in Figure 3 resulted in significant amounts of both mono- and dimethylamine standards. The ethanolamine hydrolysate showed only two major $^{3}$H peaks, and these corresponded to the $^{14}$C peaks labeled $X'_2$ and $X''_2$ (Figure 3A). These two peaks were assigned as *N*-methylethanolamine and *N,N*-dimethylethanolamine, respectively.

The hydrolysates of $^{3}$H-labeled glucosamine and galactosamine were more complex and showed four major $^{3}$H peaks. Two of these peaks corresponding to the $^{14}$C peaks labeled $X'_3$ and $X''_3$ were obtained from both hexosamines (Figure 3B,C). The two other $^{3}$H peaks from glucosamine corresponded to $^{14}$C-labeled $X'_1$ and $X''_1$,[2] in contrast to the comparable pair from galactosamine which appeared some 12–16 fractions later. The analysis technique distinguished unequivocally between methylated derivatives of glucosamine and galactosamine. Analyses of *non*hydrolyzed radiomethylated glucosamine and galactosamine gave only the $^{3}$H peaks at or near $X'_1$ and $X''_1$, and the $X_3$ peaks were reduced substantially relative to the earlier peaks in samples hydrolyzed less extensively (data not shown). We concluded that the $X'_3$ and $X''_3$ components represented hydrolysis degradation products of $X'_1$ and $X''_1$, respectively, and that these degradation products are common to the methylated derivatives of both glucosamine and galactosamine.[3] The degradation of meth-

[2] The $^{14}$C-labeled $X'_1$ peak was too small to be apparent on the scale of Figure 3 when RBC AChE radiomethylation was conducted with 10 mM [$^{14}$C]HCHO but was easily seen when the enzyme was radiomethylated with 0.2 mM [$^{14}$C]HCHO.

Table I: Relative Amounts of Amine Components Labeled by Reductive Methylation of Intact RBC AChE[a]

| RBC AChE species | $n$ | residues methylated per intact or fragmented subunit | | | | |
|---|---|---|---|---|---|---|
| | | Glu | Arg | Lys | ethanolamine | glucosamine |
| intact enzyme | 6 | 0.66 ± 0.03 | 0.34 ± 0.01 | 7.40 ± 0.20 | 1.35 ± 0.06 | 0.98 ± 0.04 |
| hydrophilic fragment | 3 | 0.63 ± 0.02 | 0.38 ± 0.03 | 7.27 ± 0.23 | 0.06 ± 0.05[b] | 0.04 ± 0.02[b] |
| hydrophobic domain fragment | 13 | 0.02 ± 0.01 | 0.02 ± 0.01[c] | 0.03 ± 0.01 | 1.31 ± 0.01 | 1.02 ± 0.01 |

[a] RBC AChE was radiomethylated with [$^{14}$C]HCHO (40 mCi/mmol), and fragments were isolated following papain digestion of either RBC AChE liposomes (two preparations) or RBC AChE–Triton X-100 micelles (seven preparations) as outlined under Materials and Methods and Figure 1. Radiolabeled amines were identified as indicated in Figures 2 and 3 and the text, and the moles of residues methylated was determined from the methyl group specific activity [see Haas & Rosenberry (1985)]. For glucosamine, the moles of $X_1$ and of $X_3$ were totaled. The moles of intact RBC AChE subunit and of hydrophilic fragment was determined as in Figure 2. Determination of the moles of hydrophobic domain fragment assumed that the total content of radiomethylated ethanolamine plus glucosamine in the fragment was the same as that observed in the intact enzyme: 2.33 ± 0.10 mol of residues per mole of fragment. Data presented are averages of $n$ analyses. [b] Hydrophilic fragments isolated from papain digests in Triton X-100 typically contained 10–20% intact enzyme. One such determination was included in these averages and contributed most of the tabulated ethanolamine and glucosamine. [c] Average of four determinations. Arginine can only be distinguished from $X'_3$ in a four-buffer amino acid analysis system (Haas & Rosenberry, 1985).

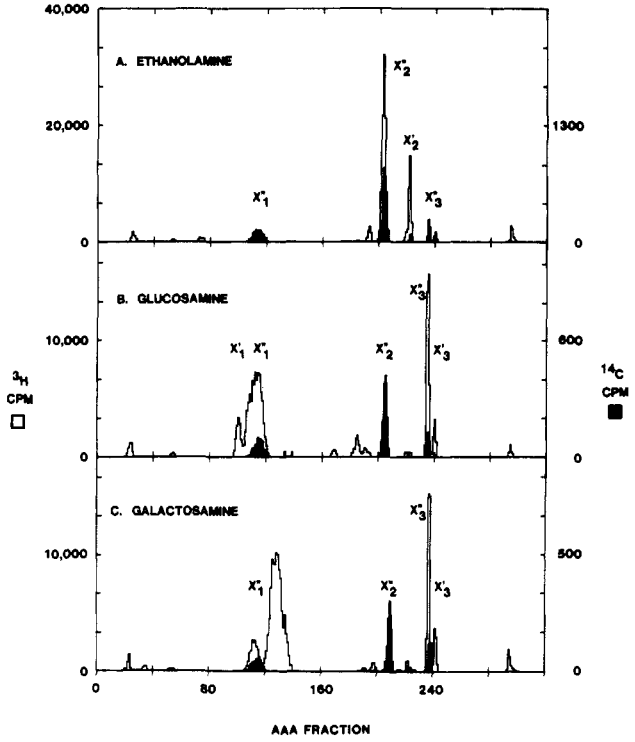


FIGURE 3: Identification of ethanolamine and glucosamine in the hydrophobic domain of RBC AChE. Ethanolamine, glucosamine, and galactosamine (1 mM) were reductively methylated with 1 mM [$^3$H]HCHO as outlined under Materials and Methods. Aliquots of each $^3$H-labeled standard were mixed with samples of $^{14}$C-labeled hydrophobic domain fragment that was produced by papain digestion of low specific activity $^{14}$C-radiomethylated RBC AChE in Triton X-100 micelles and isolated as indicated under Materials and Methods. The mixed samples were dried, hydrolyzed, and analyzed as in Figure 2. The radioactivity profiles for $^3$H (open histograms) and $^{14}$C (shaded histograms) were determined by dual-channel scintillation counting as outlined under Materials and Methods and are shown for each of the three standards. Minor $^3$H peaks that failed to match any of the X components appeared to be residual products of the methylation reagents alone. Analyzer peaks attributed to quenching amino acids (see Materials and Methods) were deleted. An apparent isotope effect suggested by a one-fraction shift in the elution positions of the $X_2$ and $X_3$ components was confirmed by analysis of mixed $^3$H- and $^{14}$C-labeled standards.

ylated glucosamine on hydrolysis permitted further confirmation of its identification in the hydrophobic domain, because the ratio of the $X_3$ to the $X_1$ components was identical for the $^3$H and $^{14}$C labels in Figure 3A. The $X'_1$ peak was assigned to *N*-methylglucosamine and the $X''_1$ peak to *N*,*N*-dimethylglucosamine. It is noteworthy that the hexosamine order of elution was mono-, un-, and dimethylated amine, in contrast to the order di-, mono-, and unmethylated amine observed for ethanolamine and all amino acids. Quantitative estimates in Table I indicate 1–2 mol of methylated ethanolamine and 1 mol of methylated glucosamine in the hydrophobic domain following radiomethylation of RBC AChE. These methylation stoichiometries assume a subunit protein mass of 70 kDa, a figure based on both the native and the denatured subunit masses after deduction for up to 15% carbohydrate [see Rosenberry & Scoggin (1984)], and thus the absolute values in Table I could easily be in error by 10–15% from this uncertainty alone.

*Radiomethylation of the Hydrophobic Domain Fragment and the Peptide Sequence of the Fragment.* Papain cleavage of a C-terminal RBC AChE hydrophobic domain fragment should result in exposure of a new N-terminal amino acid on this fragment, and we investigated whether radiomethylation would identify this amino acid. The isolated $^{14}$C-labeled fragment was methylated with 10 mM [$^3$H]HCHO, and excess methylation reagents were removed from the reaction mixture by chromatography on Sephadex LH-60 under the conditions in Figure 1C. The $^3$H- and $^{14}$C-labeled fragment eluted in the usual position which was just on the leading edge of the large peak of unincorporated $^3$H label (data not shown), and fragment recovery was intentionally reduced to minimize $^3$H contamination. Amino acid analysis of this fragment (Figure 4A) revealed $N^{\alpha}$,$N^{\alpha}$-di[$^3$H]methylhistidine as the only radiolabeled amino acid. This assignment was confirmed by methylation of the $^{14}$C-labeled fragment with 0.2 mM [$^3$H]HCHO and demonstration that the $^3$H-labeled peaks coeluted with added $N^{\alpha}$-[$^{14}$C]methylhistidine and $N^{\alpha}$,$N^{\alpha}$-di[$^{14}$C]methylhistidine standards on amino acid analysis (data not shown). The stoichiometry of radiomethylation corresponded to 0.67 mol of $^3$H-methylated histidine per mole of hydrophobic domain in Figure 4A and averaged 0.52 ± 0.09.[4]

The application of Edman sequencing to C-terminal fragments that contained the membrane-binding domains of trypanosome variant surface glycoproteins (VSGs) revealed the presence of ethanolamine in amide linkage to the C-ter-

[3] We did not make extensive efforts to identify the $X_3$ components.

[4] Averages represented three $^3$H-methylation reactions on one preparation. Methylation of two other preparations at lower [$^3$H]HCHO concentrations gave qualitative results similar to Figure 4A (data not shown). An additional preparation, initially derivatized with phenyl isothiocyanate in a concentrated pool of hydrophobic domain fragment and Triton X-100 direct from Sepharose CL-6B chromatography (Figure 1B) prior to removal of histidine and glycine by Edman degradation and methylation with 10 mM [$^3$H]HCHO, yielded only 0.05 mol of $^3$H-methylated ethanolamine per mole of hydrophobic domain fragment but showed a label distribution similar to that in Figure 4C.

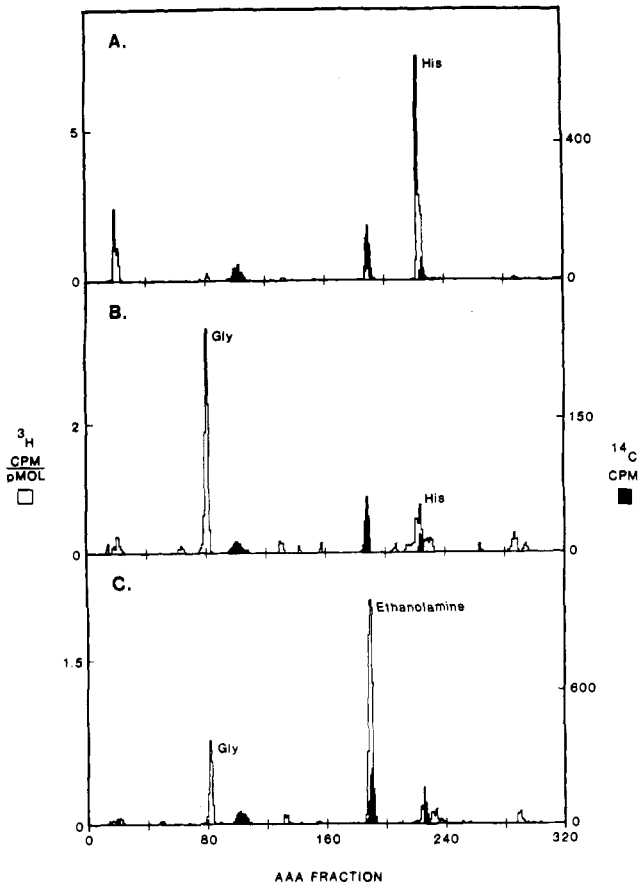


FIGURE 4: Sequencing of the hydrophobic domain fragment by sequential Edman degradation and radiomethylation analysis. (A) $^{14}$C-Radiomethylated hydrophobic domain fragment (1 nmol) prepared as in Figure 3 was reductively methylated with 10 mM [$^3$H]HCHO as outlined under Materials and Methods. The radiolabeled fragment was isolated from the methylation reaction by chromatography on Sephadex LH-60 as in Figure 1 and dried, hydrolyzed, and analyzed as in Figure 2. Radioactivity profiles for $^3$H (open histograms) and $^{14}$C (shaded histograms) were determined as in Figure 3, and the $^3$H cpm were normalized to the recovered picomoles of hydrophobic domain fragment. Labeled peaks with maxima less than twice background were deleted. Analyzer recoveries of the $^{14}$C label corresponded to 168 pmol of fragment (17%). (B) A second sample of the $^{14}$C-radiomethylated fragment in panel A (9 nmol) was coupled to phenyl isothiocyanate and isolated from excess reagent by chromatography on Sephadex LH-60 as outlined under Materials and Methods and dried in two aliquots. One aliquot (4% of the sample) was cleaved with trifluoroacetic acid to release ATZ-histidine (see Materials and Methods), dried, radiomethylated with [$^3$H]HCHO, and isolated and analyzed as in panel A. Analyzer recoveries of the $^{14}$C label corresponded to 54 pmol of fragment (14%). (C) The larger aliquot of $^{14}$C-radiolabeled sample in panel B (6.3 nmol) was cleaved with trifluoroacetic acid and dried. A second cycle of Edman degradation was conducted to release ATZ-glycine, and one-fourth of the isolated fragment was $^3$H-radiomethylated as in panel B and analyzed as in panel A. Analyzer recoveries of the $^{14}$C label corresponded to 270 pmol of fragment (17%).

minal amino acids (Holder, 1983). One cycle of Edman sequencing was applied to the RBC AChE hydrophobic domain fragment as outlined in Figure 4B to remove the N-terminal histidine, and $^3$H-methylation analysis of the modified fragment following reisolation yielded *N,N*-di[$^3$H]methylglycine (Figure 4B) at a stoichiometry of 0.36 mol of $^3$H-methylated glycine per mole of hydrophobic domain (average, 0.39 ± 0.02[4]). Two cycles of Edman sequencing removed most of the histidine and glycine, and $^3$H-methylation of the modified reisolated fragment followed by amino acid analysis revealed a $^3$H-methylated peak that coeluted with the *N,N*-di[$^{14}$C]-methylethanolamine component of the hydrophobic domain (Figure 4C). This assignment was confirmed by methylation of this modified fragment with 0.1 mM [$^3$H]HCHO and demonstration that the $^3$H-labeled peaks eluted at the positions of *N*-methylethanolamine and *N,N*-dimethylethanolamine (data not shown). The stoichiometry of radiomethylation corresponded to 0.26 mol of $^3$H-methylated ethanolamine per mole of hydrophobic domain in Figure 4C and averaged 0.20 ± 0.03.[4] The basis of the progressive reduction in $^3$H-methylation stoichiometry following each cycle of Edman sequencing was unclear. Such reductions are typical of manual Edman sequencing procedures [see Black & Coon (1982)] and may involve oxidative desulfuration of the phenylthiocarbamyl derivative, a side reaction that blocks cleavage of the ATZ-amino acid (Edman & Henschen, 1975). Nevertheless, the observation that only a single new $^3$H-methylated component emerged after each cycle of sequencing in Figure 4 argued strongly that the sequence of the RBC AChE hydrophobic domain fragment generated by papain is His-Gly-ethanolamine-Z, where Z is the remainder of the hydrophobic domain.

## Discussion

*Localization of the RBC AChE Hydrophobic Domain to the C-Terminus of the Catalytic Subunits.* In an earlier report, we demonstrated that reductive radiomethylation coupled with amino acid analysis provided accurate qualitative and quantitative estimates of known N-terminal amino acids in standard proteins with high sensitivity (Haas & Rosenberry, 1985). Radiomethylation is in fact superior to other methods of N-terminus determination for a protein cleavage study like that in Figure 2 because it will reveal loss of an original radiomethylated N-terminus even if N-terminal amino acids of the original intact and the cleaved protein are coincidentally the same. Papain digestion of radiomethylated RBC AChE resulted in quantitative retention of the original radiomethylated N-terminal amino acids in the hydrophilic enzyme fragment, indicating that the small hydrophobic domain fragment released by papain was not derived from the N-terminus. This finding extended previous observations that the radiomethylated N-termini and the [$^{125}$I]TID-labeled hydrophobic domain are found on different cyanogen bromide fragments[5] and that the amino acid content of the hydrophobic domain (Roberts & Rosenberry, 1986a) did not correspond either to the observed N-terminal amino acids or to a short N-terminal sequence determined by Edman sequencing (Haas & Rosenberry, 1985). Since the hydrophilic fragment generated by papain was nearly as large as intact RBC AChE (Dutta-Choudhury & Rosenberry, 1984; Kim & Rosenberry, 1985), the hydrophobic domain fragment appeared to be derived from one end or the other of the catalytic subunit polypeptide. We tentatively concluded that the hydrophobic domain was located at the C-terminus.

A direct test of this C-terminal localization with carboxypeptidase Y was inconclusive because this enzyme did not release significant amounts of amino acid either from intact RBC AChE or from its hydrophilic fragment (Rosenberry et al., 1984). Confirmation finally was obtained from the peptide sequencing of the isolated hydrophobic domain fragment in Figure 4. The first two sequencing cycles released histidine and glycine, in agreement with the amino acid composition of the isolated hydrophobic domain fragment (Roberts & Rosenberry, 1986a). Radiomethylation following the second sequencing cycle revealed that an ethanolamine was in amide

[5] R. Haas, L. H. Younkin, and T. L. Rosenberry, unpublished observations.

linkage to the glycine, and we conclude that this glycine must be the C-terminal amino acid of RBC AChE. Our use of Edman sequencing in combination with reductive methylation and amino acid analysis was prompted by two considerations: (1) the unusual solubility properties of the hydrophobic domain fragment resulted in its loss in the washes during conventional manual solid phase sequencing (Black & Coon, 1982); (2) quantities of the hydrophobic domain fragment were limited, and a sensitive procedure was required. Radiomethylation provided the necessary sensitivity. The smallest sample analyzed in Figure 4 involved the determination of 20 pmol of $^{3}$H-methylated glycine in a hydrolysate containing 54 pmol of the $^{14}$C-methylated hydrophobic domain fragment. The hydrophobic domain fragment was recovered by Sephadex LH-60 chromatography during each sequencing and analysis cycle, a slow procedure which progressively reduced the yields of the fragment but resulted in methylated amine profiles on the amino acid analyzer that were remarkably free of contaminants.

*Non-Amino Acid Components in the RBC AChE Hydrophobic Domain Fragment Suggest a Covalently Linked Glycolipid.* The use of reductive methylation for amine analysis in general appears attractive because of the high qualitative resolution, exemplified by the separation of glucosamine and galactosamine in Figure 3, and the high sensitivity and quantitative accuracy. Our reductive methylation data indicate that the RBC AChE hydrophobic domain contains 1 residue of glucosamine and 1–2 residues of ethanolamine with unblocked primary amine groups. An additional residue of ethanolamine appears to provide the amide linkage between the C-terminal glycine and the remainder of the hydrophobic domain. Much if not all of the hydrophobicity in the domain is contributed by 2 mol of fatty acids that can be released by methanolysis (Roberts & Rosenberry, 1985). Saturated and unsaturated species are almost equally represented, with palmitate (16:0) accounting for 70–80% of the saturated fatty acids and the relatively rare 22:4 and 22:5 comprising 50–60% of the unsaturated fatty acids. The hydrophobic domain fragment associated with Triton X-100 micelles is retained on concanavalin A affinity resin and eluted with methyl $\alpha$-mannoside but has no affinity for wheat germ agglutinin or ricin I resins (Rosenberry et al., 1986). These observations indicate that the hydrophobic domain contains mannose and/or inositol residues but not *N*-acetylglucosamine or terminal galactose. Direct chemical analysis reveals 1 mol of *myo*-inositol per mole of RBC AChE catalytic subunit (Roberts & Rosenberry, 1986b). On the basis of these data, we propose that a glycolipid covalently linked to the C-terminus comprises the RBC AChE hydrophobic domain.

*Is There a Class of Integral Membrane Proteins with C-Terminal Glycolipid Domains*? Critical experiments on RBC AChE in this report were prompted by observations on two other glycoproteins that appear to have non-amino acid components in C-terminal hydrophobic domains. Our exploration of ethanolamine and glucosamine as candidates for the radiomethylated X components in the isolated RBC AChE hydrophobic domain fragment was stimulated by a report on isolated rat brain Thy-1 (Campbell et al., 1981), an antigen of unknown function which is prevalent in rodent thymocytes and mammalian brain. C-Terminal peptides from Thy-1 appeared to have hydrophobic properties but did not contain any extended sequence of hydrophobic amino acids. Amino acid compositions of these peptides revealed glucosamine and galactosamine as well as ninhydrin-positive material tentatively identified as ethanolamine. Although the presence of a C-terminal glycolipid on all forms of Thy-1 remains controversial (Seki et al., 1985), a recent report (Tse et al., 1985) has identified ethanolamine, mannose, galactosamine, glucosamine, *myo*-inositol, phosphate, glycerol, and stearate in the hydrophobic domain of rat Thy-1 from brain and thymus. Thy-1 is released from cell membranes by PIPLC (Low & Kincade, 1985), suggesting that an inositol phosphatide may be part of the C-terminal glycolipid.

Characterization of a C-terminal glycolipid has progressed to the greatest extent with trypanosome mfVSGs. These proteins comprise 7–10% of the total trypanosome cell protein and are expressed sequentially to permit the parasite to avoid antibody-mediated destruction by the host (Borst & Cross, 1982). mfVSGs are localized in trypanosome membranes by a C-terminal glycolipid. Cleavage of a phosphodiester linkage in this glycolipid by PIPLC produces 1,2-dimyristylglycerol and generates a soluble form of the VSG (Ferguson et al., 1985a,b). Fragments of mfVSGs corresponding to the C-terminal amino acid linked to the glycolipid were isolated following digestion with proteases. One cycle of Edman sequencing applied to these fragments revealed ethanolamine as the amide linked to the C-terminal amino acid (Holder, 1983), and this observation prompted our sequencing effort on the RBC AChE hydrophobic domain fragment shown in Figure 4. Structural analyses of mfVSG glycolipid fragments have revealed one ethanolamine, zero to eight galactose (depending on the variant), one to two mannose, one glucosamine, one to two phosphate, one glycerol, and two myristate residues per glycolipid (Ferguson et al., 1985a,b; Baltz et al., 1983), and Ferguson et al. (1985b) have shown by nitrous acid deamination that the substituent in C-1 glycosidic linkage to the glucosamine is dimyristylphosphatidylinositol. Sequence analyses of trypanosome VSG cDNAs indicate that VSGs are initially translated with a 17–23 amino acid C-terminal extension consisting largely of hydrophobic amino acids, and it has been proposed that this extension is cleaved during processing to the mature mfVSGs [see Borst & Cross (1982)]. Demonstration of a similar processing event associated with Thy-1 could resolve the controversy involving a Thy-1 C-terminal glycolipid noted above.

Although complete chemical characterization of the glycolipid structures associated with trypanosome mfVSGs, Thy-1, or RBC AChE has not yet been reported, each glycolipid appears to include ethanolamine, hexosamine, inositol, and fatty acid esters. Thus, these structures represent more complex posttranslational modifications than the direct fatty acid acylations of hydroxy amino acid residues or N-termini reviewed by Schmidt (1983). Some diversity of these glycolipid structures is already apparent, as our reductive methylation data indicate at least 2 mol of ethanolamine per mole of glycolipid in contrast to the 1 mol reported for trypanosome mfVSGs. In addition, the lack of effect of PIPLC on RBC AChE [see Roberts & Rosenberry (1986a)], despite the sensitivity of other AChEs to this phospholipase (Futerman et al., 1984), raises the question of whether phosphatidylinositol is a component common to all of these structures. Despite this diversity, these C-terminal structures suggest the important concept that glycolipids can be linked to otherwise soluble proteins in a new class of posttranslational modifications designed to produce membrane-bound proteins.

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**Registry No.** AChE, 9000-81-1; ethanolamine, 141-43-5; glucosamine, 3416-24-8.


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# Complete Amino Acid Sequence of Ubiquitin from the Higher Plant *Avena sativa*†


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ABSTRACT: Ubiquitin is a 76 amino acid, eukaryotic polypeptide with several important regulatory functions that arise from its ability to become covalently ligated to cytoplasmic and nuclear proteins. The amino acid sequence of ubiquitin is remarkably conserved, being identical for all animal forms analyzed to date. Here, we present the complete amino acid sequence of ubiquitin isolated from a higher plant, oats (*Avena sativa L.*). This sequence was determined by repetitive Edman degradation of the intact molecule and of peptides derived by proteolytic digestion with trypsin or *Staphylococcus aureus* V8 protease. Comparison of this sequence with that obtained for animal ubiquitins indicates that the two forms are homologous but *not* identical. Like the animal form, oat ubiquitin contains 76 amino acid residues, no tryptophan or cysteine, and a carboxyl terminus of Leu-Arg-Gly-Gly. However, oat ubiquitin contains three amino acid substitutions at positions 19, 24, and 57.


Ubiquitin is a 76 amino acid polypeptide present in all eukaryotes. Its amino acid sequence is identical in organisms as diverse as mammals including humans (Schlesinger et al., 1975; Schlesinger & Goldstein, 1975), birds (Bond & Schlesinger, 1985), fish (Watson et al., 1978), amphibians (Dworkin-Rastl et al., 1984), and insects (Gavilanes et al., 1982), making it one of the most conserved proteins yet identified. The widespread occurrence and unparalleled sequence conservation of ubiquitin led Goldstein et al. (1975) to conclude long before its functions became evident that this protein plays an important role in eukaryotic cell physiology.

It is now known that ubiquitin has at least two functions in intracellular metabolism that are a result of its ability to become covalently ligated to other cytoplasmic and nuclear proteins [for reviews, see Hershko & Ciechanover (1982) and Finley & Varshavsky (1985)]. Ligation is via an unusual

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