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# ISOPEPTIDE BONDS IN MEMBRANE PROTEINS FROM EUKARYOTIC CELLS

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## SUMMARY

On the basis of cyanoethylation of purified plasma membrane and endoplasmic reticulum fractions derived from tissue-cultured L cells, it is estimated that $3 \cdot 10^{-3}$ to $5 \cdot 10^{-3}$ $\mu$moles lysine/mg protein participate in covalent bonds to the $\varepsilon$-amino groups. About 40% of the unreactive lysines can be accounted for by isopeptide bonds forming the dipeptide $\varepsilon$-($\gamma$-glutamyl)lysine. The dipeptide was found in membrane protein subfractions soluble in 1 mM EDTA, and in the insoluble residue remaining after exhaustive extraction with 1 mM EDTA, 0.8 M NaCl, and ethanol–ether.

## INTRODUCTION

Because membranes are involved in many important biological processes, it becomes important to isolate and characterize their molecular constituents in order to develop an understanding of membrane processes in molecular terms. While great strides have been made in isolating and characterizing the lipid components of membranes, efforts to isolate the individual protein constituents of cell membranes, particularly in eukaryotic cells, have met with great difficulty. Even in the case of the least complex and most widely studied eukaryotic cell membrane, that of the erythrocyte, the results vary depending upon the separation scheme used[1–4]. Many membrane proteins resist solubilization unless very perturbing solvents are used[5–9]. Although some constituents have molecular weights as low as 5000, a major portion of membrane proteins appear to have molecular weights in excess of 400000, even with the use of highly perturbing solvents. It is thought that constituents with such very high apparent molecular weights represent smaller protein species which either are imperfectly solubilized, or are bound together in some as yet unknown manner.

In the course of studying the cell membrane proteins of tissue-cultured L cells (derived from mouse fibroblasts), we discovered that many $\varepsilon$-amino groups of lysine

Abbreviations: EGTA, ethyleneglycol-bis-($\beta$-aminoethylether)-*N*,*N*′-tetraacetic acid.

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residues participated in covalent bonds. We were able to show that many of these bonded lysines were involved in isopeptide bonds forming the dipeptide ε(γ-glutamyl)-lysine, a dipeptide cross-link which has previously been found in stabilized fibrin clots[10,11] and hair[12]. In collagen and elastin, the ε-amino groups of lysine participate in Schiff base cross-links[13]. Various lysine cross-links may explain some of the special characteristics of membrane proteins.

## MATERIALS AND METHODS

Mouse fibroblast L cells were grown as suspension cultures in spinner bottles to a density of $5 \cdot 10^5$ to $10 \cdot 10^5$ cells/ml using minimal essential spinner medium[14] supplemented with 5% fetal calf serum, 5 g/l glucose medium, 50 units/ml penicillin G, and 50 μg/ml streptomycin. Cells were labeled by growth for 65 to 70 h in modified medium containing one-quarter the usual concentration of lysine and 0.1 μCi/ml [$^{14}$C]lysine. In some experiments cells were double labeled by growth for 65 to 75 h in modified medium containing one-quarter the usual concentrations of both lysine and glutamine and 0.1 μCi/ml [$^{14}$C]glutamine and 0.4 μCi/ml [$^{3}$H]lysine. Media containing less than 12 mg/l lysine would not sustain growth of cells.

The cells were sedimented, washed once with serum-free media, resedimented, and suspended in about 8 to 10 vol. homogenization buffer (0.25 M sucrose, 0.20 mM $MgSO_4$, and 5 mM Tris, pH 7.4). The cells were disrupted by nitrogen cavitation, and plasma membrane and endoplasmic reticulum purified by the method of Wallach and Kamat[15] except that 15% (w/w) Dextran 110 was substituted for Ficoll in the density gradients. The Dextran was less viscous, and gave lower osmotic pressure and better separation.

For some experiments, washed microsomes were fractionated by a procedure based on that of Rosenberg and Guidotti[6] for erythrocyte ghosts. Washed cells were suspended in 8 to 10 vol. homogenization buffer and disrupted by nitrogen cavitation[15]. Nuclei and cell debris were sedimented by centrifugation at 960 × *g* for 10 min. Mitochondria and lysosomes were sedimented by centrifugation at 30000 × *g* for 10 min. The pH was adjusted to 8.6 and the microsomes were sedimented by centrifugation at 105000 × *g* for 1 h. The microsomes were suspended in a small quantity of 10 mM Tris, pH 8.6, mixed, and sedimented. The microsomal pellet was then resuspended in a small quantity of 1 mM Tris, pH 8.6, and resedimented. The washed microsomal pellet (about 12 mg protein) was suspended in 19 ml 5 mM sodium phosphate buffer, pH 7.6, and dialyzed against 500 ml 1 mM $Na_2EDTA$, pH 7.6 and 70 mM β-mercaptoethanol for 24 h, and then dialyzed against a second batch of dialysis medium for an additional 24 h.

The dialyzed microsomal fraction was centrifuged at 105000 × *g* for 1 h; the supernatant was called Fraction 1. The pellet was suspended in 20 ml at 0.8 M NaCl, stirred for 18 h, and again centrifuged at 105000 × *g* for 1 h; the supernatant was called Fraction 2. The pellet was extracted two times with 10 ml portions of ethanol–ether 3:1 (v/v) at −20 °C for 24 h each. The supernatants were combined as Fraction 3, and the residue constituted Fraction 4. Fractions 1 and 2 were dialyzed against 0.25 M sucrose and 1 mM Tris, pH 8.6, for 24 h and the protein precipitated by the addition of trichloroacetic acid, final concentration 5%. The solvent was evaporated from Fraction 3 under a stream of nitrogen.

Transport ATPase ($Na^+$,$K^+$-activated ATPase) activity was determined in the presence and absence of $1 \cdot 10^{-4}$ M ouabain by the procedure of Post *et al.*[16]. NADH diaphorase was determined according to the procedure described by Wallach and Kamat[15]. Phosphate was determined by Fiske and SubbaRow's procedure[17]. Protein was determined by the method of Lowry *et al.*[18].

Isopeptide bonds were quantified as follows. Membrane fractions from labeled cells were precipitated with 5% trichloroacetic acid, and the precipitate extracted three times with ethanol–ether (1:1, v/v) and three times with ether. The precipitate was dried in a desiccator. Protein was determined either directly by weighing or by the Lowry method[18].

The cyanoethylation method of Pisano *et al.*[11] was used to measure unreactive ε-amino groups of lysine.

The enzyme digestion method was a modification of that described by Matacic and Loewy[10]. Dried membrane fractions were incubated with pronase (enzyme–protein ratio 1:10) in 0.01 M Tris buffer, pH 7.5, in the presence of a small amount of thymol to prevent growth of microorganisms. A known quantity of authentic $^3$H-labeled (γ-glutamyl)lysine was added as a marker, and to enable precise calculation of recoveries. The $^3$H-labeled dipeptide marker was synthesized by the method of Matacic and Loewy[10]. After incubation for 24 h, an amount of pronase equal to that used initially was added, the pH adjusted to 7.5, and incubation continued for an additional 24 h. Pronase then was inactivated by boiling the mixture for a minute.

The pH of the mixture was adjusted to 8.0, $MgCl_2$ was added to 5 mM final concentration, and 2 mg leucine aminopeptidase per 10 mg protein was added. After incubation for 48 h, the pH was adjusted to 2.8. The volume was measured and radioactivity determined in a small aliquot. The remainder was applied to an ion exchange column (Technicon Type A) for amino acid chromatography. The enzyme digest was eluted from the column with a sodium citrate buffer gradient: 0.2 M, pH 2.9 start to 0.8 M, pH 5.0 final. $^3$H and $^{14}$C was counted in 3.5-ml fractions of the eluate in a Packard Model 3320 scintillation spectrometer, the output data automatically punched on IBM cards, and dpm computed with an IBM 360-44 computer. Lysine concentration was measured with a modified Technicon amino acid analyzer. The chromatographic column was normally used at 60 °C. The dipeptide ε-(γ-glutamyl)lysine could be purified to a greater degree by re-running the material collected at the peak through a column at room temperature.

Radioactive amino acids were purchased from New England Nuclear Corp., enzymes from Worthington Biochemicals, tissue culture media from Grand Island Biological Co., Dextran 110 from Pharmacia, Tris salt of ATP from P–L Laboratories, ouabain and β-mercaptoethanol from Calbiochem, and ethyleneglycol-bis-(β-aminoethyl)-*N*,*N*-tetraacetic acid (EGTA) from Eastman Kodak. All other reagents were analytical grade.

## RESULTS

About 1.3% of the radioactivity of cells grown in [$^{14}$C]lysine was found in the washed microsomal fraction. When layered on Dextran 110 ($\rho$ = 1.06), material rich in plasma membranes collects as a band at the interface, and material rich in endoplasmic reticulum collects as a pellet. The plasma membrane material is charac-

terized by $(Na^+,K^+)$-activated ATPase activity and endoplasmic reticulum by NADH-diaphorase activity. The results of a typical experiment are shown in Table I. The interface band and pellet combined account for about 86% of the total radioactivity found in washed microsomal fraction. The distribution of enzymes was similar to those found for other tissue-cultured cells[15,19,20], but a higher specific activity of $(Na^+,K^+)$-activated ATPase was found in our cells than those reported previously for L cells[21,22].

TABLE I

SEPARATION OF L CELL MICROSOMES ON 15% (w/w) DEXTRAN 110

| *Fraction* | *% of protein applied* | *$(Na^+,K^+)$-activated ATPase (μmoles $P_i$/mg per h)* | *NADH diaphorase (μmoles/mg per min)* | *ATPase diaphorase* |
|---|---|---|---|---|
| Layering suspension | 100 | 3.4 | 1.1 | 3.1 |
| Upper layer medium | 3.5 | — | — | — |
| Band at interface | 24.3 | 5.6 | 0.47 | 11.9 |
| Lower layer medium | 11.9 | 3.3 | — | — |
| Pellet | 57.0 | 1.1 | 1.9 | 0.58 |

A set of control experiments was carried out using lysozyme as a model protein to determine how completely the cyanoethylation procedure used in these experiments reacts with ε-amino groups of lysine. We found that less than one ε-amino group per 10000 lysine residues failed to react. Using [$^{14}$C]lysine-labeled $T_4$ bacteriophage protein, less than one ε-amino group per 5000 lysine residues failed to react.

Membranes doubly labeled with [$^3$H]lysine and [$^{14}$C]glutamine were used to determine whether any ε-(γ-glutamyl)lysine dipeptide was missed in the assay procedure owing to inadequate hydrolysis. During the course of the experiments, glutamine was metabolized extensively and radioactivity originating from glutamine appeared in many amino acid peaks upon chromatography. Lysine was not metabolized significantly and apart from a very small amount of material appearing in the flow-through of the column, no significant amount of doubly labeled material appeared except at the ε-(γ-glutamyl)lysine dipeptide peak. The double label experiments indicate that values obtained for the dipeptide from the enzyme assay are reliable.

Cyanoethylation reveals that a significant number of lysine residues, amounting to $3 \cdot 10^{-3}$ μmoles per mg protein, in the plasma membrane and endoplasmic reticulum fractions of L cells are unreactive and probably involved in covalent bonds (Table II). Cyanoethylation of high speed supernatant proteins from L cells revealed no detectable substituted lysines. On the basis of the enzyme digestion experiments and the isolation of labeled ε-(γ-glutamyl)lysine, approx. 40% of the covalently linked ε-amino groups of lysine were involved in isopeptide bonds.

When L cell membranes were fractionated according to solubility, labeled dipeptide was found in Fraction 1 (soluble in 1 mM EDTA) and in Fraction 4 (insoluble residue after exhaustive extraction with 1 mM EDTA, 0.8 M NaCl, and lipid solvents). It is interesting to note that the relatively soluble fraction (Fraction 1) contained, if anything, more isopeptide bonds than the insoluble residue (Table III). Essentially

the same results were obtained when EGTA was used in place of EDTA. It may also be noted that the frequency of isopeptide bonds is much less in the fractionated whole microsomes than in the enriched plasma membrane and endoplasmic reticulum preparations.

TABLE II

ISOPEPTIDE BONDS IN MEMBRANE PROTEINS FROM L CELLS

Label: (a) [$^{14}$C]lysine; (b) [$^{14}$C]glutamine *plus* [$^{3}$H]lysine.

| *Sample* | *Label* | *Method of analysis* | *ε-Amino lysine bonds / mg protein* · $10^4$ | *Daltons protein / ε-amino lysine bond* | *Total lysine residues / ε-amino lysine bond* |
|---|---|---|---|---|---|
| *Plasma membrane* | | | | | |
| 1 | a | Cyanoethylation | 26.1 | 380 000 | 294 |
| 2 | a | Cyanoethylation | 30.9 | 320 000 | 248 |
| 3 | a | Cyanoethylation | 48.9 | 200 000 | 155 |
| 4 | a | Cyanoethylation | 51.5 | 190 000 | 148 |
| 5 | b | Enzyme digestion* | 17.0 | 590 000 | 300 |
| *Endoplasmic reticulum* | | | | | |
| 1 | a | Cyanoethylation | 38.6 | 260 000 | 279 |
| 2 | a | Cyanoethylation | 30.2 | 330 000 | 355 |
| 3 | a | Cyanoethylation | 34.8 | 290 000 | 310 |
| 4 | a | Enzyme digestion* | 7.5 | 2 300 000 | — |
| 5 | b | Enzyme digestion* | 6.5 | 1 500 000 | 620 |

* These data represent actual ε-(γ-glutamyl)lysine dipeptide.

TABLE III

FRACTIONATION OF [$^{14}$C]LYSINE-LABELED MICROSOMES

| *Fraction* | *% washed microsomes* | *Specific activity of lysine (dpm [$^{14}$C]lysine/μmole lysine)* | *μmoles dipeptide* / mg protein* | *Daltons protein / dipeptide residue* |
|---|---|---|---|---|
| 1 | 26.3 | $1.37 \cdot 10^5$ | $4.1 \cdot 10^4$ | 2 400 000 |
| 2 | 3.5 | N.D.** | N.D.** | N.D.** |
| 3 | 1.8 | N.D.** | N.D.** | N.D.** |
| 4 | 59.7 | $2.05 \cdot 10^5$ | $3.0 \cdot 10^4$ | 3 300 000 |

* Analysis by enzyme digestion.

** Not determined; radioactivity too low.

## DISCUSSION

It has been demonstrated that L cells grown in labeled lysine incorporate the lysine into membrane proteins which contain the dipeptide, ε-(γ-glutamyl)lysine by isolation of labeled dipeptide chromatographically after exhaustive enzyme digestion of lipid-extracted plasma membrane and endoplasmic reticulum fragments. Higher

amounts of the isopeptide bond were found in plasma membrane than in endoplasmic reticulum. As judged by the large number of bands obtained upon sodium dodecyl sulfate acrylamide gel electrophoresis[23] none of which represents more than a few percent of the total protein, membranes are composed of many different polypeptides with large range of molecular weights. We do not yet know whether isopeptide bonds are present in many or few of the various membrane polypeptides.

A beginning effort was made to answer this question by measuring the amount of dipeptide present in several fractions of cell membranes separated on the basis of solubility. The dipeptide was found in the proteins solubilized by very dilute EDTA and in the insoluble residue, and if anything, in somewhat higher amount in the relatively soluble proteins than in the insoluble residue. Further studies are under way to determine where the dipeptide can be localized in specific polypeptide bands found upon sodium dodecyl sulfate acrylamide gel electrophoresis.

Isopeptide bond formation is responsible for the propagated cross-linking of stabilized fibrin clots where about 1 to 2 ε-(γ-glutamyl)lysine dipeptides are found per 330000 daltons of fibrin[10,11]. The separation of membrane proteins into many bands on sodium dodecyl sulfate-acrylamide gel electrophoresis leads one to believe that extensive, propagated cross-linking does not exist in membranes. Rather, the isopeptide bonds are intramolecular or perhaps link closely related, specific sets of polypeptides in the case of membrane proteins. A considerable amount of membrane protein appears to have molecular weights in excess of 150000 (refs 3, 6, 24, 25); it would be of considerable interest to determine whether or not these very large proteins contain proportionately more isopeptide bonds, and consist of smaller subunits held together by isopeptide bonds.

Cyanoethylation indicates unreactive lysine bonds. Cyanoethylation of the model proteins lysozyme and $T_4$ bacteriophage protein showed that less than 1 per 10000 and less than 1 per 5000 lysine residues respectively failed to react. On the basis of these data, we conclude that it is unlikely that free but hindered ε-amino groups of lysine fail to react and that all or almost all unreactive lysines are involved in some covalent linkages. Only some of the unreactive lysines can be accounted for as ε-(γ-glutamyl)lysine; the remainder of the unreactive lysine ε-amino groups must be involved in other covalent linkages. The nature of these other covalent linkages is presently unknown.

If membranes were synthesized as discrete domains or patches and degraded as macroscopic units, one might expect "life span" kinetics for amino acid labeling experiments similar to the "life span" kinetics observed for labeling of mammalian erythrocytes. Such "life span" kinetics have not been observed for membrane proteins, but rather first order degradation with different rate constants for different proteins[26,27]. The finding in the experiments reported here that lysine is incorporated into Fraction 1 at about half the rate it is incorporated into Fraction 4 of washed microsomes (Table III) is consistent with previous observations and support the view that membrane proteins are synthesized independently of one another, and after insertion into membranes are degraded randomly.

It is interesting to speculate about the functional significance of lysine additions, and particularly isopeptide bond formation in membrane proteins. One possible function may be to confer a degree of structural organization upon some membrane proteins and even to contribute to the anisotropy of structure required for active

transport and other vectorial membrane functions. Another possibility is that at least some proteins are synthesized on polysomes as a soluble precursor protein which is modified after synthesis by substitutions on the ε-amino group of lysine. Such substitutions may be expected to increase the hydrophobicity of membrane proteins[28] and enhance their insertion into the lipid matrix of membranes.

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