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Enzymatic Basis for the Ca^{2+}-Induced Cross-Linking of Membrane Proteins in Intact Human Erythrocytes†

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ABSTRACT: The accumulation of Ca^{2+} ions in intact human erythrocytes leads to the production of membrane protein polymers larger than spectrin. The polymer has a heterogeneous size distribution and is rich in γ-glutamyl-ε-lysine cross-links. Isolation of this isodipeptide, in amounts as high as 6 mol/10^5 g of protein, confirms the idea [Lorand, L., Weissmann, L. B., Epel, D. L., and Bruner-Lorand, J. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4479] that the Ca^{2+}-induced membrane protein polymerization is mediated by transglutaminase. Formation of the polymer in the intact cells is inhibited by the addition of small, water-soluble primary amines. Inasmuch as these amines are known to prevent the Ca^{2+}-dependent loss of deformability of the membrane, it is suggested that transglutaminase-catalyzed cross-linking may be a biochemical cause of irreversible membrane stiffening.

The accumulation of Ca^{2+} ions in intact human erythrocytes leads to the formation of new high-molecular-weight protein polymers in the cell membrane which are produced by covalent bonds other than disulfides (Lorand et al., 1976a,b). Since formation of the polymer is accompanied by the disappearance of band 4.1 and also by a reduction in spectrin and band 3 materials (for nomenclature, see Fairbanks et al., 1971), at least three internal membrane proteins seem to be involved. The polymerization reaction could be induced by Ca^{2+} concentrations which are also capable of activating the intrinsic, but otherwise latent, transglutaminase of these cells, suggesting that, similarly to blood clotting (see Lorand, 1972), intermolecular γ-glutamyl-ε-lysine bridging might occur. This idea was further strengthened by the finding that polymer formation could be inhibited if, prior to and during loading with Ca^{2+}

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ions, the cells were exposed to primary amines which are known to interfere with transamidase-catalyzed cross-linking reactions (see Lorand et al., 1968a; Lorand and Nilsson, 1972).

In the present paper, we explore additional aspects of the Ca^{2+}-triggered and transglutaminase-mediated events in the intact human erythrocytes and document the de novo formation of γ-glutamyl-ϵ-lysine peptide cross-links in cell membranes following the intracellular accumulation of Ca^{2+} ions.

Materials and Methods

Red cells obtained from fresh human blood were washed at 4 °C by repeated centrifugations (3 × 1000*g* for 10 min) either in 0.16 M NaCl or in a solution containing 0.1 M KCl, 0.06 M NaCl, 10 mM glucose, and 5 mM Tris[1]-HCl (pH 7.4) and were used for experiments on the day of collection. A 5 mM stock solution of ionophore A23187 (a gift from Eli Lilly Laboratories, Indianapolis, Ind.) was prepared in dimethyl sulfoxide, and it was diluted fivefold with the washing buffer just prior to use. Histamine, aminoacetonitrile, cystamine, cysteamine, putrescine (Aldrich), glycine ethyl and methyl esters (Mann Research Laboratories), purchased as the hydrochloride salts, were freshly dissolved in water and adjusted to pH 7.4. A solution of isoniazid (Mann Research Laboratories) was prepared in the same manner. [*ring*-2-^{14}C]Histamine (Amersham-Searle) was dissolved into cold histamine, yielding a specific activity of 29.5 mCi/mmol. For transglutaminase assays in broken cells, [^{14}C]putrescine (Amersham-Searle; 60 mCi/mmol) and chemically methylated casein, i.e., *N,N'*-dimethylcasein (Lin et al., 1969), were used.

Typically, 25-μL solution of the amines at various concentrations were mixed with 1 mL of cell suspension and were allowed to incubate for 30 min at 37 °C. Then 20 μL of the ionophore (final concentration 20 μM) and 20 μL of $CaCl_2$ (to yield Ca^{2+} concentrations from 0.3 to 1.5 mM) were added. Controls without $CaCl_2$ contained equivalent concentrations of $MgCl_2$. Following incubation of the cells (1 to 18 h), the membranes were isolated and solubilized in the presence of 0.04 M dithiothreitol using the method of Fairbanks et al. (1971) and were subjected to analysis by $NaDodSO_4$ gel electrophoresis on 4.5% polyacrylamide (Steck and Yu, 1973). In the labeling experiment with [^{14}C]histamine, slices of the gels were solubilized with hydrogen peroxide (Tischler and Epstein, 1968) and were counted in a Packard scintillation spectrometer.

Purified membrane protein polymer and spectrin were obtained from ghosts isolated after treatment of packed erythrocytes (200 mL) with 1.5 mM Ca^{2+} in the presence of 20 μM of A23187, over an 18-h period at 37 °C. A 15-mL aliquot of the membrane suspension was solubilized by adding a 5-mL mixture of 4% $NaDodSO_4$, 0.1 M DTT, and 0.2 M Tris-HCl of pH 7.4. The solution was concentrated (to approximately 5 mL) by ultrafiltration using an Amicon XM100 membrane and was dialyzed against 1% $NaDodSO_4$ in 0.05 M Tris-HCl of pH 7.4 Following dialysis, 0.01 M dithiothreitol was added and insoluble materials were removed either by centrifugation (1500*g* for 10 min) or by passage through a 5 μm Millipore filter. The clear solution was then applied to a 92 × 2.6 cm column of Sepharose 4B CL (Pharmacia), eluting with the same buffer at a flow rate of 20 mL/h. Collected fractions (5 mL) were analyzed for the presence of polymer and for spectrin by $NaDodSO_4$-polyacrylamide gel electrophoresis, and appropriate fractions were pooled and concentrated to 15 mL by ultrafiltration as above and were precipitated with 25% trichloroacetic acid. The precipitated material was washed first in 10% trichloroacetic acid and then twice with 50% ethanol–acetone, once with acetone, and, finally, dried in vacuo. Occasionally, the $NaDodSO_4$ was removed by the procedure of Weber and Kuter (1971). The heterogeneity of the isolated membrane protein polymer was examined by $NaDodSO_4$ electrophoresis on 2% agarose (Moroi et al., 1975).

Amino acid compositions, following hydrolysis in constant-boiling HCl for 24, 48 and 72 h at 110 °C in vacuo, were obtained in a Durrum amino acid analyzer, and the data were corrected for hydrolytic losses. Half-cystine was determined as cysteic acid after oxidizing the proteins with performic acid (Moore, 1963).

The isolation and quantitation of the γ-glutamyl-ϵ-lysine isodipeptide from the enzymatic digest of the protein polymer were performed essentially by the method of Lorand et al. (1968a,b). The proteolytic degradation of the cross-linked protein (10 mg, dispersed in 1 mL of 0.1 M ammonium bicarbonate of pH 8) was carried out by the sequential addition of four proteolytic enzymes. Subtilisin (Novo; 1% w/w relative to substrate) caused clearing of the suspension after a 12-h incubation at 37 °C; nevertheless, two more additions of subtilisin were made at approximately 10-h intervals. After inactivation of subtilisin by heating (100 °C, 15 min), digestion was continued by addition of 0.75% w/w of both Mg^{2+}-activated leucine aminopeptidase (Worthington) and prolidase (Sigma) for 10 h. This was followed by a second addition of leucine aminopeptidase for the same duration of time. Lastly, 1% w/w of carboxypeptidase A (Worthington) was added, and this incubation was allowed to proceed for about 12 h. The entire digest of about 1.5 mL was then passed through a 0.45-μm Millipore filter, lyophilized, and taken up in a pH 2.2, 0.2 N citrate buffer. A similarly prepared and incubated mixture of the proteolytic enzymes themselves, without polymer added, served as control. Analysis and collection of γ-glutamyl-ϵ-lysine were performed on a Beckman 120C amino acid analyzer using a Custom AA-15 resin column (0.9 × 55 cm, equilibrated in 0.2 N citrate, pH 3.31, at a flow rate of 75 mL/h at 55 °C) from which the dipeptide elutes between leucine and tyrosine (Lorand et al., 1968a,b; Williams-Ashman et al., 1972). In parallel runs the dipeptide was collected, taken to dryness, and hydrolyzed in constant-boiling HCl for 24 h at 110 °C, in order to measure the ratio of glutamic acid to lysine contents.

The maximal number of cross-links to which lysine side chains might contribute was estimated by treating the subtilisin digest of the polymer with acrylonitrile and measuring the residual lysine content after total acid hydrolysis (Pisano et al., 1969; Williams-Ashman et al., 1972).

When analyzing cross-links directly in the cell membranes themselves, rather than in the purified polymer, very similar procedures were followed. After solubilization of the erythrocyte ghosts (15 mL) in 1% $NaDodSO_4$, 25 mM dithiothreitol, and 0.05 M Tris-HCl at pH 7.4 and concentrating (to 5 mL) with an Amicon XM-100 filter, the proteins were precipitated with trichloroacetic acid, washed, and dried as described for the polymer above. The average amino acid composition of the membrane was measured after hydrolyzing 2 mg of the dried material with 2 mL of constant-boiling HCl for 24 h at 110 °C. For the purpose of cyanoethylation (Pisano et al., 1969), 4-mg aliquots of the dried membrane proteins were suspended in 1 mL of 0.1 M ammonium bicarbonate of pH 8 and were digested with 0.04 mg of subtilisin for about 16 h, prior to treatment with acrylonitrile. The cyanoethylated

[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

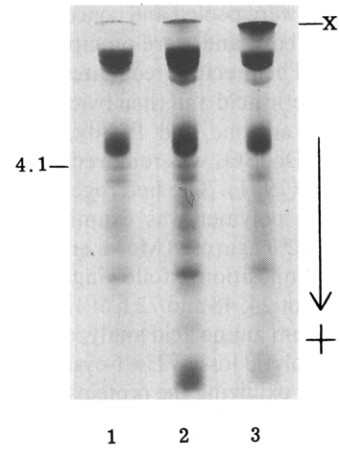

FIGURE 1: Inhibition of membrane protein polymerization by aminoacetonitrile. Fresh erythrocytes were incubated for 3 h at 37 °C with 20 μM A23187 ionophore and (gel 1) 1.5 mM $MgCl_2$, (gel 2) 1.5 mM $CaCl_2$ in the presence of 10 mM aminoacetonitrile or (gel 3) 1.5 mM $CaCl_2$ only. Following incubation, membranes were isolated, solubilized in 1% $NaDodSO_4$–40 mM DTT, and subjected to polyacrylamide gel (4.5%) electrophoresis. Polymer is denoted as X.

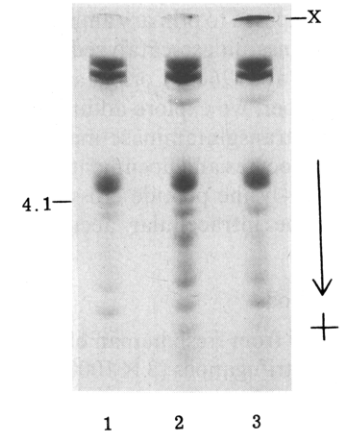

FIGURE 2: Inhibition of polymer formation by cystamine. The procedure outlined in Figure 1 was followed, except that aminoacetonitrile was replaced by 10 mM cystamine.

material was lyophilized and hydrolyzed (Williams-Ashman et al., 1972), and 40 μg of protein equivalent was placed on the Durrum amino acid analyzer for measuring residual lysine content.

Transamidase activity was measured by the analytical method of Lorand et al. (1972a). The tube containing washed packed cells was immersed in dry ice–ethanol and was brought to 37 °C immediately after freezing. Aliquots of 25 μL of the red cell lysate were mixed with 20 μL of *N,N'*-dimethylcasein (2% in 50 mM Tris-HCl of pH 7.5) and 10 μL of [^{14}C]putrescine (1.6 mM). The enzyme reaction was initiated by adding 10 μL of $CaCl_2$ at various concentrations. Reactions were terminated after 10, 20, and 30 min by spotting 5-μL aliquots of the mixtures onto Whatman 3MM filter papers (1 cm^2) and immersing in 10% trichloroacetic acid. Samples were prepared for scintillation counting as previously described (Lorand et al., 1972a; Curtis and Lorand, 1976).

Results

Inhibition of Membrane Protein Polymerization by Synthetic Amines Using Intact Red Cells. Incubation of fresh human erythrocytes in a Ca^{2+}-containing medium, with A23187 ionophore present, leads to the formation of non-disulfide-bonded protein polymers in the cell membrane (Lorand et al., 1976a,b) which can be readily detected by electrophoresis of the membrane constituents after reduction with dithiothreitol and solubilization in $NaDodSO_4$. In most experiments, the polymers are so large ($\geq 10^6$; as in Figure 1, marked by X) that they can barely penetrate polyacrylamide (4.5%) gels. As judged from Coomassie blue staining, formation of the polymer is accompanied by the disappearance of the 4.1 band and by a reduction in the amount of proteins in the spectrin and band 3 regions. The glycoprotein profile, developed by "stains-all" (King and Morrison, 1976), however, does not seem to change in the membrane upon loading of the cells with Ca^{2+}.

It is well known from investigations on the cross-linking of fibrin (Lorand et al., 1968a and 1972c; Lorand and Nilsson, 1972) that extraneously added primary amines can be of considerable use in probing the question as to whether non-disulfide type of protein polymerizations proceed through a transglutaminase-catalyzed amide exchange, involving formation of γ-glutamyl-ϵ-lysine cross-links. The most obvious sign of interference by a synthetic amine is inhibition of the polymerization reaction itself. This is accomplished by virtue of the fact that the amine competes against ϵ-lysines of the protein. In experiments with intact cells, however, the situation is certain to be more complex, and inhibition will depend on whether the amine is able to penetrate the cell membrane and whether it can gain access to the acyl-enzyme intermediate during the process of the intrinsic cross-linking of organized membrane proteins. Thus, it was of particular significance to find that a number of amines could actually inhibit the Ca^{2+}-triggered cross-linking in intact erythrocytes. Aminoacetonitrile (Figure 1), cystamine (Figure 2), and cysteamine proved to be most effective. Incubation with 10 mM cystamine, for example, for 30 min prior to and then during loading of the cells with as much as 1.5 mM Ca^{2+} at 37 °C, prevented polymer formation for periods of up to 3 h and, at the same time, the 4.1 band remained virtually intact. Appreciable inhibition of polymer formation was also obtained by treating cells with either histamine or glycine methyl or ethyl ester,[2] but at 10 mM concentrations none of these seemed to be as potent as the three compounds above, and they were not able to prevent the disappearance of band 4.1 which appears to be the most sensitive sign of change in the membrane protein profile for Ca^{2+}-loaded cells. Putrescine (at 10 mM) was a rather poor inhibitor of polymerization in the intact erythrocyte.

Cystamine is clearly not a pure amine type of inhibitor. It is known to undergo a disulfide exchange with the active-center thiol groups of similar enzymes, i.e., guinea pig liver transglutaminase and human fibrinoligase (C. Svahn and L. Lorand, unpublished results), causing their inactivations. Thus, we believe that inhibition of polymer formation by cystamine is due in large extent to its direct inactivating effect on transglutaminase.

The Intrinsic Labeling of Some Membrane Proteins in Intact Erythrocytes Using [^{14}*C*]*Histamine.* Histamine, mainly because of the availability of the ^{14}C isotope at acceptable levels of specific activity, was selected to examine the question whether the incubation of intact cells with this inhibitory amine (Lorand et al., 1976a) could label specific membrane proteins in the course of Ca^{2+} loading. The rationale is, of course, quite

[2] Isoniazid which can act as an amine donor in transglutaminase-catalyzed reactions (Lorand et al., 1972b) also inhibited membrane protein polymerization in the intact red cell.

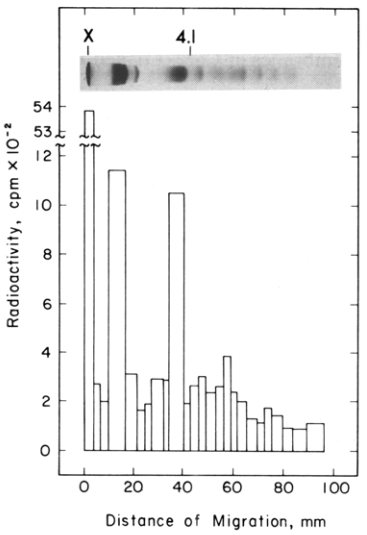

FIGURE 3: Intrinsic labeling of membrane components by [^{14}C]histamine in intact erythrocytes. Fresh cells were incubated with 1.5 mM $CaCl_2$ and 20 μM ionophore, in the presence of 5 mM [^{14}C]histamine, for 6 h at 37 °C. Following incubation, membranes were isolated and analyzed as in Figure 1. Bars indicate the distribution of radioactivity in polyacrylamide gel slices. In the absence of Ca^{2+}, no radioactivity could be found in the gel.

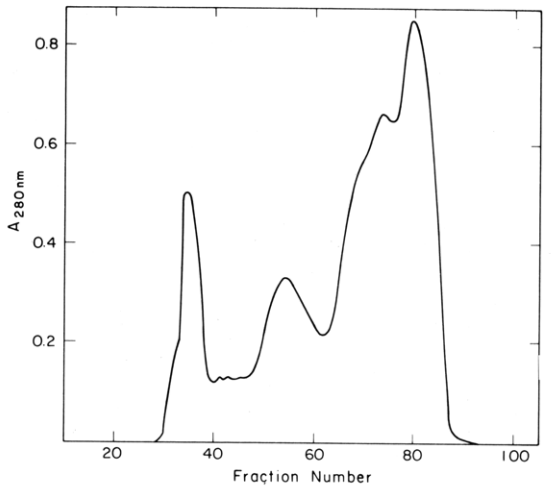

FIGURE 4: Elution profile of membrane proteins from Sepharose 4B CL following incubation of red blood cells with 1.5 mM $CaCl_2$ and 20 μM ionophore for 18 h at 37 °C. For details, see Materials and Methods section.

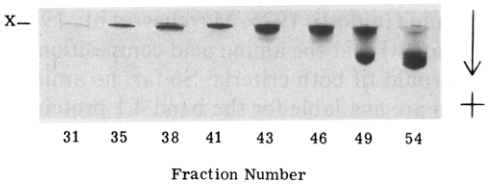

FIGURE 5: $NaDodSO_4$–polyacrylamide (4.5%) gel electrophoretic analysis of material obtained from the experiment shown in Figure 4. Fractions (as marked) were pretreated with DTT (40 mM) prior to electrophoresis. Besides those shown, no other protein bands were visible.

analogous to that employed for titrating the γ-glutamine cross-linking sites in fibrin through the functioning of the Ca^{2+}-dependent transamidase itself (Lorand and Ong, 1966a,b; Lorand and Chenoweth, 1969; Lorand et al., 1972b,c). It is shown in Figure 3 that the loading of erythrocytes with Ca^{2+} (1.5 mM) in the presence of externally added [^{14}C]histamine (5 mM) does, indeed, give rise to membrane protein labeling. Moreover, the isotope distribution pattern is highly selective in the sense that, apart from labeling the growing polymer, the radioactive histamine becomes incorporated into proteins of the spectrin and band 3 regions only. The observation clearly suggests that these particular membrane proteins contain γ-glutaminyl functionalities which, upon activation of the intracellular enzyme by Ca^{2+}, become blocked by reaction with histamine in lieu of participating in an amide exchange with ϵ-lysine side chains in proteins.

Estimation of the Number of Lysine Residues Maximally Involved in the Cross-Linking of Membrane Proteins in Ca^{2+}-Loaded Erythrocytes. When proteins are reacted with acrylonitrile, it is possible to select conditions such that all free lysine side chains become cyanoethylated. Thus, if total acid hydrolysis of the modified protein yields any lysine at all, it is thought to derive from various types of N^{ϵ}-acyllysines which were unavailable for reaction with acrylonitrile (Pisano et al., 1969). Some or all of the acrylonitrile-resistant lysines may be due to γ-glutamyl-ϵ-lysine cross-links so that the residual lysine content of acid hydrolysates of cyanoethylated proteins may be used as an index for evaluating the maximal amount of such isopeptide bridges.

We have prepared membranes from cells which were exposed to 20 μM ionophore at 37 °C for 18 h either in the presence of 1.5 mM Mg^{2+} or the same concentration of Ca^{2+}. In order to secure more complete access of all ϵ-lysine side chains to the acrylonitrile reagent, the membranes were treated with subtilisin prior to cyanoethylation, as described under Materials and Methods. Following reaction with acrylonitrile and total acid hydrolysis, membranes isolated from Ca^{2+}-loaded cells gave a residual lysine content of about 3–4 mol per 100 000 g of protein. By contrast, membranes of the control (i.e., Mg^{2+}-treated) cells, examined under identical conditions, yielded less than 0.2 mol of residual lysine per 100 000 g of protein.

Characterization of the Polymer Formed in Intact Cells during the Accumulation of Ca^{2+} and Isolation of the γ-Glutamyl-ϵ-lysine Isopeptide Cross-Links. When the cell membrane of Ca^{2+}-loaded erythrocytes (following treatment with 20 μM ionophore and 1.5 mM Ca^{2+} for 18 h at 37 °C) was dispersed in 1% $NaDodSO_4$ containing 0.025 M dithiothreitol and was chromatographed on Sepharose 4B CL as described under Materials and Methods, an elution profile such as shown in Figure 4 was obtained. The first major peak (fractions 30–43) is characteristic for cells exposed to Ca^{2+} and, as seen by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5), these fractions correspond to polymeric proteins. Fraction 49 contained approximately equal amounts of polymer and spectrin. The behavior of the polymer fractions on $NaDodSO_4$–polyacrylamide gels indicates a multiple distribution of molecular weights; fraction 31 does not penetrate even the top regions of the gels, but proteins from consecutive fractions enter 4.5% polyacrylamide to a considerable degree. This heterogeneity of the composition of the polymer is more readily demonstrable by $NaDodSO_4$ electrophoresis in 2% agarose (Moroi et al., 1975) which, as shown in Figure 6, revealed that the polymer comprised several distinct components of different molecular weights. So far, we have been unable to obtain a sharper resolution for these individual components on agarose, and this, in itself, may indicate a heterogeneity of size distribution within each of the bands seen on the electrophoretic profile.

The amino acid composition of the pooled polymeric material obtained from the Ca^{2+}-loaded intact erythrocytes (i.e., fractions 30–43 in Figure 6), and that of spectrin (fraction 55),

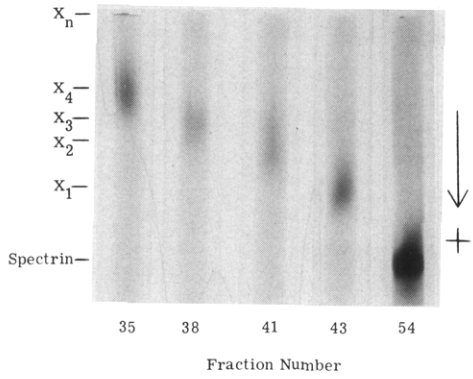

FIGURE 6: $NaDodSO_4$–agarose (2%) gel electrophoretic profile of the fractions presented in Figure 5. Polymeric species are denoted as X_1 to X_n.

is given in Table I. The main differences pertain to proline and glutamic acid contents, suggesting that a membrane constituent containing significantly more proline and less glutamic acid than spectrin participates in polymer formation. Reports in the literature show (Tanner and Boxer, 1972; Yu and Steck, 1975; Ho and Guidotti, 1975; Marchesi et al., 1977, personal communication) that the amino acid composition of the band 3 protein would fit both criteria. So far, no amino acid analytical data are available for the band 4.1 protein.

Degradation of the polymer by the sequential application of proteolytic enzymes, as outlined under the Materials and Methods section, would not affect peptides comprising amino acid residues in linkages not involving α-carbonyl groups. Thus, if γ-glutamyl-ϵ-lysine was present, the elution profile for the total enzymatic digest of the polymer should and did, in fact, show (Figure 7) the presence of a peak at 305 min in the Beckman 120C analytical system described (Lorand et al., 1968a,b; Williams-Ashman et al., 1972) which is the position where γ-glutamyl-ϵ-lysine emerges. From the measured ninhydrin color value of the synthetic reference peptide and from the amino acid composition of the polymer, we calculate that the polymer contains 5.9 mol of γ-glutamyl-ϵ-lysine per 100 000 g of protein. From cyanoethylation performed on the subtilisin digest of the polymer, a value of 6.8 mol of acrylonitrile-resistant lysine per 100 000 g protein was obtained, in rather close agreement with the actual amount of dipeptide cross-links isolated by the direct approach.

The material obtained from the total enzymatic digest as γ-glutamyl-ϵ-lysine, when taken to dryness and hydrolyzed in constant-boiling HCl, gave a Glu/Lys ratio of 1.16. The reference dipeptide treated in an identical manner, including initial passage through the Beckman 120C analyzer, gave a Glu/Lys ratio of 1.11.

Ca^{2+} Sensitivity of Transglutaminase in Erythrocyte Lysates. The results presented so far establish the fact that Ca^{2+} accumulation in the intact human red cell gives rise to the formation of membrane polymers with a high frequency of γ-glutamyl-ϵ-lysine cross-links. Both this circumstance and the inhibition of the polymerization reaction by an amine with the concomitant labeling of specific membrane proteins point to the participation of an intrinsic transglutaminase in the Ca^{2+}-induced intracellular events. Figure 8 presents data which show that this enzyme in the freshly lysed erythrocyte environment occurs in an inactive form. As measured by the incorporation of [^{14}C]putrescine into *N,N'*-dimethylcasein (Lorand et al., 1972a), without the addition of Ca^{2+}, no uptake of the isotope by proteins can be seen. The enzyme, however, is readily activated by adding Ca^{2+} and, as the data in Figure 8 pertaining to a single donor show, an apparent half-saturation value of 0.3 mM was obtained. Similar values were also obtained with pooled blood cells.

TABLE I: Amino Acid Composition of Polymer[a] and Spectrin[b] Isolated from Membranes of Ca^{2+}-Loaded Human Erythrocytes.

Amino acid	Quantity (mol %) Polymer	Spectrin
Lys	6.6	6.8
His	2.3	2.8
Arg	5.3	6.0
Asp	9.5	10.2
Thr[c]	5.0	4.4
Ser[c]	6.7	5.7
Glu	14.9	18.4
Pro	4.7	2.7
Gly	6.6	5.1
Ala	8.2	8.7
Val[d]	6.3	5.2
Met	2.3	2.1
Ile[d]	4.0	3.8
Leu[d]	10.7	11.8
Tyr	2.6	2.3
Phe	3.7	3.3
cysteic acid[e]	0.8	0.7

[a] Fractions 30–43 in Figure 6. [b] Fraction 55 in Figure 6. [c] Extrapolated to zero time of hydrolysis. [d] Values pertain to 72 h of hydrolysis. [e] Measured separately.

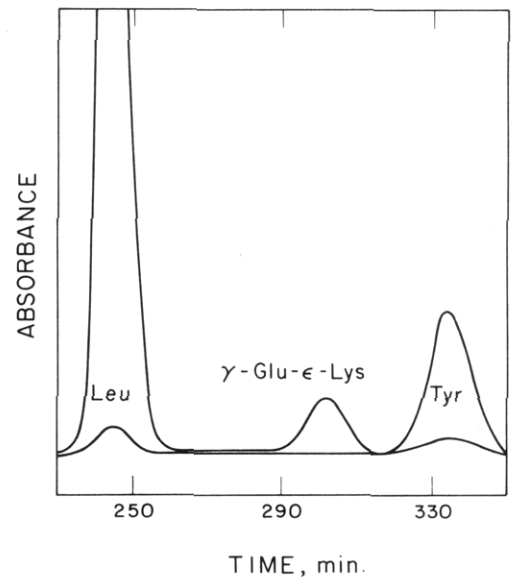

FIGURE 7: Isolation of γ-glutamyl-ϵ-lysine isodipeptide from the enzymatic digest of the polymer obtained from the membranes of Ca^{2+} loaded erythrocytes. For details, see Materials and Methods. The lower tracing represents the pattern obtained when the enzymes used for digestion were incubated without addition of polymer. The γ-Glu-ϵ-Lys peak obtained from 0.72 mg of polymer corresponds to a recovery of 40 nmol of the isodipeptide.

Discussion

Earlier work from this laboratory (Lorand et al., 1976a,b) already strongly suggested that the Ca^{2+}-dependent formation of membrane protein polymers by covalent linkages other than disulfide, occurring in broken as well as in intact erythrocytes, was a transglutaminase-mediated event. With regard to the broken red cell system, confirmatory evidence has since been obtained by King and Morrison (1977) and by Anderson et al. (1977). The present work (see also Siefring and Lorand, 1977) deals with intact fresh human erythrocytes in which membrane

protein polymerization was induced by incubating cells with Ca^{2+} in the presence of ionophore A23187. Formation of the membrane polymer, which is accompanied by the disappearance of the 4.1 band as well as by a reduction in the normal amounts of spectrin and band 3 proteins (Lorand et al., 1976a,b), can be prevented by extraneously added primary amines (Figures 1 and 2) which are known to serve as substrates for transglutaminases. Moreover, as predicted for catalysis by transglutaminase, an inhibitory amine could be used to selectively label the polymer and also the spectrin and band 3 constituents of the membrane, marking these as specific substrates for the intracellular Ca^{2+}-triggered reaction (Figure 3).

The nature of the polymer obtained from membranes of Ca^{2+}-loaded erythrocytes is of considerable interest because it bears directly on this unique assembly process within the intact cells. Our foremost concern in the present work was to present evidence for the existence of γ-glutamyl-ϵ-lysine links in the polymer. The reported data indicate a relatively high average frequency of isopeptide bridging (ca. 6 mol/100 000 g of protein, utilizing approximately 10% of all lysines) in this membrane component. Though this is not as high a frequency as can be seen in the most cross-linked structure of this type (see Williams-Ashman et al., 1972; clotted seminal plasma; ca. 30 mol/100 000 g of protein, corresponding to about 30% of all lysines), it is considerably greater than that found in fibrin (see Lorand et al., 1968a,b; Matacic and Loewy, 1968; Pisano et al., 1969; ca. 1.8 mol/100 000 g, utilizing only about 3% of all lysines).

Resolution of the isolated membrane polymer into at least five distinct molecular-weight groups is also significant. The largest could not enter 2% agarose (Figure 6) and should thus be considered to have a size greater than 1.5×10^6. The presence of the other four smaller species suggests that they may be intermediates in what appears to be a graded, rather than concerted, enzymatic fusion of membrane proteins. As far as amino acid composition is concerned, the bulk polymer isolated from intact normal erythrocytes seems to differ from the one obtained by Lux and John (1977) from the cells of splenectomized individuals. However, there is a possibility that the latter may have included some disulfide-bonded material which was not cross-linked by γ-glutamyl-ϵ-lysine. The composition of the high-molecular-weight species seen in experiments with 2–5 mM Ca^{2+} in lysed systems (Carraway et al., 1975) agrees rather well with that of the polymer described. In view of our earlier work (Lorand et al., 1976a,b), and also in view of recent reports by King and Morrison (1977) and by Anderson et al. (1977), there is every reason to believe that the material obtained from experiments with broken cells is, in fact, identical with the polymer we isolated from Ca^{2+}-loaded intact erythrocytes.

The demonstration (Figure 8) that the activity of the latent transglutaminase of erythrocytes is regulated by Ca^{2+} gives a particularly relevant dimension to our studies. Half-maximal velocities are obtained by concentrations of Ca^{2+} which have actually been found in either old (LaCelle et al., 1973) or abnormal red cells (LaCelle, 1971; Eaton et al., 1973; Palek, 1973). Accumulation of Ca^{2+} in erythrocytes is known to give rise to loss of membrane deformability (Kirkpatrick et al., 1975) and to an irreversible transformation of cell shape from discocyte to echinocyte (Weed and Chailley, 1973). The transglutaminase-catalyzed cross-linking of membrane proteins may be a key for explaining these Ca^{2+}-induced permanent alterations of cell structure in biochemical terms. Current work, with transglutaminase inhibitors as primary tool, is aimed at examining this proposition. Using the micropipet

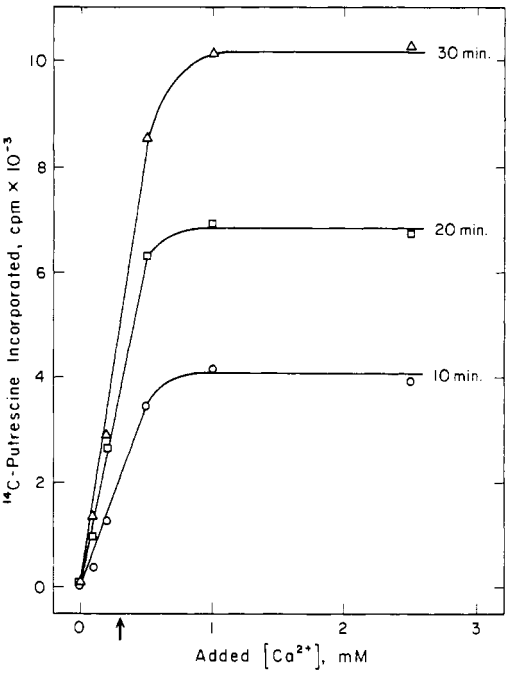

FIGURE 8: Ca^{2+} dependence of transglutaminase activity in fresh human erythrocyte lysate. Details are given under Materials and Methods. Arrow indicates Ca^{2+} requirement for half-maximal activity of the enzyme.

method for measuring membrane deformability in individual cells, it was possible to show (Smith et al., 1977) that amines, which interfered with transamidation, inhibited the development of the Ca^{2+}-triggered viscoelastic changes in the cell membrane. Furthermore, the presence of an amine can also prevent the echinocytic transformation from becoming irreversible (Siefring et al., 1978).

The use of transglutaminase inhibitors, which penetrate whole cells, opens up possibilities for probing the question as to whether the formation of the γ-glutamyl-ϵ-lysine containing membrane polymer is in any way related to red cell survival in the circulation. Other pertinent pathophysiological issues will, no doubt, include the irreversible deformation of sickle-cell membranes (Lux et al., 1976) and formation of protein deposits (e.g., Heinz bodies; Jacob, 1970). From the fact that the intracellular accumulation of Ca^{2+} ions occurs only in old and in energy-depleted cells, it would appear that the transglutaminase-mediated process of membrane stiffening is mainly relevant to dying cells, and it might be possible to extrapolate from our experiments with erythrocytes to other cell types. In nucleated cells, Ca^{2+} may not have to be carried in from the outside, but might be released into the cytoplasm from mitochondrial or other stores in the process of cell death. Intracellular transglutaminases might thus play a general role in the completion of cell cycles. It is interesting in this regard that the formation of the keratinizing envelope in skin cells (Rice and Green, 1977; Abernethy, et al., 1977) seems to be a transglutaminase-catalyzed reaction.

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