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Formation of γ-Glutamyl-ε-Lysine Bridges Between Membrane Proteins by a Ca^{2+}-Regulated Enzyme in Intact Erythrocytes

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A rise in the intracellular concentration of Ca^{2+}-ions in human erythrocytes causes the formation of high-molecular-weight membrane protein polymers, cross-linked by γ-glutamyl-ε-lysine side chain bridges. Cross-linking involves proteins at the cytoplasmic side of the membrane (band 4.1, spectrin, and band 3 materials) and the reaction is catalyzed by the intrinsic transglutaminase. This enzyme is regulated by Ca^{2+}-ions and it exists in a latent form in normal cells. The protein polymer, isolated from the membranes of Ca^{2+}-loaded intact human red cells, is heterogeneous in size and may contain as many as 6 moles of γ-glutamyl-ε-lysine cross-links per 100,000 gm of protein.

Synthetic compounds, which either compete against the ε-lysine cross-linking functionalities of the protein substrates (eg, histamine, aminoacetonitrile, cystamine) or directly inactivate the transamidase (eg, cystamine), inhibit the membrane polymerization reaction in intact human erythrocytes.They also interfere with the Ca^{2+}-induced irreversible shape change from discocyte to echinocyte and inhibit the irreversible loss of membrane deformability. Thus, the transamidase-catalyzed production of γ-glutamyl-ε-lysine cross-links in the membrane may be a common denominator in these cellular manifestations.

Key words: fibrin cross linking, erythrocyte transamidase, spectrin, cytoskeleton

SOME CHARACTERISTICS OF Ca^{2+}-LOADED ERYTHROCYTES

The experimental approach of rapid loading of erythrocytes with Ca^{2+} by using the specific ionophore A23187 proved to be of considerable advantage in our efforts to assess the molecular basis of the Ca^{2+}-induced irreversible alterations of shape and loss of membrane deformability. Inasmuch as general terms such as "Ca^{2+}-loaded" or "energy-depleted"

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cells may have different meanings for different investigators, it may be best to specify conditions at the outset. Experiments were performed with freshly washed human red blood cells (hematocrit 10–70%), typically over periods of 30 min to 18 h at 37°C, with 10–20 μM ionophore and 0–1.5 mM added concentration of Ca^{2+}. Controls always contained Mg^{2+}, equimolar to the Ca^{2+} employed. Loss of K^+ from the cells was counteracted by using a buffer containing 0.1 M KCl, 0.06 M NaCl, 10 mM glucose, and 5 mM Tris-HCl (pH 7.4). It should be pointed out that in spite of inclusion of glucose the intracellular adenosine triphosphate (ATP) concentration dropped to less than 20% of normal by 2 h of incubation with 1.5 mM Ca^{2+}. Thus, it is important to bear in mind that within the context of the experiments described Ca^{2+}-loaded cells are also low in ATP. The loss of ATP is attributable to the presence of Ca^{2+}, because cells incubated with ionophore and Mg^{2+} had essentially normal levels of ATP.

For purposes of the present discussion, attention should be directed to three important manifestations resulting from the intracellular accumulation of Ca^{2+}-ions in human erythrocytes: irreversible echinocytic transformation [1, 2], irreversible loss of membrane deformability [3, 4], and formation of membrane protein polymers cross-linked by γ-glutamyl: ε-lysine peptide bridges [5–9]. These nondisulfide-linked polymers can be readily detected by electrophoresis of the membrane constituents after reduction with dithiothreitol (DTT) and solubilization in sodium dodecyl sulfate (SDS) (Fig. 1), and their formation may be a common denominator in the irreversible cellular manifestations referred to above. In most experiments the polymers are so large ($\geqslant 10^6$, as in Fig. 1, marked by X) that they can barely penetrate polyacrylamide (4.5%) gels. As judged by

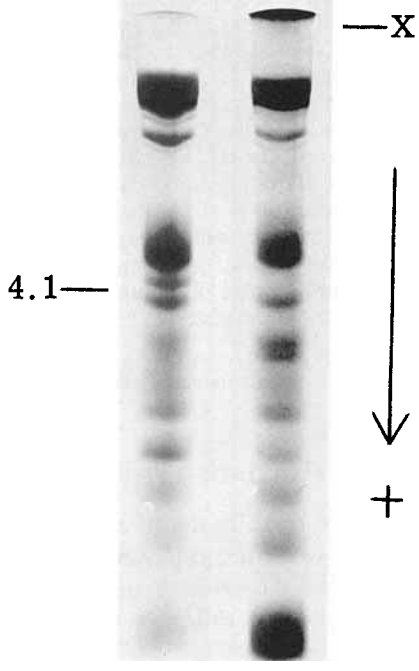


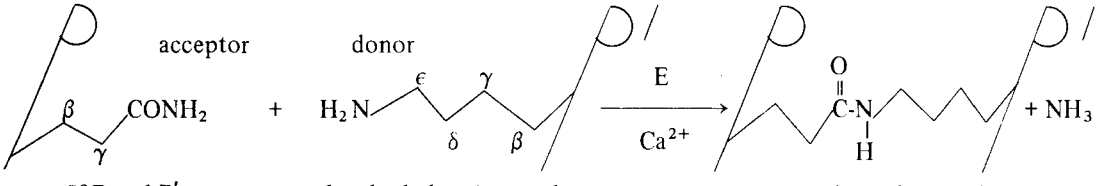
Fig. 1. Polyacrylamide (4.5%) SDS (0.2%) gel electrophoresis of membrane proteins obtained from fresh erythrocytes following incubation (3 h, 37°C) with ionophore (20 μM) and 1.5 mM $CaCl_2$ (gel on right) and 1.5 mM $MgCl_2$ (gel on left).

Coomassie blue staining, polymer production is accompanied by the disappearance of the 4.1 band, and by a lowering of the amount of proteins in the spectrin and band 3 regions (for nomenclature, see Fairbanks et al [10]), suggesting that polymerization involves at least these three membrane protein constituents. Polymer formation was observed at added concentrations of Ca^{2+} as low as 0.15 mM.

The chemical nature of the nondisulfide cross-links in the membrane polymer and the manner in which they arise are obviously of great interest. Past research in our laboratory on blood clotting [11–14] provided the conceptual as well as the technical framework for the erythrocyte studies presented here, and a brief review of the enzyme chemical background might be helpful in appreciating various aspects of the problem.

TRANSAMIDASE-CATALYZED CROSS-LINKING OF PROTEINS

In its simplest form, the dimerization of two protein molecules (P and P′) by a transamidase (transglutaminase*) of the endo-γ-glutamine:ε-lysine transferase type [14] may be outlined as follows:

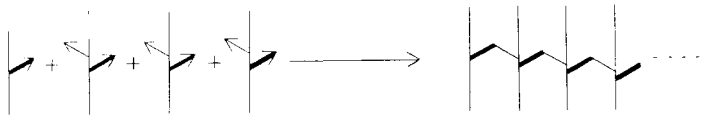


If P and P′ represent molecules belonging to the same protein species, a homologous dimer is obtained, whereas if they are of different types, a hybrid dimer ensues. From a thermodynamic point of view, as written, the amide exchange reaction would be expected to be readily reversible, but in reality – as in clot formation, where polymerization leads to a phase transition – reversibility may be hard to demonstrate. There is clear evidence from studies on fibrin that, of the large number of glutaminyl and lysyl residues of the protein, only a select few react with the specific enzyme [15] and are involved in the actual cross-linking event as acceptor and donor functionalities, respectively. It is also interesting to recall that relatively minor alterations of the protein substrate, as occurring in the fibrinogen ⟶ fibrin transformation, can affect reactivity towards the cross-linking enzyme.

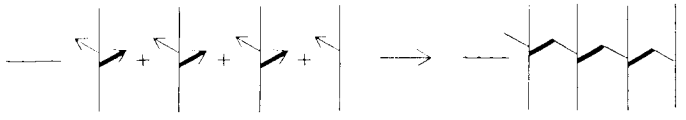
A given protein is considered bifunctional if it carries enzyme-specific acceptor as well as donor cross-linking functionalities. If in shorthand notation a bifuctional protein is symbolized as ↖↗ [where the | vertical line, regardless of the protein species, represents the polypeptide backbone, the ↗ heavy arrow represents the acceptor glutamine, and the ↖ light arrow represents the electron donor lysine side chains], the possibility of an infinite degree of linear polymerization by γ-glutamyl-ε-lysine bridges may be indicated as:

*The name "transglutaminase" (given by Clarke et al, Arch Biochem Biophys 79:338, 1958) is actually a misnomer because glutamine (with its α-NH_2 group free) is not even a substrate for these types of enzymes.

There might be proteins which carry acceptor or donor functionalities exclusively. Accordingly, they could only serve either as chain initiators or terminators, and their presence in a mixture might give rise to the following possibilities:

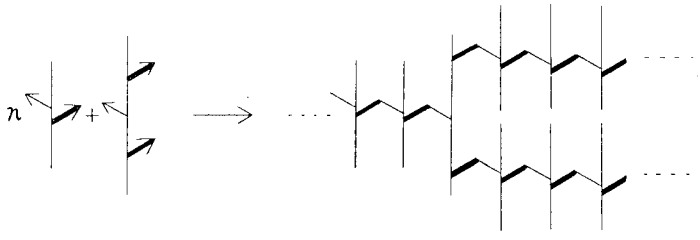


or



or even

The minimum requirement for branching would be that a reacting protein substrate be present either with two acceptor or two donor functionalities:



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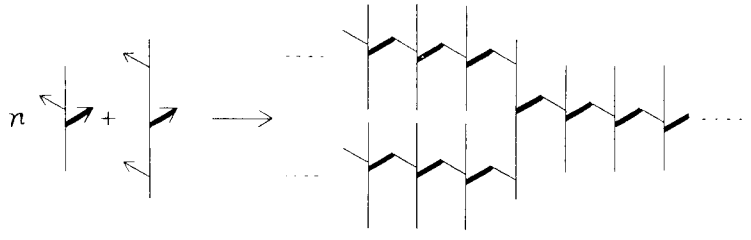


This brief analysis may suffice to show that the pattern of fusion can be varied and that cross-linked proteins of the type formed in the erythrocyte membrane during accumulation of intracellular Ca^{2+} (Fig. 1) may in fact represent diverse populations of growing polymers of not very well definable size and composition.

The enzymic reaction pathway is far more complex than could be inferred from the simple presentation of a nucleophilic displacement, written for dimerization as:

$$\text{P-CONH}_2 + \text{H}_2\text{N-P}' \longrightarrow \text{P-CONH-P}' + \text{NH}_3$$

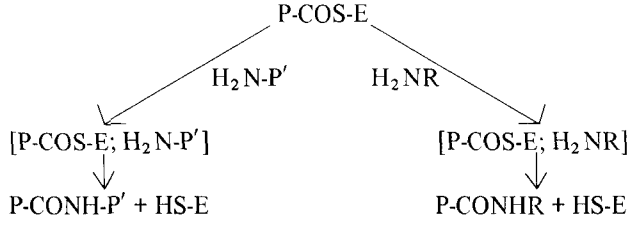
As suggested for the cross-linking of fibrin, the reaction is thought to proceed through an acylenzyme intermediate which is formed between the γ-carbonyl group of the glutamine in P and the cysteine thiol center of the enzyme (HS-E). The thiolester intermediate (P-COS-E) then undergoes an aminolytic deacylation by reacting with the ϵ-NH_2 function of lysine in P′. Specific binding steps operate with regard to both protein substrates and involve the formation of Michaelis complexes between E and P prior to acylation of the enzyme, and between P′ and the acylenzyme intermediate prior to deacylation:

Acylation: $P\text{-}CONH_2 + HS\text{-}E \rightleftharpoons [P\text{-}CONH_2; HS\text{-}E] \rightarrow P\text{-}COS\text{-}E + NH_3$

Deacylation: $P\text{-}COS\text{-}E + H_2N\text{-}P' \rightleftharpoons [P\text{-}COS\text{-}E; H_2N\text{-}P'] \rightarrow P\text{-}CONH\text{-}P' + HS\text{-}E$

Net reaction: $P\text{-}CONH_2 + H_2N\text{-}P' \rightarrow P\text{-}CONH\text{-}P' + NH_3$

In addition to chelating Ca^{2+} with ethelenediaminetetraacetic acid disodium salt (EDTA) or ATP, or inactivating the enzyme by Zn^{2+} [16], mercurials, or disulfides [17], the above type of formulation of the pathway suggests that primary amines – if they satisfy the specificity requirements of the enzyme – might interfere with the formation of the P-CONH-P′ product. Indeed, we found that such synthetic compounds could inhibit cross-linking rather effectively even in the complex biological system of clotting in whole blood [13, 18]. The inhibitory amine (H_2NR) competes against H_2N-P′ in the deacylation step by virtue of the fact that it serves as an alternate substrate:



The more efficiently the acylenzyme intermediate can be diverted towards forming P-CONHR (on the right), the less cross-linked protein product P-CONH-P′ is formed (on the left). It should be emphasized that the inhibitor becomes incorporated into P, specifically blocking and (with appropriate labeling of R) uniquely identifying its reactive glutamine sites.

IDENTIFICATION OF γ-GLUTAMYL-ϵ-LYSINE CROSS-LINKS IN THE MEMBRANE POLYMER OF Ca^{2+}-LOADED CELLS

The protein polymer formed as a result of intracellular accumulation of Ca^{2+}-ions in erythrocytes was isolated by means of gel filtration chromatography following reduction of the membrane materials in 0.025 M dithiothreitol and dispersion in 1% SDS. The appearance of peaks emerging early from the Sepharose 4B CL column (represented by fractions emerging before 46 in Fig. 2) is a characteristic feature of the chromatographic profile of cells exposed to Ca^{2+}. As inferred from considerations of particle size, this region contained the polymeric products and this was confirmed by electrophoretic analysis of the various fractions using either SDS-polyacrylamide (4.5%) or SDS-agarose (2%). Both of these techniques, but particularly the latter, indicated that "the polymer"

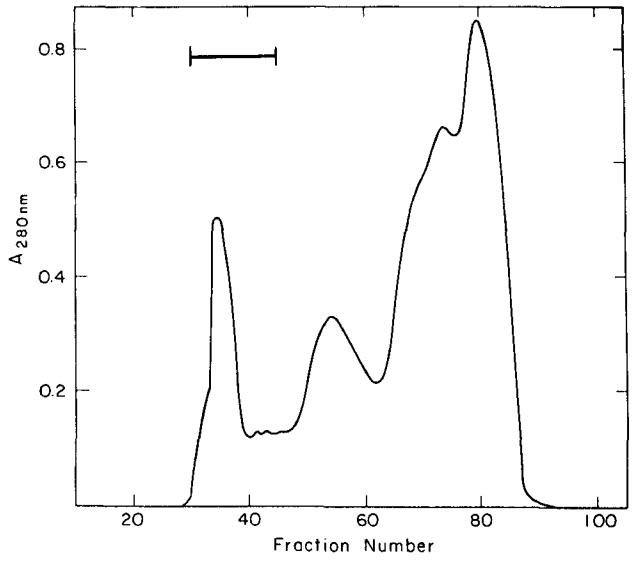


Fig. 2. Elution profile of membrane proteins from Sepharose 4B CL following incubation of red blood cells with 1.5 mM $CaCl_2$ and 20 μM ionophore for 18 h at 37°C. For details, see Siefring et al [9]. Horizontal bar represents polymer-containing fractions.

comprised several distinct components of different molecular weights and that probably even each component itself was somewhat heterogeneous in size. As discussed above, such a heterogeneity was not unexpected and it suggested that polymerization was a graded rather than a concerted, fusion of membrane proteins proceeding by way of a number of oligomeric intermediates. The identification of γ-glutamyl-ϵ-lysine dipeptide bridges in the isolated bulk polymer was carried out by procedures first developed in our laboratory for measurements of this isodipeptide in cross-linked fibrin [13, 19]. Advantage was taken of the fact that the γ-peptide linkage is resistant to hydrolysis by known proteolytic enzymes. Thus the protein could almost totally be digested by sequential treatment with proteases (subtilisin, leucine aminopeptidase, prolidase, and carboxypeptidase A) without impairing the γ-glutamyl-ϵ-lysine structure. The direct isolation, identification, and quantitation of this unique dipeptide could then be performed using the Beckman 120 C chromatographic system in the manner previously described, so that the isopeptide emerged between leucine and tyrosine (Fig. 3). From the measured ninhydrin color value of a synthetic reference peptide and from the amino acid composition of the polymeric protein, a γ-glutamyl-ϵ-lysine cross-bridge content of about 6 per 100,000 gm of protein could be calculated. This value represents a relatively high average cross-linking frequency involving approximately 10% of all lysines in the protein material. It should be mentioned in passing that the enzymic cross-linking of fibrin, though producing a much lower frequency of γ-glutamyl-ϵ-lysine bridging, contributes significantly to the viscoelastic stiffening of the clot structure [20, 21].

EFFECT OF TRANSAMIDASE INHIBITORS ON MEMBRANE PROTEIN POLYMERIZATION IN INTACT ERYTHROCYTES

Earlier work from this laboratory showed that the transamidase-catalyzed cross-linking of fibrin could be effectively inhibited by primary amines. Inhibition by such compounds is so specific that the "clotting time," which is a reflection on the overall process

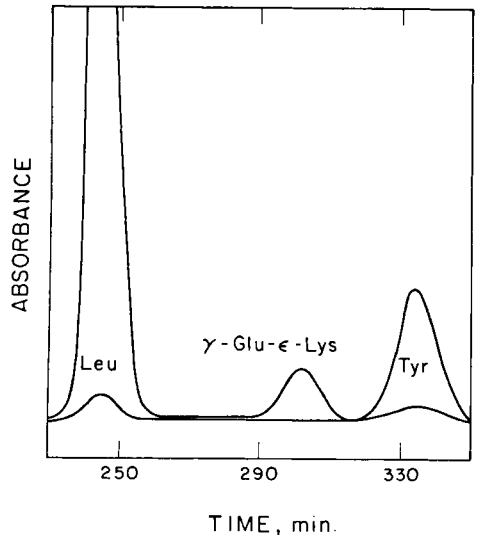


Fig. 3. Isolation of γ-glutamyl-ε-lysine isodipeptide from the enzymic digest of the polymer obtained from the membranes of Ca^{2+}-loaded erythrocytes. For details, see Siefring et al [9]. The lower tracing represents the pattern obtained when the enzymes used for digestion were incubated without addition of polymer. The γ-Glu-ε-Lys peak obtained from 0.72 mg of polymer corresponds to a recovery of 40 nmoles of the isodipeptide. The equimolar Glu and Lys content of the material in this peak was confirmed by further acid hydrolysis and amino acid analysis.

of fibrin aggregation, is not even delayed. The best synthetic inhibitors carry a bulky substituent attached to an aminoalkane residue essentially the equivalent of a lysine side chain in proteins. This type of amine specificity, exemplified by the structural features in dansylcadaverine, seems to be a common requirement for all endo-γ-glutamine:ε-lysine transferases. Though there are several amines which inhibit fibrin cross-linking at concentrations less than 10^{-4}M and which also yield K_i's of about 10^{-5}M with liver transglutaminase, their use with whole erythrocytes is fraught with difficulties. Apart from enzyme specificity, inhibition in this system would also depend on whether the amine could pass through the cell membrane to the cytoplasmic interface and whether it could gain access to the acylenzyme intermediate during the process of the intrinsic cross-linking of presumably organized membrane protein components. Compounds with large hydrophobic constituents (such as the dansyl group in dansylcadaverine) would hardly be suitable. Nevertheless, in an apparent compromise between the specificity requirement of the transamidase and passage through the cell membrane, it was still possible to select some amines which inhibited the Ca^{2+}-triggered cross-linking in intact erythrocytes. Aminoacetonitrile, glycine methyl or ethylester, histamine, cysteamine and cystamine serve as typical examples and Figure 4 provides some illustrations. At 10 mM concentrations, all of these amines prevented polymer formation for periods of up to 2 h during loading of cells with 1.5 mM Ca^{2+} at 37°C. Neither sarcosine methylester (ie, N-methylglycine methylester), an analogue of glycine methylester, nor $^{\alpha}$N-dimethylhistamine, an analogue of histamine, inhibits membrane protein polymerization. Thus the presence of a primary amino group seems to be essential.

Cystamine is obviously in a special category and inhibition by this compound cannot be ascribed to its primary amino groups alone. It is known from studies with pure transamidating enzymes (C.-M. Svahn and L. Lorand,unpublished results) that cystamine

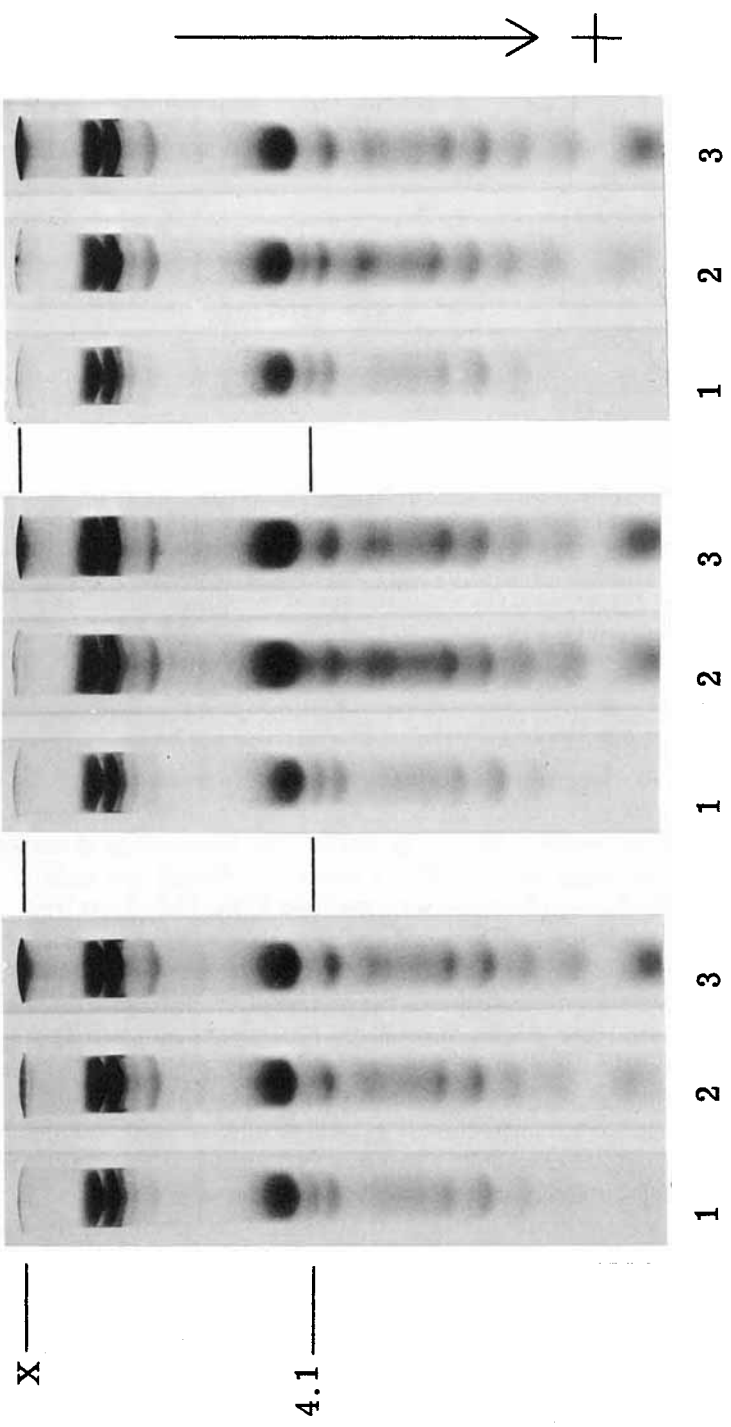


Fig. 4. Inhibition of membrane protein polymerization by amines. Fresh erythrocytes were incubated for 3 h at 37°C with 20 μM A23187 ionophore and 1.5 mM $MgCl_2$ (gel 1), 1.5 mM $CaCl_2$ (gel 2) in presence of 10 mM of an amine (histamine in panel A, aminoacetonitrile in B, and cystamine in C), or 1.5 mM $CaCl_2$ only (gel 3). Following incubation, membranes were isolated, solubilized in 1% SDS–40 mM DTT and subjected to polyacrylamide gel (4.5%) electrophoresis. Polymer is denoted as X.

undergoes a specific disulfide exchange reaction with the active center cysteine of these enzymes, causing their inactivation as:

$$\text{E-SH} + \text{H}_2\text{NCH}_2\text{CH}_2\text{S-SCH}_2\text{CH}_2\text{NH}_2 \longrightarrow \text{E-S-SCH}_2\text{CH}_2\text{NH}_2 + \text{HSCH}_2\text{CH}_2\text{NH}_2$$

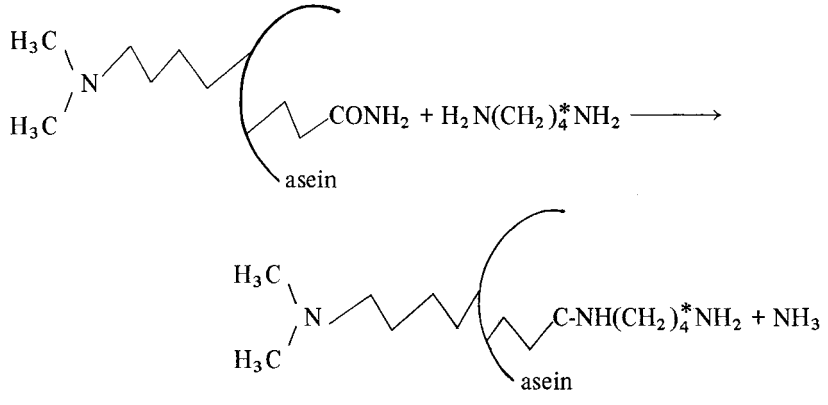
Thus there is reason to believe that inhibition of polymer formation by cystamine is due in large extent to its direct inactivating effect on transglutaminase itself.

The disappearance of band 4.1 seems to be the most sensitive sign of the changes occurring in the membrane protein profile of cells during the intracellular accumulation of Ca^{2+}-ions. Of the inhibitors tested, at 10 mM concentration, thus far cystamine was the most effective in preventing the disappearance of the 4.1 band.

When the Ca^{2+}-loading of erythrocytes is carried out in the presence of a radioactive amine (^{14}C-histamine), selective labeling of membrane proteins occurs, in agreement with the postulated mode of action of amine type inhibitors as discussed before. Thus far, we have been able to demonstrate the covalent incorporation of the labeled amines into spectrin and band 3 material, as well as the growing polymer itself.

Ca^{2+}-REGULATION OF ERYTHROCYTE TRANSAMIDASE

The transglutaminase activity of red cell hemolysates can be conveniently assayed by the filter paper technique developed in our laboratory for measuring the covalent incorporation of radioactive amines (eg, ^{14}C-putrescine) into casein derivatives [22]. Regarding the latter, N,N-dimethylcasein is very useful [23] because the ϵ-lysine groups are blocked and the protein substrate can only function as an acceptor in the enzymic reaction:



It is seen from the data in Figure 5 that virtually no transglutaminase activity can be found in fresh erythrocyte lysate in the absence of externally added Ca^{2+}. Thus, within the normal red cell milieu, the enzyme may be assumed to exist in a "latent form." However, transamidase activity becomes easily detectable by raising the Ca^{2+} concentration to about 5×10^{-5}M and the enzyme exhibits half-maximal velocity at about 3×10^{-4}M of Ca^{2+}. It should be mentioned that the Ca^{2+} dependence of the "latent-to-active transglutaminase" transition coincides with Ca^{2+} concentrations found in old cells [24], sickle cells [25, 26], or cells with impaired metabolism [27].

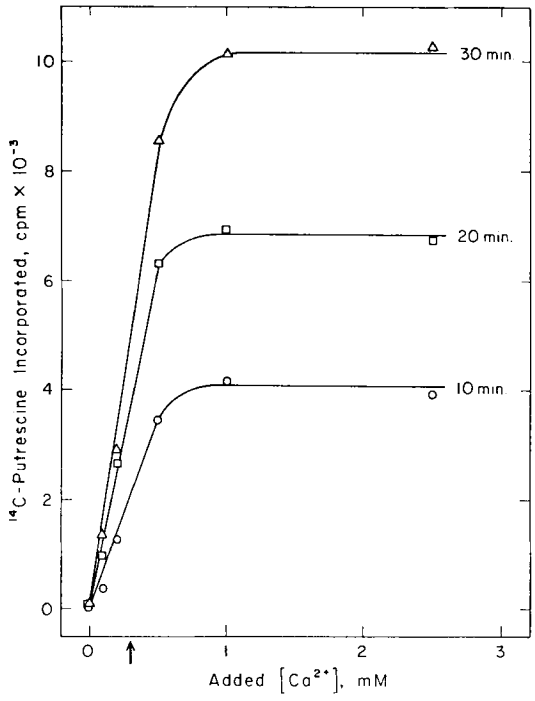


Fig. 5. Ca^{2+} dependence of transglutaminase activity in fresh erythrocyte lysate obtained from an individual donor. For details see Siefring [9]. Arrow indicates Ca^{2+}-requirement for half-maximal activity of the enzyme.

EFFECT OF TRANSAMIDASE INHIBITORS ON THE Ca^{2+}-INDUCED LOSS OF MEMBRANE DEFORMABILITY AND ECHINOCYTIC TRANSFORMATION IN ERYTHROCYTES

With specific transglutaminase inhibitors as primary tools, efforts are being directed towards examining the relationship of the enzyme-dependent cross-linking of membrane proteins to the cellular phenomena of irreversible membrane rigidification and irreversible echinocytic transformation. Though the results are still quite preliminary, a connection emerges already as a strong possibility.

In collaboration with Dr Paul LaCelle and Mr Brian Smith of the University of Rochester Medical School, the effect of histamine was examined on the Ca^{2+}-dependent irreversible loss of membrane deformability [28] using the micropipette methodology. The elastic membrane extension for cells incubated for 1 h in 0.05 M $CaCl_2$ (in the presence of 10 μM ionophore A23187) was only about 32% of control, whereas still 93% extension was obtained in the presence of histamine (80 mM). $^{\alpha}$N-dimethylhistamine, which as mentioned before does not contain a primary amino group and is not a transamidase inhibitor, did not interfere with the loss of membrane deformability.

Transglutaminase inhibitors also appear to have a rather dramatic effect on the Ca^{2+}-induced irreversible shape change in erythrocytes [29]. It is shown in Figure 6 that the echinocytic transformation is irreversible in the sense that the majority of the cells (as documented in Table I) retain this shape even after the removal of Ca^{2+} and of ionophore by washing with serum albumin and EDTA according to Sarkadi et al [30]. If fixation of the echinocytic shape took place by cross-linking of membrane proteins through

γ-glutamyl-ε-lysine bonds, the presence of a suitable transglutaminase inhibitor during the echinocytic transformation should allow the shape change to become reversible. Indeed, the results (Fig. 6 and Table I) clearly show that if an inhibitory amine is added before loading of the cells with Ca^{2+}, although the echinocytic shape prevails as long as Ca^{2+} is present, once this ion and the ionophore are removed a significant proportion of the cells return to a form which approximates that of normal discocytes and cupped cells. It is interesting to note that this apparent reversal of shape occurs without a need for augmenting the intracellular concentration of ATP.

CONCLUSIONS

The intracellular accumulation of Ca^{2+}-ions in human erythrocytes initiates a cascade of events by activating a latent transamidating enzyme of the endo-γ-glutamine:ε-lysine transferase type. Following the Ca^{2+}-dependent conversion into the active form, this enzyme causes the fusion of certain membrane proteins (P, P'. . .) by γ-glutamyl-ε-lysine cross-bridges (X):

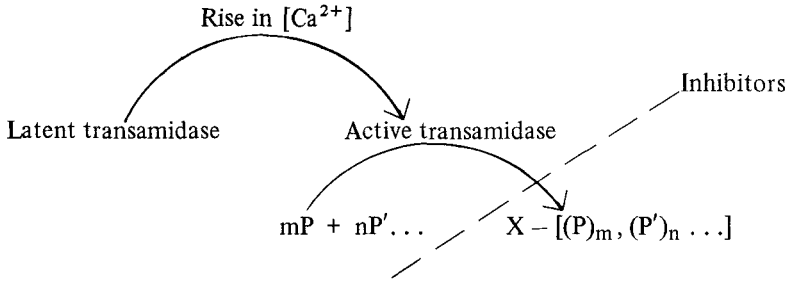


TABLE I. Effect of Transamidase Inhibitors on the Reversibility of Ca^{2+}-Induced Shape Change

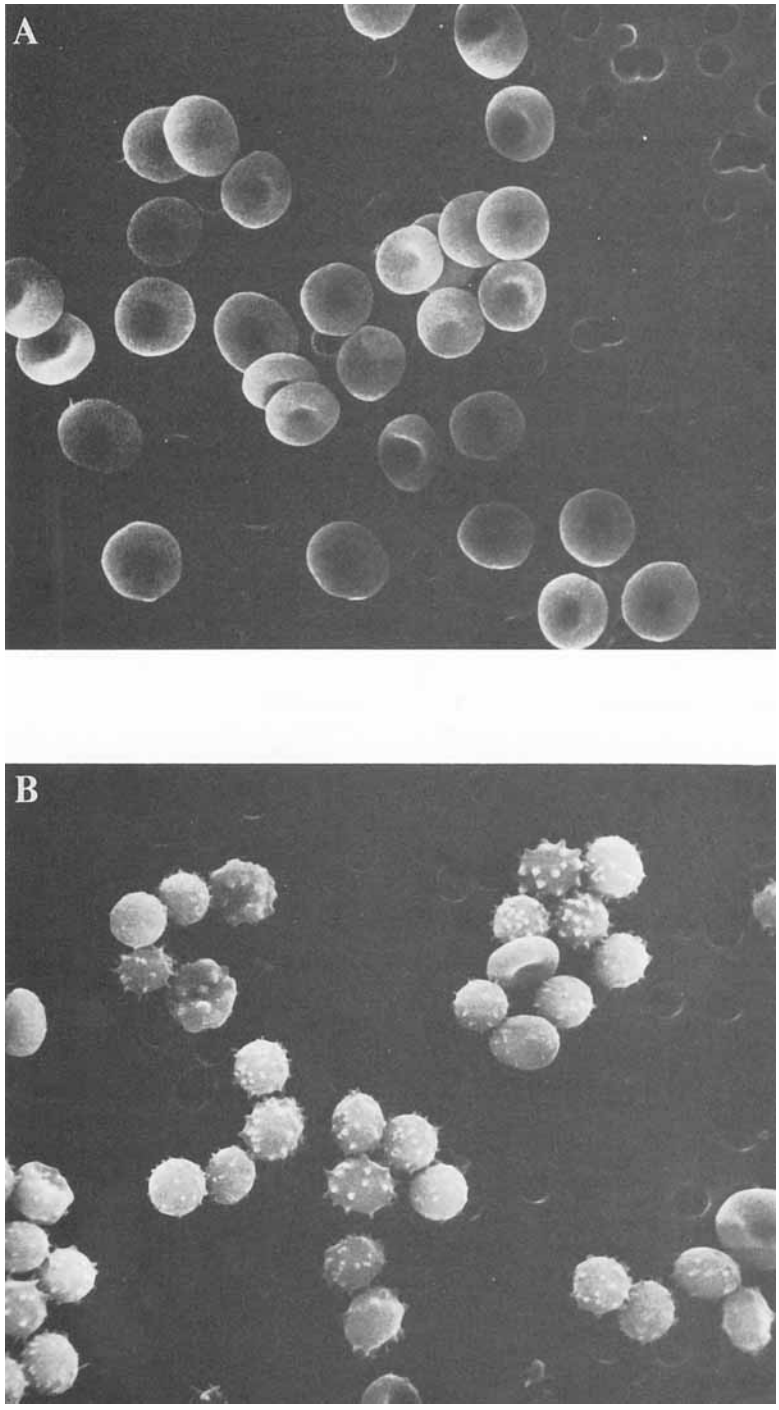
I[a]	II	Before wash		After wash with BSA-EDTA[b]	
2 h, 37°C	2 h, 37°C (20 μM A23187, 0.4% DMSO)	Echinocytes[c] (%)	ATP[d] (nmoles/gm Hb)	Echinocytes[c] (%)	ATP[d] (nmoles/gm Hb)
	Plus 1.5 mM $MgCl_2$	15	2,885	5	2,830
	$CaCl_2$	90	460	77	500
Plus 20 mM H_2NCH_2CN	$CaCl_2$	90	230	40	200
$H_2NCH_2CH_2$-(imidazole)	$CaCl_2$	85	490	38	620
$H_2N(CH_2)_2SS(CH_2)_2NH_2$	$CaCl_2$	88	285	30	50

[a]Incubation with buffer A: 0.1 M KCl, 0.06 M NaCl, 10 mM glucose, 5 mM Tris-HCl, pH 7.4. Hematocrit 50%.

[b]Wash: 40 volumes of 0.4% BSA–1 mM EDTA in buffer A for 5 min at 4°C, then 2 h at 37°C, followed by 40 volumes of 0.05% BSA–1 mM EDTA in buffer A for 2 × 1 h at 37°C.

[c]Monitored by counting ca. 2,000 cells and refers to echinocytes type II-IV and spheroechinocytes (for classification, see Bessis [31]).

[d]ATP was measured as described by Beutler [32].



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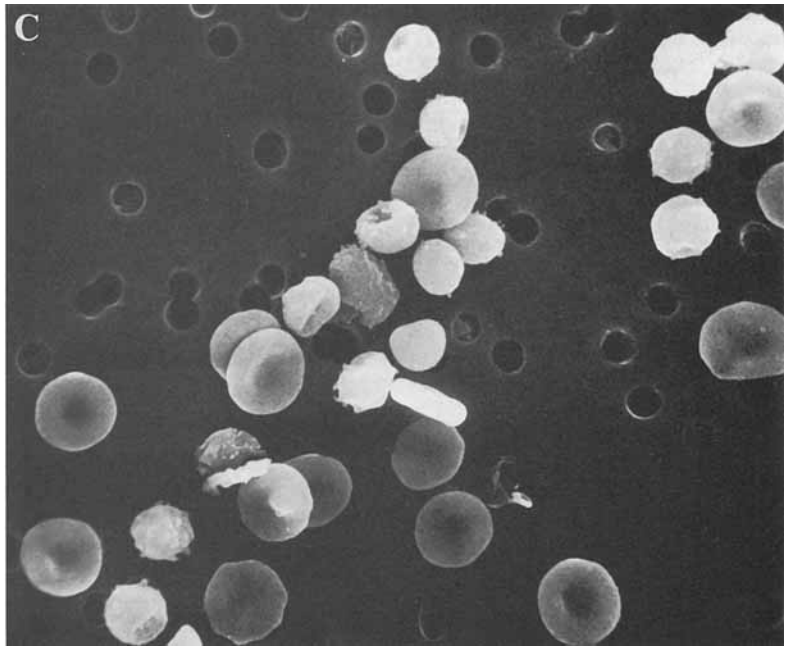

Fig. 6. Scanning electron microscopic photographs of human erythrocytes incubated at 37°C for 2 h with 20 μM ionophore and 1.5 mM $MgCl_2$ (top, left), 1.5 mM $CaCl_2$ (bottom), or 1.5 mM $CaCl_2$ plus 20 mM cystamine (above). Electron microscopic examination was performed on the cells after washing in bovine serum albumin (BSA) [28] and EDTA on Nucleopore membrane N300CPR with holes of 3-μ diameter. For details see Table I.

The demonstration that specific transamidase inhibitors can interfere with the Ca^{2+}-induced irreversible loss of membrane deformability and with the irreversible fixation of echinocytic shape underscores the possible significance of this enzymic cross-linking mechanism.

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