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Transglutaminases

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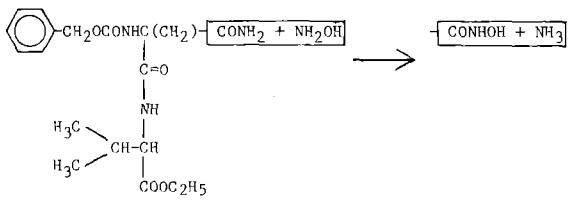
Summary

This paper is intended as a background to the topic of transglutaminases, while focusing on current ideas regarding the biological roles of these enzymes. Specifically, the following topics are discussed: geometry of forming γ-glutamyl-ε-lysine cross-linked structures; energetic considerations; the γ-glutamyl-ε-lysine cross-link; amine incorporation assays; artefactual incorporation of amines in cells and tissue homogenates; synthetic substrate systems; regulation of transglutaminase activities; strategies for probing transglutaminase-mediated events in biological systems; the blood clotting paradigm; transglutaminase and cell aging: the Ca^{2+}-enriched human erythrocyte; transglutaminase and cell activation: the thrombin-stimulated human platelet and the fertilized sea urchin egg.

Introduction

This paper is intended as a background to the topic of transglutaminases, while focusing on current ideas regarding the biological roles of these enzymes (see also Refs. 1 and 2).

The 'transglutaminase' designation was first used by Heinrich Waelsch and co-workers about thirty years ago (3) to describe a Ca^{2+}-dependent transamidating activity present in the liver and in some other tissues of the guinea pig, which could be measured by the covalent incorporation of amines (e.g. histamine, putrescine) into proteins (e.g. casein), and also by a reaction between $^{\alpha}N$ and $^{\alpha}C$-blocked glutamine peptides (such as benzyloxycarbonylglutaminylvaline ethylester) and hydroxylamine (4):



The transglutaminase name is an obvious misnomer, since it is *not* free glutamine but only compounds with γ-glutaminyl residues occupying endo-positions which serve as substrates[1].

In the absence of small molecular weight amines, a cross-linking between endo-γ-glutaminyl and endo-ε-lysyl residues of proteins may take place. This results in the formation of γ-glutamyl-ε-lysine or ε(γ-glutamyl)lysine isopeptide side-chain bridges, suggesting the more descriptive 'endo-γ-glutamine:ε-lysine transferase' name. The Enzyme Commission recommends the 'R-glutaminyl-peptide:-amine-γ-glutamyl-transferase' designation (EC 2.3.2.13).

With protein substrates, transglutaminases can thus catalyze the two nucleophilic displacement reactions shown in Fig. 1, leading either to protein cross-linking (as in reaction A) or to the incorporation of small primary amines into the protein substrate (as in reaction B)[2].

The biological significance of the cross-linking of

[1] Lysine also shows very little substrate activity.

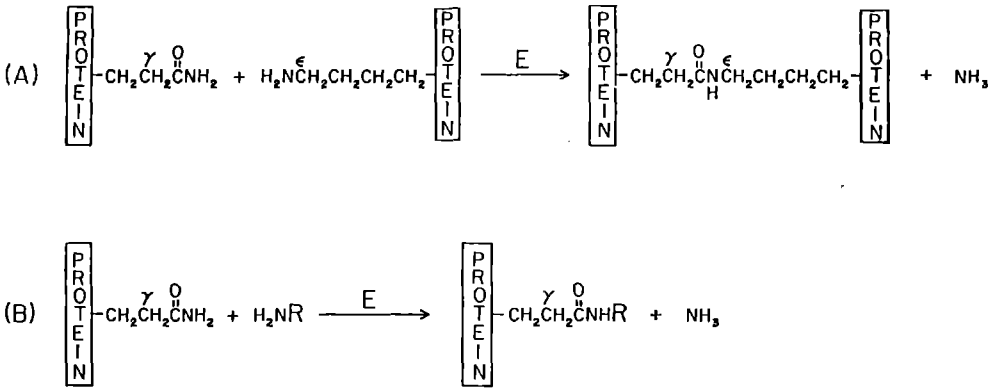


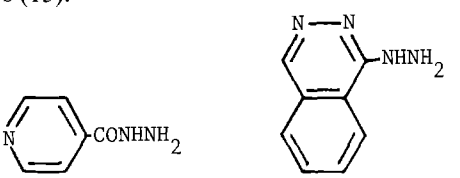
Fig. 1. Two known biological reactions are catalyzed by transglutaminases: (A) cross-linking of proteins, creating γ-glutamyl-ε-lysine side chain peptides, and (B) incorporation of small molecular weight amines into γ-glutamine sites of proteins. Reactions (A) and (B) compete against each other.

protein units by even a few γ-glutamyl-ε-lysine bridges is nowhere more obvious than in human blood clotting where a disorder of fibrin stabilization may give rise to life-threatening hemorrhage (for a current review, see Ref. 7). As shown in Fig. 2, the low elastic modulus of plasma clots, inadequate to stem the loss of blood in patients with hereditary deficiency of fibrin stabilizing factor (Factor XIII), can be augmented as much as five-fold by supplementation with the purified zymogen of the cross-linking enzyme (8). Conversely, the elastic modulus of the clot formed in normal human plasma can be reduced five-fold when competing small molecular weight amines are added to prevent the development of ε-lysyl bridges.

Dramatic physical changes accompany the transglutaminase-mediated cross-linking of protein units in other biological systems as well. Clotting of *Homarus* plasma (9) or the generation of copulation plug derived from the seminal fluid of rodents (10) can be cited as examples relating to *extra-cellular* phenomena, whereas the irreversible rigidification of cell membrane in CA^{2+}-enriched human erythrocytes (11) and in keratinocytes (12) are the results of the cross-linking of proteins by *intra-cellular* transglutaminases.

In sharp contrast with reaction A, the biological consequences of reaction B in Fig. 1 are far less obvious. Nevertheless, the expectation may well be justified that the post-translational modification of proteins by small molecular weight primary amines, be it a drug or a natural polyamine, is indeed an important function for transglutaminases.

As far as drugs are concerned, isoniazid, used in antituberculosis therapy, and hydralazine, used as an anti-hypertensive agent, are the best studied examples (13):



Current textbooks of pharmacology offer no enlightenment, yet the possibility of transglutaminase-dependent modification of proteins by such drugs may carry farreaching consequences: a given enzyme might become more or less active following the covalent incorporation of a drug into its structure; turnovers of proteins could be affected; antigenic properties might be altered which could conceivably lead to a loss of tolerance against one's own proteins and precipitate an autoimmune response. That the latter could be an important consideration is suggested by the fact that in several hemorrhagic disorders following the sudden appearance of some inhibitor of fibrin stabilization (7), there was a history of treatment with isoniazid, and the circulating inhibitor was an IgG of great specificity directed either against Factor XIII itself

[2] There are some reports suggesting the possibility that small molecular weight acyl compounds such as benzyloxycarbonyl-glutaminylvaline ethylester (5) and t-cinnamoylthiocholine (6) might also react with proteins in a transglutaminase-dependent manner, presumably by amidating ε-lysine sites.

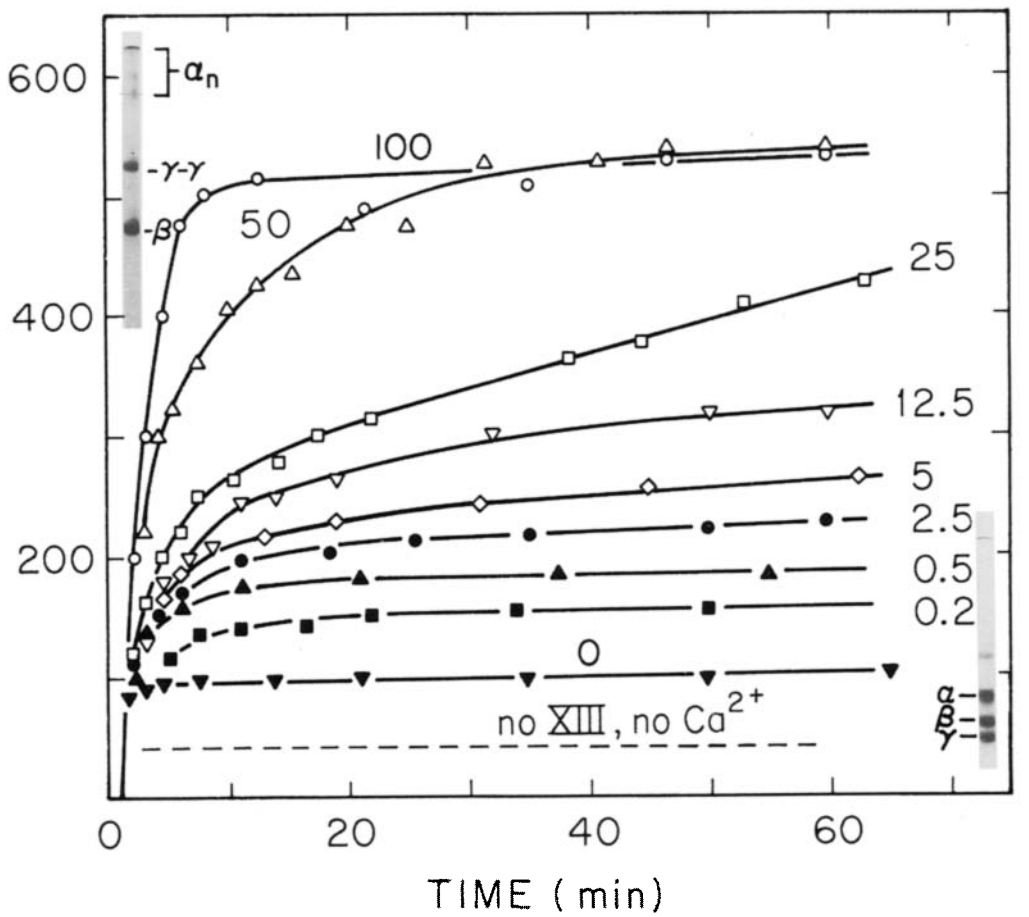
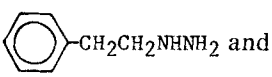


Fig. 2. Increase in the elastic modulus (G) of clot in a Factor XIII-deficient plasma, as a function of supplementing with measured doses of the purified Factor XIII zymogen (8). Added concentrations of the latter, based on active site titrations with iodoacetamide, ranged from 0.2×10^{-9} M to 100×10^{-9} M and are indicated on the graph ($\times 10^9$) for each experiment. The line marked 0 (▼) at about 100 dynes/cm² represents the strength of the clot formed in Factor XIII-deficient plasma, with thrombin and Ca^{2+} added but without supplementation with purified Factor XIII. The broken line at about 40 dynes/cm² is the elasticity of the clot obtained in Factor XIII-deficient plasma just with the addition of thrombin. Fibrinogen content was 1.88 mg/ml. The inserts show the electrophoretic profiles for reduced clots in sodium dodecylsulfate for the 65 min sample from the deficient plasma (▼; lower right) and for the 60 min sample from the 10^{-7} M Factor XIII supplemented material (○; upper left hand).

(14, 15) or against the fibrinogen/fibrin substrate (16). Considering the structures of other drugs such as phenelzine,  and a variety of monoamine oxidase inhibitors (Lorand, unpublished results) and what we know about the amine specificity of transglutaminase (17), the iatrogenic issue is a very serious one, indeed[3].

[3] Recently some more compounds such as actinomycin D and adriamycin were added to this list (18).

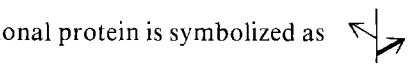
The post-translational modification of protein structures by naturally occurring polyamines (histamine, putrescine, spermidine, spermine) or by their monoacetylated derivatives is an equally intriguing possibility. Alterations in enzyme activities could ensue (19), and, in fact, it has recently been reported that ornithine decarboxylase was 'down-regulated' by the covalent incorporation of putrescine (20). However, this claim by Russell regarding the inactivation of ornithine decarboxylase has been seriously questioned (21). As shown by Lockridge (22) and confirmed later by Birk-

bichler et al. (23), purified guinea pig liver transglutaminase is capable of incorporating amines into its own structure. However, even with 2–3 moles of histamine incorporated per mole of transglutaminase, no noticeable change in activity was found (22) in the benzyloxycarbonylglutaminylglycine and hydroxylamine substrate system. It is, of course, always possible that activity towards some protein substrate might have been affected.

Geometry of forming γ-glutamyl-ε-lysine cross-linked structures

In its simplest form, the dimerization of two protein molecules (P and P′) by a transglutaminase may be outlined as shown in Scheme A.

If P and P′ represent molecules belonging to the same protein species, a *homologous ensemble* is obtained, whereas if they are of different types, a *heterologous ensemble* ensues. A homopolymeric example is a pure, stabilized fibrin clot (24); whereas the lobster plasma clot (9, 25)[4], rat copulation plug (see chapter by Williams-Ashman), collagen-fibronectin (26) and the α_2-plasmin inhibitor-fibrin (27, 28) combinations exemplify heteropolymeric structures of this type. The polymers formed in Ca^{2+}-enriched human erythrocytes (29, 30) are also fused heterologous structures.

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

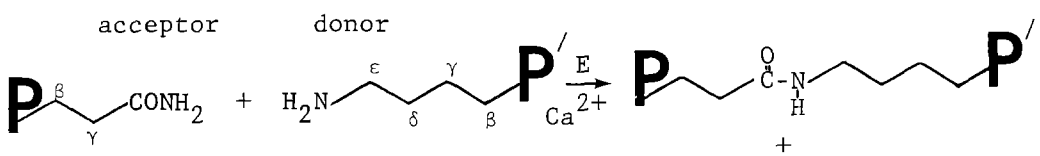
There might be proteins which carry acceptor or donor functionalities exclusively. Accordingly, they could only serve either as chain initiators (marked I) or terminators (T), and their presence in a mixture might give rise to the possibilities shown in Scheme C.

The combinations of collagen with fibronectin (26) and of α_2-plasmin inhibitor with fibrin (27, 28) are thought to be of the latter, dimeric type.

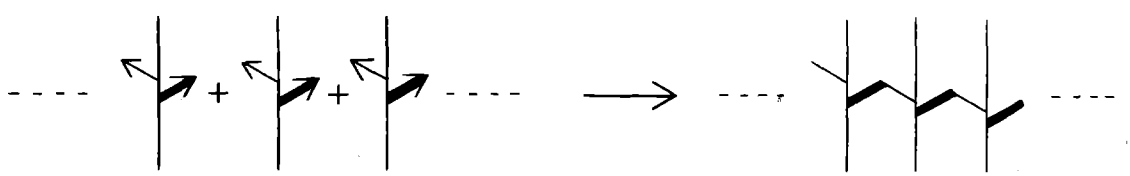
The minimum requirement for branching would be that a reacting protein substrate possesses either two acceptor or two donor functionalities as in Scheme D.

Little is known about the natural geometric arrangement of constituent protein units in any of the biologically occurring polymeric ensembles. Enzyme-dependent titration and labelling of γ-glutamyl sites (see reaction B in Fig. 1) with dansylcadaverine or with ^{14}C-glycine ethylester led to the finding that the cross-linking sites in fibrin were situated near the C-terminus of γ-chains and the mid-portion of the wandering α-chain domain of

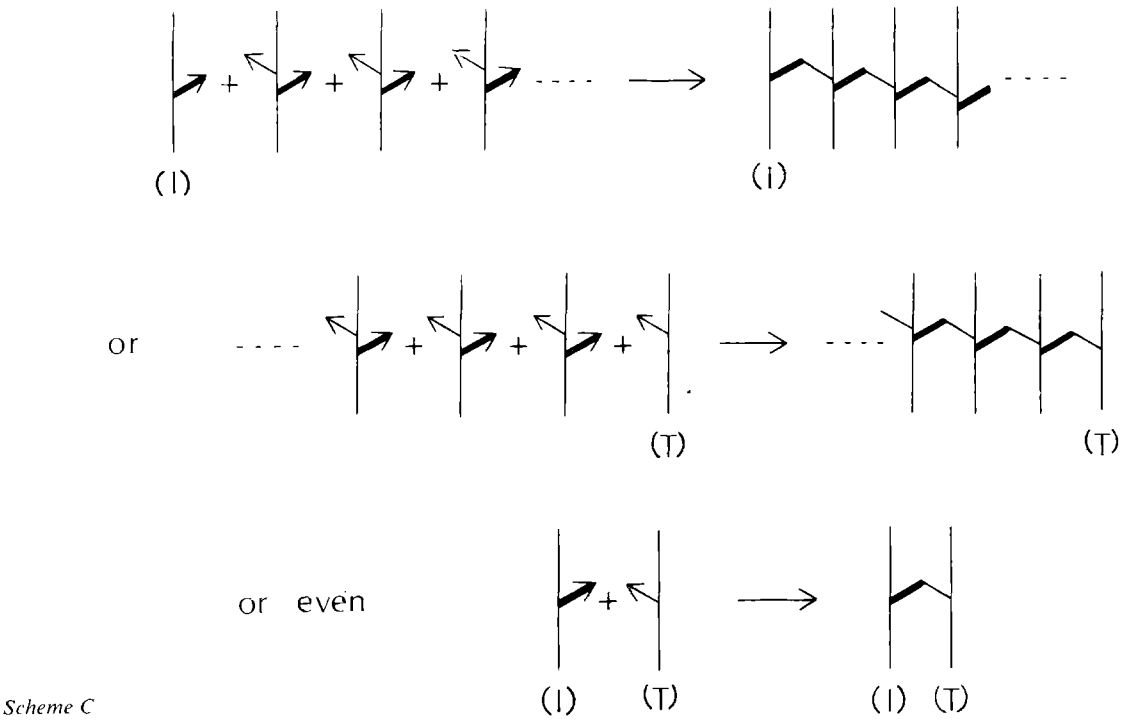
[4] We have recently shown (Myhrman, Bruner-Lorand and Lorand, unpublished results) that actually more than one protein (i.e., not just fibrinogen) in the plasma of the *Homarus americanus* is involved in clotting.



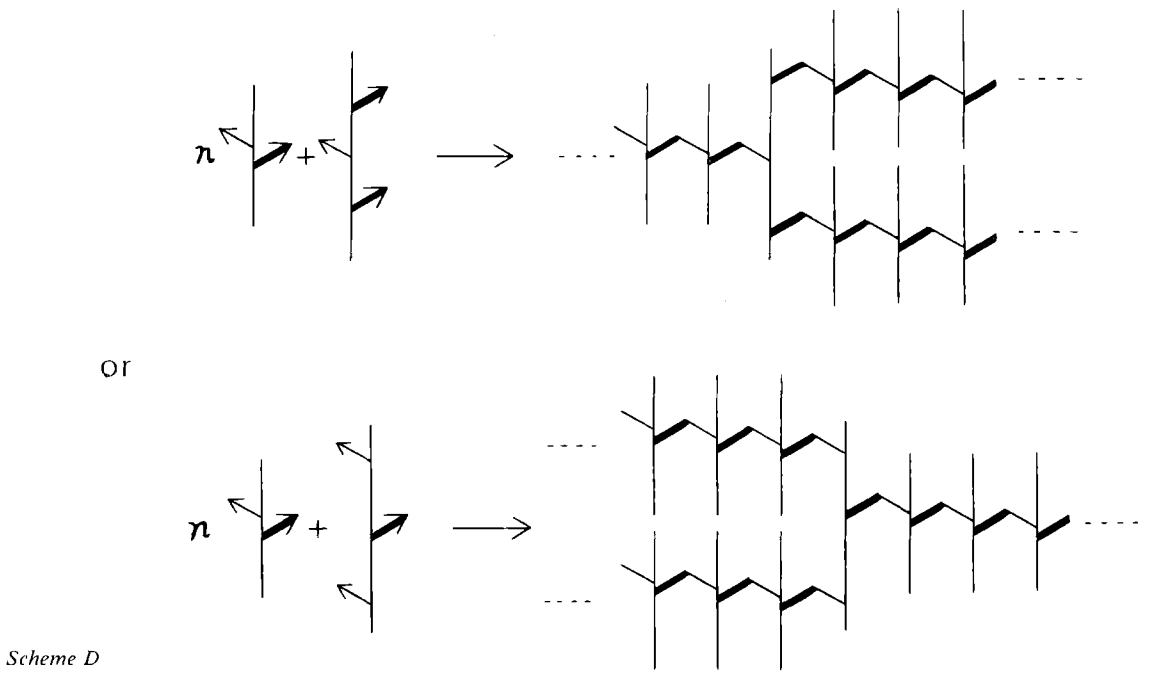
Scheme A



Scheme B



Scheme C



Scheme D

the protein (31–34) (Fig. 3). Reciprocal cross-linking of γ-chains generates an end-to-end γ-γ' dimer in the direction of the fiber axis, whereas formation of peptide cross-links between α-chains gives rise to polymeric α-chain units, promoting side-by-side polymerization.

The site-specific, transglutaminase-directed labelling of proteins in γ-glutamine residues by dan-

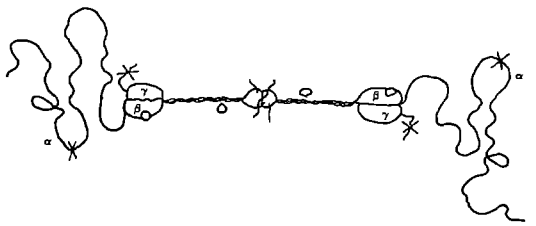


Fig. 3. Cross-linking sites in human fibrin. Fibrinogen has an $(\alpha_2\beta_2\gamma_2)A_2B_2$ composition, where A and B represent the fibrinopeptides at the N-termini of the α and β chains on the central nodule of the molecule (called E domain). These are the peptides cleaved by thrombin during clotting [i.e. $(\alpha_2\beta_2\gamma_2)A_2B_2 \rightarrow \alpha_2\beta_2\gamma_2 + 2A + 2B$] which, however, leaves the linear arrangement of nodules essentially unchanged. The Factor $XIII_a$-reactive cross-linking sites of the protein (marked by X) are located near the C-terminal ends of the short γ-chains and in the midsection of the C-terminal, wandering portion of the long α-chain, both protruding from the two distal nodules (called D domains). Amine substitution, as mediated by Factor $XIII_a$, occurs much faster with fibrin than with fibrinogen, indicating that removal of fibrinopeptides serves to unmask the cross-linking sites of the protein. Furthermore, the reaction takes place in an ordered manner with substitution of γ-sites proceeding faster than that of α-sites.

sylcadaverine (35) or by other labelled amines has become a general strategy for probing the cross-linking domains of proteins. Human plasma fibronectin, for example, is known to contain two regions reactive towards fibrinoligase[5]; one near the C-terminal, inter-chain disulfide bridge in the molecule and another involving the third residue from the N-terminal end (36–40).

No methods are available for disassembling γ-ϵ cross-linked polymeric structures without destroying the backbones of the constituent units themselves. In other words, we have no means of preferentially cleaving the γ-ϵ peptide bond without breaking α peptides. In complex hybrid systems, such as found in the membranes of Ca^{2+}-enriched human erythrocytes and in keratinocytes, even the identification of constituent proteins (not to mention an evaluation of the stoichiometry of combination) proved to be a very laborious task. However, immunochemical methods were found to be the most helpful (41). The approach is based on the idea that γ-ϵ linked polymers still retain some of their antigenic determinants of their individual protein constituents and that the probing of shared epitopes by crossed immunoelectrophoresis or by Western blotting can reveal the composition of the ensemble. By methods of this type, it could be shown that the cross-linked polymer of Ca^{2+}-enriched human red cells comprised band 3, spectrin, ankyrin, band 4.1 protein, and possibly also hemoglobin and catalase (Bjerrum, Hawkins, Griffin and Lorand, unpublished results).

[5] Synonymous with coagulation Factor $XIII_a$ or plasma transglutaminase.

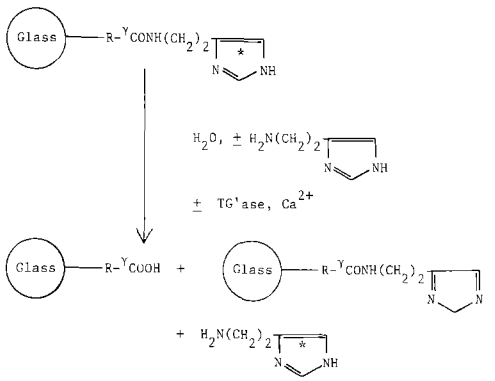
Energetic considerations

The reactions, as written in Fig. 1, would proceed with very little change in free energy ($\Delta G^\circ \sim 0$) and should be considered reversible. In both instances a

$$\gamma\text{-amide bond is broken: } -\overset{\overset{O}{\|}}{C}-NH_2$$

and a γ-amide bond is made: $-\overset{\overset{O}{\|}}{C}-\underset{\underset{H}{|}}{N}-$. In the physiological milieu, of course, the NH_3 leaving group would undergo immediate protonation: $NH_3 + H^+ \rightarrow NH_4^+$, which would promote reaction to the right. However, even by adding large amounts of extraneous amines, reversibility for protein cross-linking reactions, as shown in Fig. 1A, is often difficult to demonstrate because of clotting or phase transitions. Actual reversal has been reported only for the Factor $XIII_a$-catalyzed dimerization of fibrin with α_2-plasmin inhibitor (27).

However, a back exchange of protein-bound amides can be more easily shown. In the example given in Fig. 4 (42), first succinylated β-lactoglobulin was covalently attached to arylamine glass beads, then reacted with ^{14}C-histamine in the presence of guinea pig liver transglutaminase and Ca^{2+} which modified available γ-glutamine sites on β-lactoglobulin (R). After unbound radioactivity was removed by washing, the beads were mixed with transglutaminase in the presence of cold histamine and Ca^{2+}, and the release of radioactivity into the supernatant was measured. It can be seen that the delabelling was transglutaminase-dependent and that it was augmented four-fold when histamine was added (i.e., hydrolytic delabelling was about 25% of the total achievable with an amine present). These reactions are illustrated in Scheme E.



Scheme E

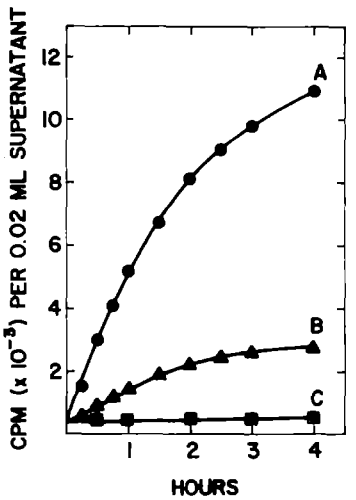


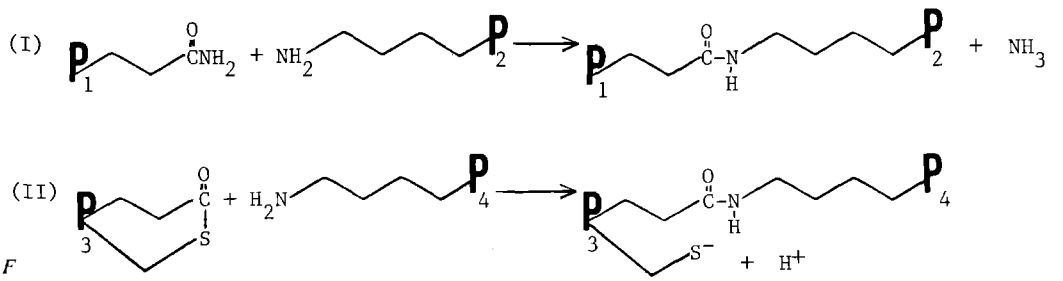
Fig. 4. Progression curve for transglutaminase-catalyzed removal of label from ^{14}C-histamine-succinylated-β-lactoglobulin-arylamine glass (42). The reaction mixture (0.15 ml) contained 53 mM Tris-HCl (pH 7.5), 2.4 units transglutaminase/ml, and 20 mg ^{14}C-histamine-succinylated-β-lactoglobulin-arylamine glass (5 500 cpm/mg). Incubation was at room temperature (23 °C). The radioactivity in 0.02 ml samples of supernatant was determined. *A:* Complete system with 50 mM cold histamine. *B:* Complete system without added cold histamine. *C:* Same as *A*, but without enzyme. The 4 hr value of 11×10^3 cpm represents the release of 75% of the total bound radioactivity.

The γ-glutamyl-ε-lysine cross-link

As indicated in relation to Fig. 2, even the few γ-ε side chain bridges in a blood clot (ca. 1 mole γ-ε peptide/80 000 g protein) (35, 43–45) have a profound effect on physical properties. The frequency of such cross-links varies greatly from one system to the next, which is probably a reflection on biological requirements. The guinea pig copulation plug (10) and the cornifying envelope in keratinocytes (12) represent the highest order of cross-linking of this type, involving as much as 30% and 18% of lysyl residues, respectively.

Characterization and quantitation of the γ-glutamyl-ε-lysine dipeptide is still a laborious task since it can only be identified following the total digestion of the polymer by a series of proteolytic enzymes. More significantly, a single analytical system alone does not yield unambiguous results. The choice is between employing two or more independent systems for isolating the dipeptide on the basis of different principles of separation, or further analyzing the purified product by hydrolysis or derivatization. In our laboratory, we developed three different chromatographic systems (35, 43, 46) and, presently, we rely on the parallel examination of proteolytic digests by a direct ion exchange chromatographic procedure and by an HPLC technique following pre-column derivatization with o-phthalaldehyde (46). Each system must have the capacity of resolving trace quantities of the γ-glutamyl-ε-lysine dipeptide against at least a thousand times higher concentration of all other products of protein digestion.

It must be remembered, however, that not all γ-glutamyl-ε-lysine producing reactions are due to transglutaminase. In addition to the reaction catalyzed by this enzyme (I), there is at least one other well-known possibility (II) for forming such side-chain bridges between protein units (47, 48) (see Scheme F).



Scheme F

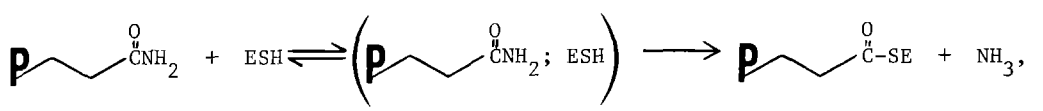
Though both I and II would give rise to γ-ϵ peptides, there are a number of differences between the two reactions: (i) Reaction I proceeds only in the presence of transglutaminase, also requiring Ca^{2+}, whereas no enzyme is needed in reaction II. (ii) In reaction I, formation of the γ-ϵ peptide is accompanied by the liberation of NH_3, whereas in reaction II the leaving group is a cysteine thiol which remains protein bound on P_3. (iii) Different Glx residues are involved in the two reactions; a glutamine in I and an internal glutamyl thioester in II. As exemplified by α_2-macroglobulin (49), both types of Glx functionalities could be present in the same protein. (iv) Small molecular weight amines (H_2NR) could replace the ϵ-NH_2 lysyl residues (in P_2 and P_4) in both I and II and could become incorporated into P_1 and P_3, while inhibiting cross-linking to other proteins. However, there may be considerable differences in H_2NR specificities. From what we know about the pathway of transglutaminase (E-SH)-catalyzed reactions, deduced from studies with synthetic substrates to be discussed later, we can postulate the existence of an acylenzyme intermediate for reaction I as in Scheme G, and the amine specificity depends on the affinity of the small molecular weight nucleophile for the acylenzyme intermediate, as shown in Scheme H.

Thus, amine incorporation by transglutaminase always follows a Michaelis-Menten type of saturation kinetics (17, 35). By contrast, we are unaware of any report indicating a prior complexation step for the incorporation of amines by α_2-macroglobulin, the C3 or the C4 components of complement, all of which contain similar γ-glutamyl thioester bonds (47). In these situations, the velocity of amine incorporation may depend only on a bi-molecular encounter between these proteins and the H_2NR. Due to the different nature of the two reactions, even amines of similar pK_a's (e.g., methylamine and dansylcadaverine) can be expected to differ as reactants in I and II.

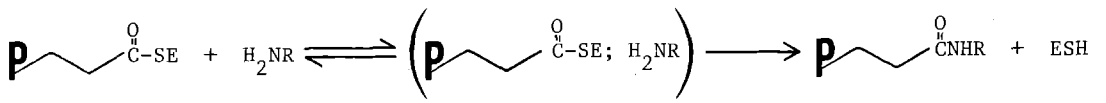
Amine incorporation assays

The history of research on transglutaminases is intimately tied to assays based on the incorporation of isotopically labelled amines (e.g., putrescine, histamine, spermine, cadaverine) into trichloracetic acid-precipitable proteins (3). In spite of its many pitfalls, the Ca^{2+}-dependent incorporation of amines remains the cornerstone for detecting and measuring transglutaminase activities in tissue homogenates. Various modifications of the original methodology are in use. A significant improvement was the blocking of the ϵ-lysine residues of test proteins (e.g., casein) so that no self cross-linking of the protein substrate could occur; acetylation, succinylation (50), dimethylation (51) or guanidylation (52) were found to be useful in this regard. Filter paper (53) and chromatographic techniques (54) are employed for the efficient and simultaneous handling of a large number of test samples and for the rapid separation of protein-bound radioactivity from free amine.

The synthesis of dansylcadaverine in this laboratory (35, 55) opened up new avenues for studying transglutaminases by fluorescence methodologies. Dansylcadaverine has excellent affinity for all acylenzymes of this kind ($K_{M,app}$, $\sim 10^{-4} - 10^{-5}$ M at pH 7.5); its incorporation into casein and into many other proteins is accompanied by a large blue-shift and by an increase in the quantum yield of dansyl group emission. These circumstances made it possible to devise continuous rate assays (50) for measuring transglutaminase activities and to apply his-



Scheme G



Scheme H

tological manipulations for the staining of enzymes in tissue slices (56)[6]. The dansylcadaverine reaction also permitted activity staining of transglutaminases on electrophoretic gels (58, 59). As such, purified guinea pig liver transglutaminase, which had previously been represented to comprise only a single enzyme, could be resolved by non-denaturing electrophoresis and dansylcadaverine staining into two differently charged components[7] (Fig. 5). In fact, *multiple forms* of transglutaminases can be detected in many tissues. In guinea pig prostate alone, four different transglutaminases could be identified (Fig. 6) and three activities could be discerned in homogenates of the sea urchin egg (61).

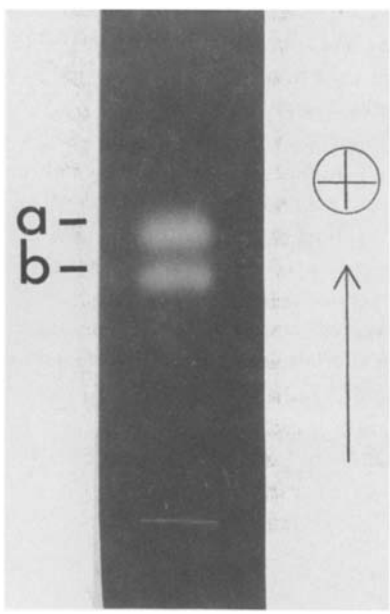
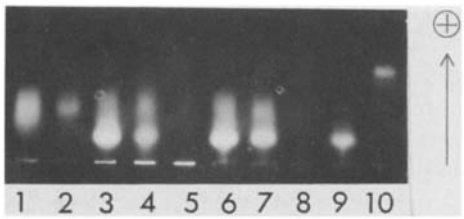


Fig. 5. Multiple forms of transglutaminase in fresh guinea pig liver homogenate as revealed by dansylcadaverine-specific fluorescent activity staining following non-denaturing electrophoresis (59).

[6] A recent report deals with other fluorescent and colored derivatives of cadaverine for such purposes (57).

[7] Such preparations of guinea pig liver transglutaminase are also known to contain a proteolytic contamination (60).



Lobe	Lanes		
	Fluid	Homogenate	High Speed Supernatant
Anterior	1	3	6
Dorsal	2	4	7
Lateral		5	8
Seminal Vesicle			10

Lane 9. Purified transglutaminase I from anterior lobe

Fig. 6. Multiple forms of transglutaminases in the guinea pig prostate, as shown by activity staining with dansylcadaverine (139).

Artefactual incorporation of amines in cells and tissue homogenates

Following incubation of human peripheral lymphocytes with isotopically labelled putrescine, activation by phytohemagglutinin caused incorporation of label from putrescine into cell proteins (preferentially into one of 18 000 MW) (62, 63). However, as it was later shown, this incorporation was not due to transglutaminase action, but to the formation of protein-bound hypusine by an unrelated sequence of events (64).

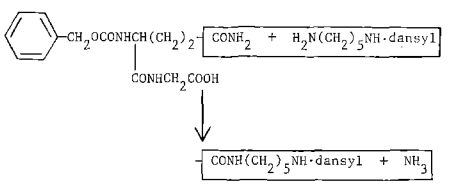
Spurious incorporation of a label originating from amines may also be encountered if the amine (e.g., putrescine) is oxidized in the tissue to an aldehyde. Among other products, a Shiff base could ensue. In situations where interference by amine oxidases is suspected (e.g., in pathological serum samples[8] or when serum is collected during pregnancy) (65, 66), specific inhibitors of the oxidase must be included in the test system.

Another possibility for the artefactual, transglutaminase-independent incorporation of amines (putrescine, histamine, etc.) into proteins is contained in a recent report regarding the action of myeloperoxidase which converts RNH_2 to $RNCl_2$ in tissues. The latter products can react with proteins very rapidly (67).

[8] In connection with assaying Factor XIII in a patient's plasma, we have recently encountered (Lorand and Velasco, unpublished data) an anomalously high incorporation of label from ^{14}C-putrescine, which could be abolished by the addition of methylglyoxalbis(guanylhydrazone), an inhibitor of diamine oxidase.

Synthetic substrate systems

Efforts to synthesize a large number of $^{\alpha}$N-blocked glutaminyl peptides (68) followed the path set by the early work of Waelsch and collaborators (4, 69). The water soluble compound, benzyloxycarbonylglutaminylglycine (though its affinity even for guinea pig liver transglutaminase is quite poor; $K_{M,app} = 0.05$ M), proved to be useful for detecting transglutaminases. Sensitivity can be greatly improved if, instead of employing it with hydroxylamine, it is used in combination with dansylcadaverine (54):

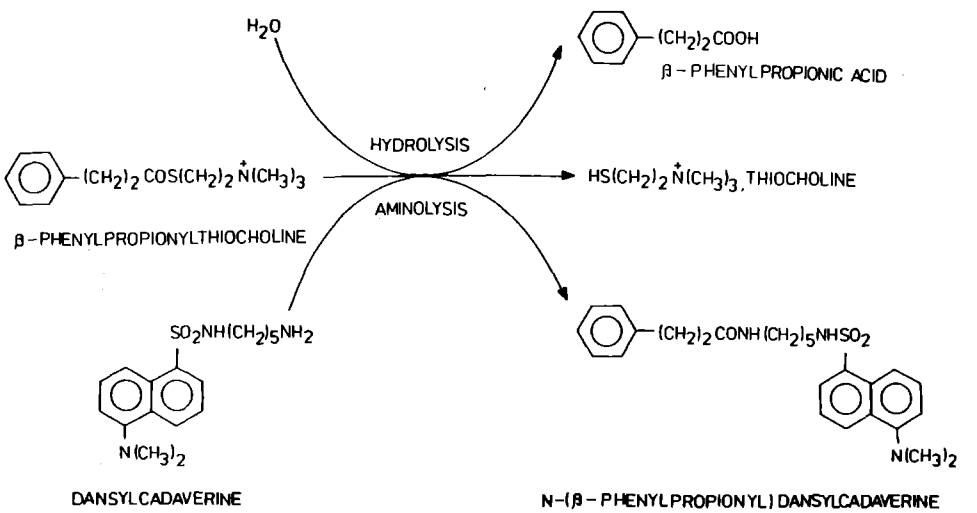


Synthetic substrates have been most helpful from the point of view of elucidating the pathway of catalysis of transglutaminases. Significant progress was made in recent years with regard to two such enzymes: guinea pig liver transglutaminase and human Factor $XIII_a$. Folk and co-workers used nitrophenylesters such as p-nitrophenylacetate or trimethylacetate (60, 70–73), whereas Lorand and collaborators synthesized a variety of thioesters such as t-cinnamoyl- or β-phenylpropionyl thiocholine (58, 74–77) for their studies. The thiocholine derivatives are not only more water soluble and very much more stable than the p-nitrophenylesters[9], but the thioesters are also considerably more specific as substrates. In addition, they offer a wide range of analytical possibilities, including fluorescence. This is illustrated in Scheme I for the β-phenylpropionylthiocholine-dansylcadaverine substrate pair where the formation of thiocholine can be measured by on-line reaction with 5,5′-dithiobis(2-nitrobenzoic acid), production of the acid can be followed in a pH′ stat and the water-insoluble coupling product can be quantitated by fluorescence, following continuous extraction into a heptane phase layered above the aqueous reaction mixture. Chromatographic procedures are also available for measuring residual dansylcadaverine as well as the formation of the amide product (54). Hydrolysis of transcinnamoylthiocholine can be studied by spectral changes in the UV (74).

Some kinetic data are given as illustration in Tables 1 and 2. The former relates to the steady-state hydrolysis of a number of thioesters by human Factor $XIII_a$, whereas Table 2 pertains to the question of amine specificity, based on the acceleration of thiocholine production in the presence of different amines by this enzyme.

Amine specificity is one of the most characteristic aspects of transglutaminase-catalyzed reactions, as it has been repeatedly emphasized in publications from this laboratory since 1968 (35). Affinity for the side chains of amines can be demonstrated (i) by

[9] p-Nitrophenylesters are akin to anhydrides, with a free energy of hydrolysis of $\Delta F°$ at pH 7 = 13 010 cal mole^{-1} (78).

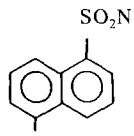
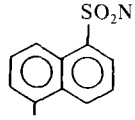
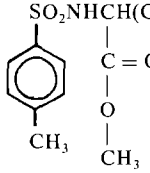


Scheme I

Table 1. Kinetic constants for the non-enzymatic and for the fibrinoligase-catalyzed steady-state hydrolysis of thiol esters at pH 7.5 and 25 °C (75).

	Non-enzymatic	Enzymatic		
	$k^{hyd}_{m,app}$	k^{hyd}_{cat}	$k^{hyd}_{cat}/K^{hyd}_{m,app}$	
	$sec^{-1} \times 10^6$	$M \times 10^3$	sec^{-1}	$M^{-1}\ sec^{-1} \times 10^{-3}$
$C_6H_5(CH_2)_2COS(CH_2)_2N(CH_3)_3$ + I- β-Phenylpropionylthiocholine iodide	1.3	0.29	0.21	0.74
$C_6H_5(CH_2)_2COS(CH_2)_2N(CH_3)_2 \cdot HCl$ 2-Dimethylaminoethanethiol β-phenylpropionate hydrochloride	58.5	0.18	0.13	0.74
$C_6H_5CH = CHCOS(CH_2)_2N(CH_3)_3$ I- *trans*-Cinnamoylthiocholine iodide	1.3	0.10	0.08	0.80
$C_6H_5CH = CHCOS(CH_2)_2N(CH_2C_6H_5)(C_2H_5)_2$ + Br- 2-Benzyldiethylaminoethanethiol *trans*-cinnamate bromide	8.0	0.25	0.08	0.32

Table 2. Enhancement of the steady-state formation of thiocholine in the reactions of five different amines with 1.5 mM β-phenylpropionyl-thiocholine iodide, at 50 mM calcium chloride, E_0 = 0.12 μM, pH 7.5, and 25 °C[a] (75).

Amine substrates	v^{am}_{lim}/E_0	$K^{am}_{m,app}$
	sec^{-1}	$M \times 10^4$
 $N(CH_3)_2$ *N*-(5-Aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide; dansylcadaverine	1.7	8
 $N(CH_3)_2$ *N*-(5-Amino-3-thiapentyl)-5-dimethylamino-1-naphthalenesulfonamide; dansylthiacadaverine	1.7	4
 *N*-α-*p*-Tosyl-L-lysine methyl ester	1.4	11
$NH_2(CH_2)_4NH_2$ Putrescine	2.1	77
$CH_3(CH_2)_3NH_2$ *n*-Butylamine	1.2	430

[a] Limiting aminolytic enhancement rates (v^{am}_{lim}) were calculated from Lineweaver-Burk transforms of data after subtracting the purely hydrolytic velocity. Amine concentrations required to bring about half of the limiting velocities are denoted as $K^{am}_{m,app}$.

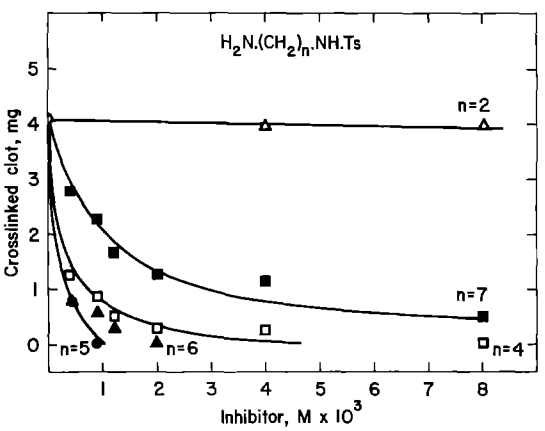


Fig. 7. Specificity of inhibition of fibrin cross-linking (at pH 7.4) with regard to side chain length in compounds of the tosyl · $NH(CH_2)_nNH_2$ series, with n = 2, 4, 5, 6, and 7 (35).

measuring interference with the biological formation of γ-ε cross-linked polymeric structures (Fig. 7) (35); (ii) by competitively inhibiting the incorporation of a labelled amine substrate such as ^{14}C-putrescine or histamine (17) into casein (Table 3); or (iii) as shown in Table 2 (75), by examining reactions in fully synthetic substrate systems.

Parameswaran and Lorand (77) recently synthesized the acceptor substrate, dansyl-(N-methyl-β-alanyl)thiocholine:

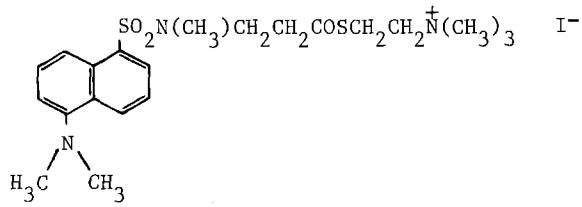
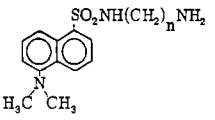
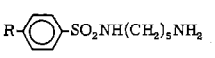
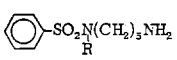


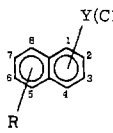
Table 3. Apparent relative affinities of amines for guinea pig liver transglutaminase, as determined by competitive inhibition kinetics for the incorporation of labelled putrescine into N,N'-dimethylcasein at pH 7.5, 37 °C (17, 53). Affinities are given in relation to dansylcadaverine (III) for which the reciprocal apparent inhibition constant ($K_{i,app}^{-1}$) is referred to as 100.

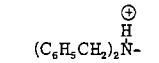
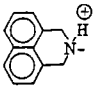
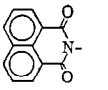
Compound	Structure	Relative affinity measured	Relative affinity corrected[a]
A.			
	n		
I	3	2	2
II	4	60	60
III	5	100	100
IV	6	44	44
B.			
	R		
V	-H	28	28
VI	$-CH_3$	25	25
VII	$-OCH_3$	23	23
VIII	-I	35	35
IX	$-NO_2$	34	34

[a] Corrected for difference of the pK_a of the primary ammonium ion of the compound relative to that of dansylcadaverine.

C.	Ar-Y-$(CH_2)_5NH_2$			
	Ar	Y		
VI	p-$H_3CC_6H_4$-	SO_2NH	25	25
X	p-$H_3CC_6H_4$-	NHCO	6	6
XI	2,4-$(NO_2)_2C_6H_3$-	SNH	47	47
XII	2,4-$(NO_2)_2C_6H_3$-	SO_2NH	105	105
XIII	2,4-$(NO_2)_2C_5H_3$	NH	20	20
IX	p-$NO_2C_6H_4$-	SO_2NH	34	34
XIV	p-$NO_2C_6H_4$-	NHCO	24	24

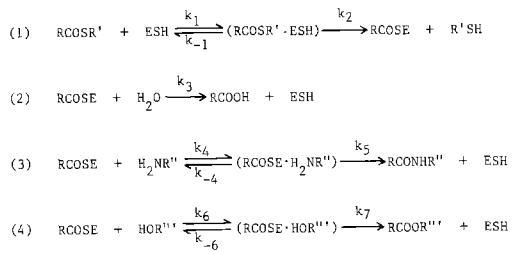
D.			
	R		
V	-H	28	28
XV	$-CH_3$	24	24
XVI	$-C_2H_5$	29	29
XVII	$-CH(CH_3)_2$	17	17
XVIII	$-CH_2C_6H_5$	31	31

E.			
	R	Y (-SO_2NH-)	
XIX	-H	1-	37	37
III	5-$N(CH_3)_2$	1-	100	100
XX	5-$(CH_3)_2NCH_2$-	1-	41	41
XXI	5-$(CH_3)_2N(CH_2)_3$-	1-	54	54
XXII	-H	2-	40	40
XXIII	6-CH_3-N- 	2-	57	57

F.	R-$(CH_2)_5NH_2$		
	R		
XXIV		156	16
XXV		422	42
XXVI		33	33

G.	R-$SO_2NHCH_2CH_2$-Y-$CH_2CH_2NH_2$			
	R	Y		
VI	p-$H_3CC_6H_4$-	CH_2	25	25
XXVII	p-$H_3CC_6H_4$-	S	181	18
XXVIII	p-$H_3CC_6H_4$-	$C(CH_3)_2$	2	2
VII	p-$H_3COC_6H_4$-	CH_2	23	23
XXIX	p-$H_3COC_6H_4$-	S	103	10
XXX	2,4,6-$(CH_3)_3C_5H_2$-	CH_2	63	63
XXXI	2,4,6-$(i\text{-}C_3H_7)_3C_6H_2$-	CH_2	95	95
XXXII	2,3,4-$Cl_3C_5H_2$-	S	198	20
XXXIII	p-C_6H_4-N=NC_6H_4-	S	223	22
XIX	naphthalene-1-	CH_2	37	37
XXXIV	naphthalene-1-	S	214	21
XX	5-$(CH_3)_2NCH_2$-naphthalene-1-	CH_2	41	41
XXXV	5-$(CH_3)_2NCH_2$-naphthalene-1-	S	183	18
III	5-$(CH_3)_2N$-naphthalene-1-	CH_2	100	100
XXXVI	5-$(CH_3)_2N$-naphthalene-1-	S	767	77
XXXVII	5-$(CH_3)_2N$-naphthalene-1-	$C(CH_3)_2$	11	11
XXXVIII	5-$(CH_3)_2N$-naphthalene-1-	SO	21	2
XXXIX	5-$(CH_3)_2N$-naphthalene-1-	SO_2	20	2

which allowed yet another approach for studying nucleophile specificities in reactions with amines as well as with alcohols (Table 4). The latter reaction also made it possible to obtain a meaningful pH profile for the first time for human Factor $XIII_a$ (Fig. 8). The steady-state kinetic analysis led to the conclusion that the reaction proceeded along an acylenzyme intermediate pathway. Since the active center is known to be a cysteine thiol (79, 80)[10], the reaction with the enzyme (ESH) can be illustrated by equations 1–4:



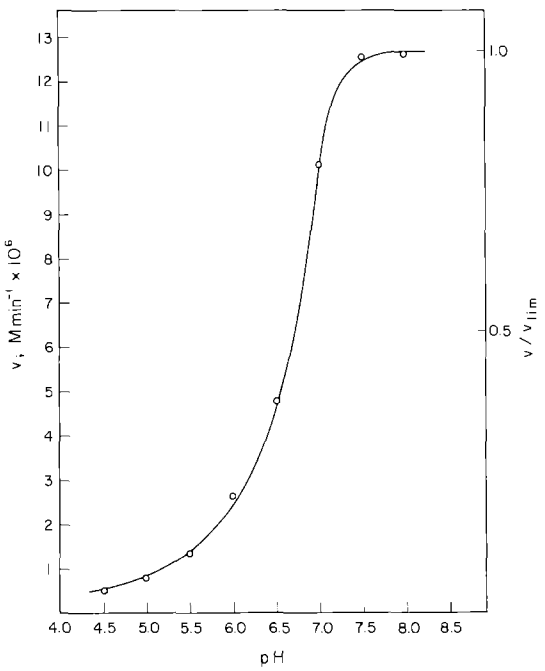


Fig. 8. Effect of pH on the steady-state velocity of methanolysis of [dansyl-(N-methyl-β-alanyl)]thiocholine iodide (S_1) by fibrinoligase in 0.1 M sodium acetate, 27 °C, and 30 mM calcium chloride (77). The reaction was carried out in 0.1 ml of aqueous phase containing 0.1 M sodium acetate, 1.86 mM S_1, 1.24 M methanol, 0.5% N,N-dimethylformamide, thrombin-activated plasma Factor XIII ($e_0 = 0.36\ \mu M$), and finally 30 mM calcium chloride.

[10] The claim of 'half of the sites' type of reactivity for Factor $XIII_a$ by Chung et al. (81) cannot be supported by investigations from this laboratory (80).

The acylenzyme intermediate[11] (RCOSE) formed in eq. 1 could either react with water (as in eq. 2), with an amine (as in eq. 3) or with an alcohol (as in eq. 4), giving rise to three different products. The kinetic specificity of amines is governed by their affinities for the acylenzyme intermediate (i.e., by $K_b = k_{-4}/k_4$) as well as by their reactivities (i.e., k_5), and the k_5/K_b expression is a suitable index of amine specificity.

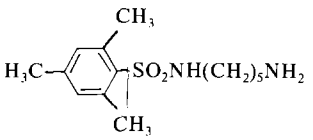
If one subscribes to the view that kinetic analysis of specificity corresponds to a 'template mapping' of the environment of active center domains of an enzyme, the conclusion is justified that the acceptor and the donor substrates must fit into rather tight crevices on Factor $XIII_a$ and on transglutaminases. It is as if the carbonyl group of the first substrate (i.e., γ-position in glutamine) had to span a fixed distance from some surface anchoring toward the catalytic sulfhydryl group, and as if the amine functionality of the second substrate (i.e., ε-position in lysine) also had to reach a certain length to be able to react with the acylenzyme intermediate. An illustration of these concepts is given in Fig. 9. For example, dansylcadaverine would be bound through its aryl moiety to some site, defined as a 'secondary binding site' (59), on the surface of the acylenzyme and with the pentylamine side chain accommodated in a crevice to orient it for reaction with the activated carbonyl group of the first substrate.

Regulation of transglutaminase activities

In spite of the fact that some of the enzymes discussed show a great deal of specificity for preferred protein substrates (e.g., Factor $XIII_a$ reacts with fibrin nearly ten times faster than with fibrinogen) (33, 82), transglutaminases could not have evolved without some regulatory safeguards. Activation of an enzyme of this type at the wrong time and wrong place could have disastrous consequences. Thus, in nature they are found as inactive *zymogens* or as *latent enzymes*. Elucidating the mechanisms of activation of zymogens and latent forms is essential for the understanding of biological controls.

[11] A recent publication by Folk (60) describes the isolation of the trimethylacetyl intermediate of guinea pig liver transglutaminase in small yield.

Table 4. Steady-state kinetic constants for the fibrinoligase (human plasma Factor $XIII_a$; $E_0 = 0.3$ to 0.72×10^{-6} M) catalyzed reactions of dansyl-N-methyl-β-alanylthiocholine iodide (S_1) with amines or alcohols as second substrates (S_2) in the presence of 30 mM $CaCl_2$ (27 °C; 0.5% dimethyl formamide) (77).

S_2	K_M ($M \times 10^3$) for		k_{cat} (s^{-1})	k_{cat}/K_M (for S_2) ($s^{-1}\ M^{-1}$)
	S_1	S_2		
amines				
CH_3NH_2	1.1[a]	9.1[a]	0.45[a]	50[a]
	1.1[b]	12.5[b]	0.5[b]	40[b]
$CH_3(CH_2)_3NH_2$	1.1[c]	13.3[c]	0.18[c]	14[c]
$(CH_3)_3COC(=O)NH(CH_2)_5NH_2$	1.1[a]	7.7[a]	0.8[a]	104[c]
t-Boc-cadaverine	1.1[a]	0.95[a]	0.64[a]	674[a]
 (mesitylenesulfonyl)cadaverine	0.9[a]	0.05[a]	0.21[a]	4200[a]
	K_M ($M \times 10^3$)	K_M (M)		
alcohols				
CH_3OH	1.5[d]	0.4[d]	0.72[d]	1.8[d]
	1.3[e]	0.4[e]	1.14[e]	2.85[e]
CH_3CH_2OH	0.6[d]	0.8[d]	0.2[d]	0.25[d]
	1.3[e]	1.1[e]	0.48[e]	0.44[e]
	0.7[f]	0.8[f]	0.35[f]	0.45[f]
	1.4[g]	1.1[g]	0.37[g]	0.34[g]
$CH_3(CH_2)_3OH$	0.9[d]	0.65[d]	0.15[d]	0.23[d]

[a] 0.05 M Tris-HCl, pH 7.5 at $\mu = 0.15$.
[b] 0.1 M sodium acetate, pH 7.5, at $\mu = 0.21$.
[c] 0.05 M *N*-methylmorpholine hydrochloride, pH 7.5, at $\mu = 0.14$.
[d] 0.1 M sodium acetate, pH 7.0, at $\mu = 0.19$.
[e] 0.05 M *N*-methylmorpholine hydrochloride, pH 7.5, at $\mu = 0.12$.
[f] 0.05 M Pipes-Na^+, pH 7.5, at $\mu = 0.2$.
[g] 0.05 M Pipes-Na^+, pH 7.0, at $\mu = 0.2$.

There are examples for *sequestering* the enzyme from the substrate, with contact occurring between them only as the need arises. An interesting case in point is the clotting of the seminal fluid of rodents, where enzyme and substrates are secreted by two different lobes of the prostate and the two become mixed only in the ejaculate (see chapter by Williams-Ashman). Another example of compartmentalization is found in *Homarus* blood clotting where the transglutaminase, which is responsible for catalyzing the polymerization of several plasma proteins, is contained within the amebocytes and is released only as the cells disintegrate (Fig. 10). Interestingly, human platelets, unlike the *Homarus* cells, do not release transglutaminase by contact activation.

Whether examples can be found for the sequestration of transglutaminase from its substrate within the same cell, and whether redistribution of the enzyme from one sub-cellular compartment to another might take place as a result of stimulation (e.g. from the membrane bound to soluble phase), is an interesting question.

The conversion of Factor XIII (fibrin stabilizing factor) in vertebrates to Factor $XIII_a$ (fibrinoligase) is the best studied model for *zymogen activation* thus far. In as much as both the cross-linking enzyme and the main target substrate (fibrin, derived from fibrinogen) are generated in the fluid phase (plasma) of the same tissue (blood), the zymogen form must have represented the best evolutionary solution poised for rapid activation. Details of the regulation of Factor XIII conversion are so interesting that we shall have to discuss them later under a separate heading. Here, it should suffice to state that the zymogen (Z) first undergoes a *limited proteolysis* with thrombin, but that the hydrolytically

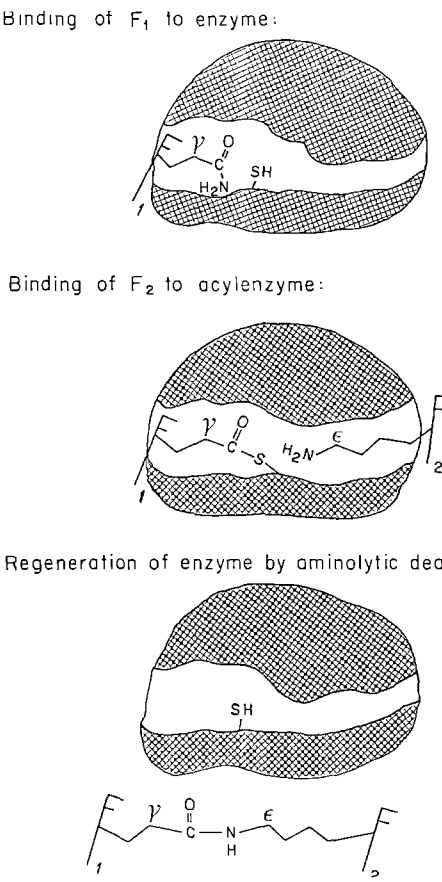


Fig. 9. Intermediate steps for the fibrinoligase (Factor $XIII_a$)-catalyzed cross-linking of two fibrin molecules (F_1 and F_2) by a γ-glutamyl-ϵ-lysine peptide bond.

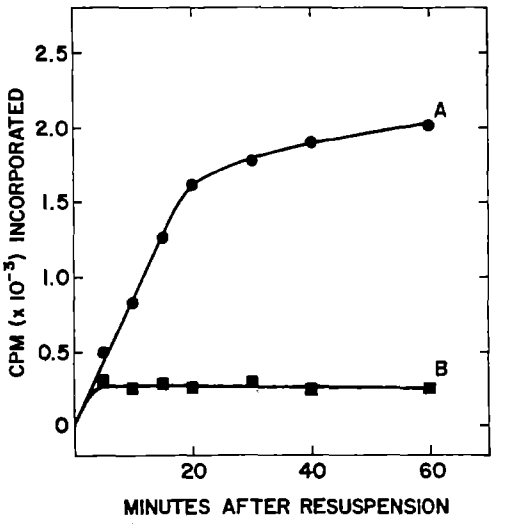


Fig. 10. Time-dependent release of lobster hemocyte transglutaminase under clotting conditions (42). Plasma (10 ml) was extracted from a chilled lobster into a chilled syringe. It was divided into two equal portions and centrifuged at 12 000 × g for 10 min at 0 °C in plastic centrifuge tubes. The pellets were resuspended with 2 ml of 0.4 M NaCl–20 mM $CaCl_2$. One tube (A) was maintained at 23 °C (i.e. at clotting condition); the other (B) was kept at 0 °C. Release of the enzyme was followed by taking 20 μl samples of supernatant for assaying by the filter paper method of Lorand et al. (53), using β-lactoglobulin as test protein for the incorporation of ^{14}C-putrescine.

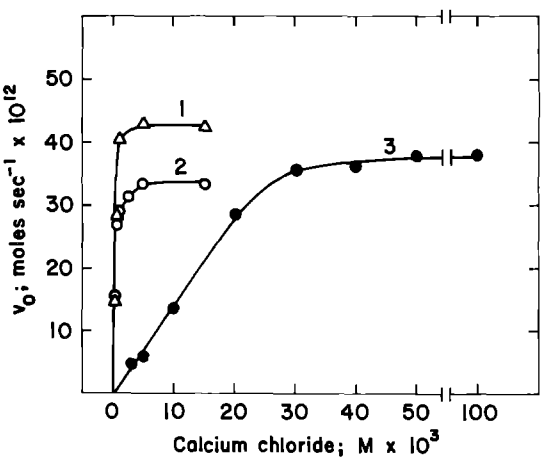


Fig. 11. Apparent Ca^{2+} ion requirement for three transamidating enzymes (76). The aqueous reaction mixtures contained 8.2 mM β-phenylpropionyl-thiocholine iodide, 1.6 mM dansylcadaverine, 50 μg of platelet transglutaminase/ml (△), 0.50 μM guinea pig liver transglutaminase (○), or 0.32 μM thrombin-activated human Factor XIII (●), and $CaCl_2$ at the concentrations shown on the abscissa, added last to initiate amide formation (ordinate), which was measured from the steady-state increase of fluorescence in n-heptane, layered above the aqueous phase.

modified zymogen (Z′) is still inactive. The active center-cysteine of the enzyme (E) is unmasked only in the presence of Ca^{2+}. As such, this type of activation model is represented by a sequence of:

$$Z \xrightarrow{\text{protease}} Z' \xrightarrow{Ca^{2+}} E.$$

Ca^{2+} seems to be a *universal activator* for all latent transglutaminases studied thus far (Latent enzyme $\xrightarrow{Ca^{2+}}$ Active enzyme), and an example for three such enzymes is given in Fig. 11. The Ca^{2+}-requirement of plasma Factor XIII is a special case and will be discussed separately. The intracellular transglutaminases show an apparent requirement for half-maximal activation in the 10^{-4} M to 5×10^{-4} M Ca^{2+} range, and many different cells are known to accumulate this much Ca^{2+} during their life cycles. As a rule, however, this is

not the Ca^{2+}-concentration where calmodulin is most effective in regulating enzyme activities (83); nevertheless, there is a recent abstract by Puszkin et al. (84) suggesting a relationship between transglutaminase and calmodulin.

The Ca^{2+} needed for transglutaminase activation may enter the cell from the outside, or it may arise by virtue of redistribution from intra-cellular stores of Ca^{2+}, e.g. by destruction of mitochondria. Augmentation of free Ca^{2+} may also be caused by a drop in concentration of a metabolite (e.g., ATP) which would otherwise bind Ca^{2+}. In addition, *Zn^{2+} ions* are known to compete against Ca^{2+} and can inhibit transglutaminase with a K_i of about 10^{-7} M (Figs. 12 and 13) (85); thus, alteration of the Ca^{2+}/Zn^{2+} ratio in some cells could be a critical factor.

Once transglutaminase is generated, various *activators* and *inhibitors* could exercise a regulatory role. So far, we have no idea as to the nature of such compounds in the biological milieu, but *in vitro* experiments might serve to highlight some possibilities. Since transglutaminases operate by a cysteine thiol active center mechanism (ESH), disulfide-containing metabolites (RSSR; e.g., cystamine),

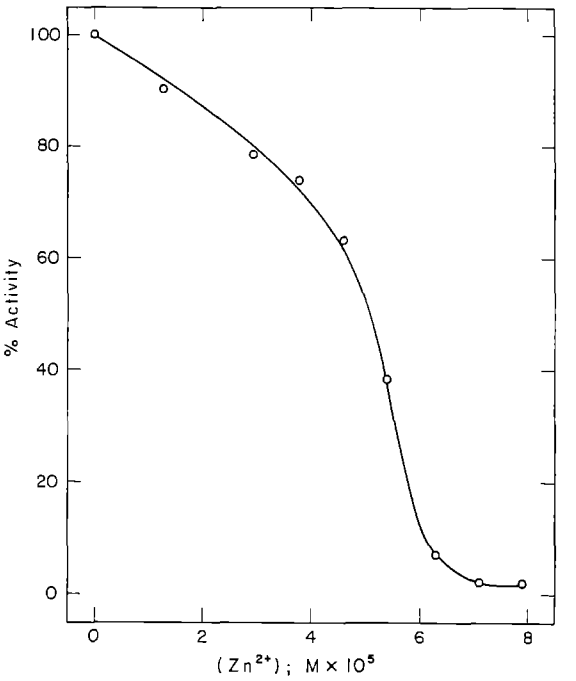


Fig. 13. Inhibition of guinea pig liver transglutaminase by Zn^{2+}-ions (85). Amide formation between β-phenylpropionylthiocholine and dansylcadaverine was measured at pH 7.5, $\mu = 0.25$, 25 °C at an enzyme concentration of 1.8×10^{-7} M in the presence of various concentrations of Zn^{2+}.

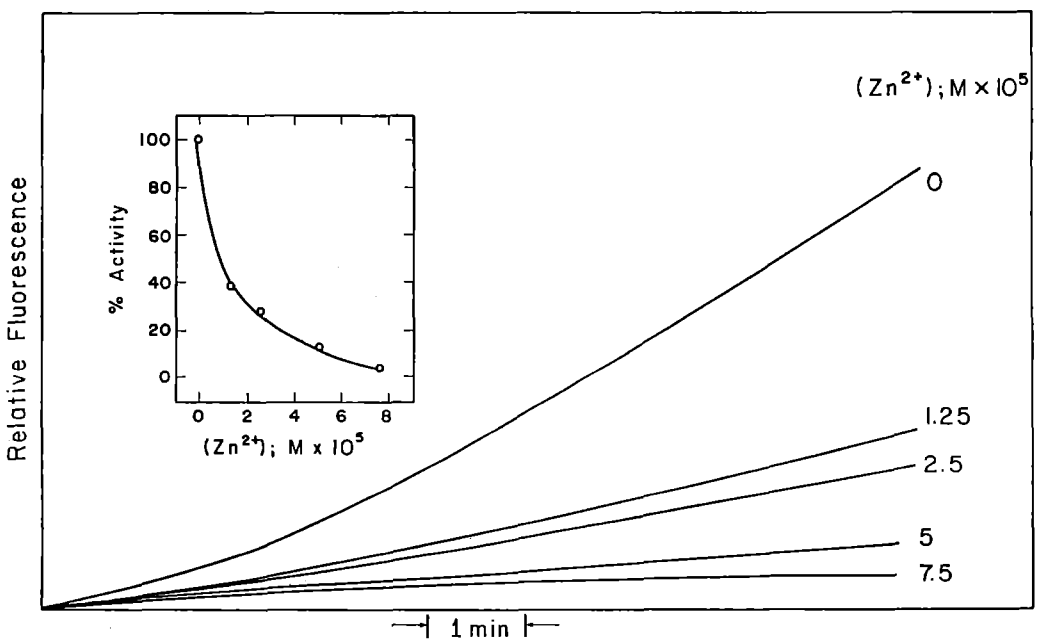
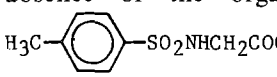


Fig. 12. Inhibition of human fibrinoligase (Factor $XIII_a$) by Zn^{2+}-ions (85). Amide formation between β-phenylpropionylthiocholine and dansylcadaverine was measured at pH 7.5, $\mu = 0.37$, 25 °C at an enzyme concentration of 1.6×10^{-7} M, in the presence of various concentrations of Zn^{2+}.

similarly to the regulation of phosphofructokinase (86), could block enzyme activity by a disulfide exchange reaction: ESH + RSSR → ESSR + RSH, where ESSR represents a reversibly inactivated form of transglutaminase. An illustration for this type of inactivation is given in Fig. 14 (unpublished work of Svahn, Credo and Lorand).

A remarkable example of *activation by a solute* is shown in Fig. 15 regarding the non-secretory transglutaminase of the guinea pig prostate gland. In the absence of the organic solute tosylglycine:  , the enzyme was essentially inactive, but a hundred-fold activation occurred in the presence of the solute. The activity of epidermal transglutaminase could be increased by heating at 56 °C or by treatment with dimethylsulfoxide (87).

In addition to such examples for 'enzyme-level' control, there are indications that transglutaminase activity may also be regulated at the *'substrate level'*, and this may take different forms. The possibility of proteolytic unmasking of the amine incorporating γ-glutamine sites has already been referred to in connection with the finding that fibrin was a much better substrate for Factor $XIII_a$ than the parent protein, fibrinogen. In fact, the removal of only fibrinopeptide A by thrombin: $(\alpha\beta\gamma)_2(A)_2(B)_2 \xrightarrow{\text{thrombin}} (\alpha\beta\gamma)_2(B)_2 + 2A$ is sufficient to enhance the reactivity of the protein substrate toward Factor $XIII_a$, the specific cross-linking enzyme (but not toward liver transglutaminase!). Such a regulation has a clear implication for coordinating the physiological blood clotting event in an ordered manner (i.e., to give preference to the cross-linking of fibrin over that of fibrinogen; see Fig. 16), and may be taken as a

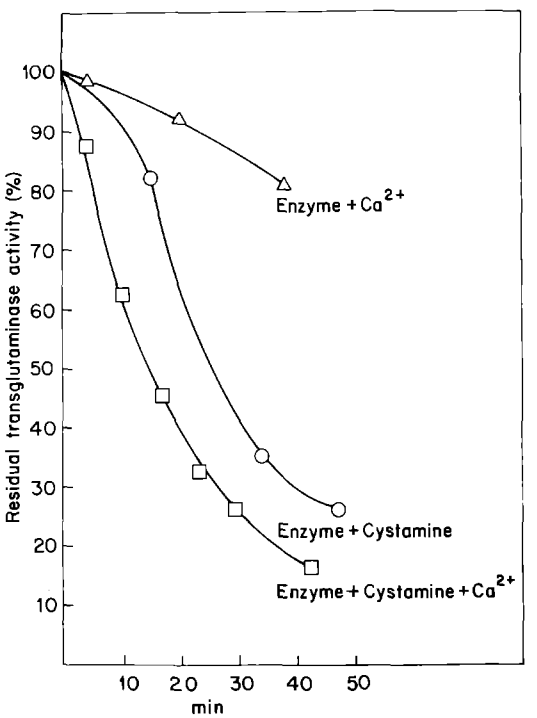


Fig. 14. Inhibition of guinea pig liver transglutaminase by incubation (time on abscissa) with cystamine (10^{-4} M) at pH 7.5 (Svahn and Lorand, unpublished results). When indicated, 17 mM Ca^{2+} was also present during incubation. Residual enzyme activity was measured by formation of the fluorescent amide product in the reaction of β-phenylpropionylthiocholine with dansylcadaverine.

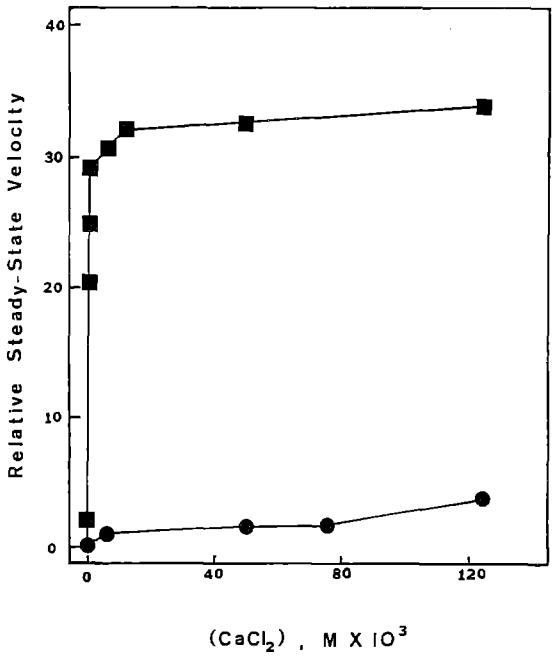


Fig. 15. Effect of tosylglycine on the activity of non-secretory guinea pig prostate transglutaminase (139). Steady-state velocities for amide formation were determined at 30 °C in reaction volumes of 120 μl containing 50 mM Tris-acetate of pH 7.0, 3.5 mM β-phenylpropionylthiocholine, 1.13 mM dansylthiacadaverine, 0.036 mg/ml enzyme, appropriate amounts of sodium chloride to maintain a constant ionic strength of 0.5 and varying concentrations of calcium chloride (●). Formation of the amide product was followed also in the presence of 96 mM p-tosylglycine (■).

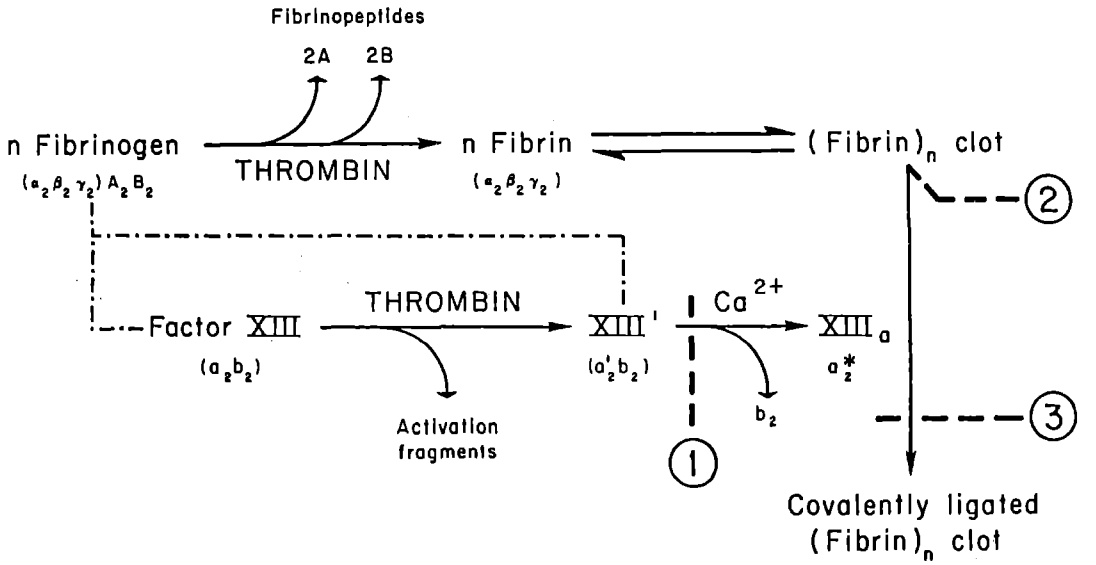


Fig. 16. Outline of the clotting reaction in normal human plasma. Disorders of fibrin stabilization can be genetic, such as Factor XIII-deficiency, or acquired due to the appearance of circulating inhibitors directed against one of the steps in the sequence marked with broken lines (– – –) as ①, ②, and ③. For a discussion of the various molecular diseases, consult Lorand et al. (7). The dotted lines (– · – · –) refer to the regulation exerted by fibrinogen on the conversion of the Factor XIII zymogen, in regard to enhancing the rate of release of activation fragments by thrombin and the lowering of Ca^{2+} requirement.

sign/of co-evolution between the cross-linking enzyme and its protein substrate (33).

Apart from limited proteolytic activation, there is also a possibility that 'substrate-level' control could be exercised through the *prior covalent modification* of the protein serving as target for transglutaminase action. Phosphorylation (e.g., —**O**—PO_3H_2), methylation (e.g., —**COO**CH_3) or acetylation (e.g., —**NHCO**CH_3) of various side chains of the substrate (in bold letters) might alter its reactivity towards transglutaminase. It may be more than coincidence that many of the best protein substrates for transglutaminases, i.e., casein, β-lactoglobulin, or histones, are also among the best substrates of other enzymes which post-translationally modify proteins (88, 89).

Specific *interaction between a* particular *solute* (L) and the protein *substrate* (S) might render the latter (L · S) more reactive toward a transglutaminase: $L + S \rightleftharpoons L \cdot S$. This type of regulation was first recognized by Williams-Ashman et al. (90) who showed that the addition of polyanions, by simulating the effect of bulbourethral gland secretion, greatly enhanced the reactivity of seminal vesicle secretion proteins (but not that of casein!) towards the clotting enzyme.

Fluctuations in the *intra-cellular concentrations of polyamines* (spermidine, spermine, putrescine and cystamine) or their monoacetylated forms might play a regulatory role by competing against the cross-linking of proteins, as suggested by the equations in Fig. 1.

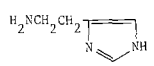
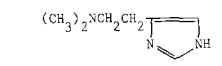
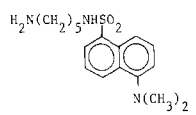
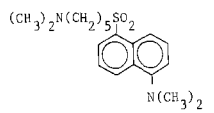
Strategies for probing transglutaminase-mediated events in biological systems

When the participation of transglutaminase in a biological reaction is suspected, the first step is often to try to interfere with the phenomenon by the addition of one of the synthetic amine substrates. This approach was introduced in connection with the study of fibrin stabilization during the process of blood coagulation (35) and, in general, the following rules should apply.

(i) Specificity for the *primary amino* group functionality of the inhibitor should be demonstrated. If dansylcadaverine is inhibitory, it is expected that the tertiary amino group containing N-dimethyl analogue[12] (Table 5) would be less so,

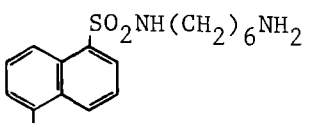
[12] Because of widely differing solubilities, dansylamidopentanol is not an appropriate control for dansylcadaverine.

Table 5. Amine substrates for probing transglutaminase activities in biological systems and recommended control compounds.

Substrates	Controls
glycine ethylester $H_2NCH_2COOCH_2CH_3$	sarcosine ethylester $CH_3NHCH_2COOCH_2CH_3$
histamine 	$^{\alpha}$N-dimethylhistamine 
dansylcadaverine 	dimethylated dansylcadaverine 

and the same could be said for comparing the effects of histamine and $^{\alpha}$N-dimethylhistamine, or that of glycine ethylester and sarcosine ethylester. Such controls are all the more important in cellular systems, where inhibition might simply be due to interference by the hydrophobic dansylamidoalkyl moiety with some membrane function or to the changing of pH in an intra-cellular compartment (e.g., lysomotropic effect), which could be elicited by primary and tertiary amines equally well.

There are other complications with the use of amine inhibitors in cell biology. Even a compound with as favorable a K_M ($\sim 10^{-5}$ M) for transglutaminase as dansylcadaverine cannot be considered so restricted in its mode of action as to be assumed to interfere with this enzyme exclusively. There is some information already available that dansylcadaverine can inhibit a variety of metabolic processes [ketosteroid reductase (91); glycoprotein synthesis (C. G. Curtis, personal communication); phosphatidylcholine synthesis (92)] and the list is almost certain to grow. In addition, considering the fact that some commercially marketed 'calmodulin inhibitors' (W-5 and W-7 of Rikaken Co., Japan):



Y = H or Cl

are structurally quite similar to dansylcadaverine and other synthetic substrates of transglutaminase (17), one wonders if dansylcadaverine and its analogues might not also act as calmodulin inhibitors[13].

(ii) *Incorporation of the amine* inhibitor into target proteins must be demonstrable. Under ideal conditions, all the enzyme-reactive γ-glutaminyl acceptor sites of the protein can be modified. Various amine isotopes are available for this purpose, and other synthetic amines may be studied by virtue of their fluorescence or color, or because antibodies are easily obtained against some substituents (e.g., dansyl or dinitrophenyl). Though a necessary requirement, the covalent attachment of the label to proteins by itself is not sufficient for concluding that incorporation was mediated by transglutaminase. Such a difficulty was encountered when work with lymphocyte activation showed that the putrescine-derived radioactive label was converted into hypusine and not, as previously thought, into γ-glutamylspermidine (62, 64).

Transglutaminase-catalyzed reactions between proteins and amines produce amides; thus, acid hydrolysis (5.7 N HCl, 24 hr, 100°) should permit recovery of the amine. Furthermore, total enzymatic digestion, as in the analysis of the γ-glutamyl-ϵ-lysine cross-linking peptide (46), should produce a labelled γ-glutamyl-amide.

Apart from the hypusine pathway (64), putrescine could also undergo oxidation to aldehyde which, in turn, might react with proteins in a transglutaminase-independent manner. When such a possibility is transglutaminase-independent manner. When such a possibility is suspected, diamine oxidase inhibitors (such as methylglyoxalbis (guanylhydrazone) or aminoguanidine) could be employed. As discussed already, spurious incorporation of an amine label could also arise from the action of myeloperoxidase (67).

(iii) *Other inhibitors* of transglutaminases fall into various categories:

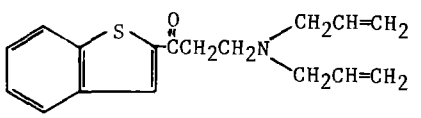
(a) Active site-directed compounds may block the essential cysteine thiol group of the enzyme in various ways. Disulfides, such as cystamine (Fig. 14), participate in an exchange reaction as shown by Svahn, Credo and Lorand (unpublished results)

[13] In cell biology, we are forced to realize that the specificity of an inhibitor is inversely proportional to our knowledge regarding its mode of action.

of the: RSSR + ESH ⇌ (RSSR; ESH) → RSSE + RSH type. Other compounds, e.g., halomethyl ketones (93) or blocked diazooxonorvaline or norleucine derivatives (94), are also inhibitory. However, these are not naturally occurring substances, nor is it known whether they are more selective in reacting with transglutaminase than iodoacetamide or N-ethylmaleimide would be.

(b) As shown in Fig. 12, Zn^{2+} appears to be a good inhibitor of transglutaminase by virtue of competing against Ca^{2+}.

(c) Lorand, Barnes, Michalska, Hawkins, and Bruner-Lorand (unpublished results) recently explored a novel, *non-competitive inhibitor* (95) for the purpose of preventing protein cross-linking reactions in Ca^{2+}-enriched human erythrocytes and in thrombin-activated human platelets:



Both reactions were abolished by adding less than 10^{-3} M of the inhibitor to the medium. Though again there are no data regarding selectivity, the approach of using such non-competitive inhibitors may be very useful in the future.

(iv) In addition to the previous approaches, *isolation and identification of γ-glutamyl-ε-lysine* cross-linked polymers is important in studying the participation of transglutaminase in a biological reaction (12, 35, 96). However, one should always keep in mind the possibility that such cross-links may also arise without the involvement of transglutaminase.

The blood clotting paradigm

It is now possible to reconstruct, in molecular terms, the sequence of events leading to the formation of a γ-ε cross-linked (ligated) fibrin clot in normal plasma (Fig. 16) (7). The regulatory aspects of generating the cross-linking enzyme (fibrinoligase, Factor $XIII_a$) from the circulating zymogen (fibrin stabilizing factor, Factor XIII) offer unique insights into possible control mechanisms regarding transglutaminases in general. Elucidation of the physiological pathway of clot formation in normal plasma, together with the development of ancillary methodologies, also made it possible to diagnose novel molecular hemorrhagic diseases related to various defects of fibrin stabilization (7, 97).

The plasma zymogen comprises two types of subunits (98) of about 80 000 MW each with a presumed molecular weight of ~320 000: a_2b_2. Activation can be effected simply by treating the zymogen with high concentrations of Ca^{2+} (37 °C, pH 7.5, $\mu = 0.3$): $a_2b_2 \rightarrow a_2^\circ + b_2$, when the active center cysteine on the catalytic *a* subunits is unmasked coincidentally with dissociation from the carrier *b* subunits. Addition of chaotropic anions (p-toluenesulfonate, thiocyanate, iodide, bromide) can reduce the Ca^{2+}-requirement to about 0.05 M (99, 100).

The physiological pathway of enzyme generation, however, is thrombin dependent. The a_2b_2 zymogen is first modified in a limited proteolytic step (101, 102):

$$a_2b_2 \xrightarrow{\text{thrombin}} a_2'b_2 + \text{activation peptides}$$

to yield an $a_2'b_2$ ensemble which, however, is still devoid of catalytic activity. Nevertheless, the hydrolytic removal of the N-terminal peptide fragment from the *a* subunit leads to a loosening of the heterologous association between *a* and *b* subunits, as seen by the fact that they can be dissociated at the physiological ionic strength of $\mu = 0.15$ merely by the addition of 10–20 mM $CaCl_2$, at pH 7.5, 37 °C in 10 min (80). However, since normal plasma contains only 1.5 mM Ca^{2+}, some other regulatory feature still had to be uncovered and it was found that fibrinogen (ϕ), at the physiological concentration of about 10^{-5} M, exerted a profound effect on the

$$a_2'b_2 \xrightarrow[\phi]{Ca^{2+}} a_2^* + b_2$$

transition by lowering the Ca^{2+}-requirement to 1.5 mM (99). A peptide fragment corresponding to a sequence in the mid-portion of the α-chain of fibrinogen (residues 242–424) displayed the same activity as the native protein (103).

In addition to interacting with the hydrolytically modified ($a_2'b_2$) species, fibrinogen was also found to regulate the rate of release of the N-terminal activation peptide from the parent zymogen (104):

$$a_2b_2 \xrightarrow{\text{thrombin}} a_2b_2 + \text{activation peptides (AP)}.$$

Using an HPLC system for measuring AP release, a k_{cat}/K_M of 10 μM^{-1} min^{-1} was obtained (at

$\mu = 0.15$, pH 7.4 in phosphate buffered saline and 37 °C). The addition of human fibrinogen caused a marked increase, in a dose-dependent fashion, in the rate of release of AP. In the absence of fibrinogen, 0.57 nM human α-thrombin, for example, released 50% of AP from 0.1 μM Factor XIII in 150 min, whereas in the presence of 0.45 μM fibrinogen the time required for 50% release was only 4 min.

From an evolutionary point of view, the clotting systems of *Homarus* plasma (9, 25, 105) and of the rodent seminal fluid (10) could be considered to be more primitive, because they depend only upon the encounter of the latent or the sequestered transglutaminase with the protein substrates in the presence of Ca^{2+}.

Transglutaminase and cell aging. The Ca^{2+}-enriched human erythrocyte

Due to the operation of an efficient, outward directed Ca^{2+} pump, the Ca^{2+} concentration in normal human red blood cells is kept below 10^{-6} M. When the pump fails (as in aged cells, sickle cells or in some hereditary metabolic diseases), the intra-cellular Ca^{2+} may rise as high as 2×10^{-4} M (106–109), which would be sufficient to activate transglutaminase to an appreciable extent. The human erythrocyte enriched by Ca^{2+} in the presence of ionophore (A23187) is a suitable experimental model. If Ca^{2+} loading is allowed to proceed for periods longer than a few minutes, the cell suffers a permanent shape change (110) (see also Fig. 17) and a loss of membrane deformability (111). Parallel with these physical manifestations, the proteins of the membrane also undergo profound structural changes.

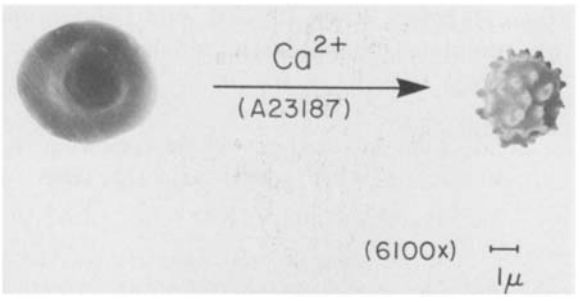


Fig. 17. Irreversible shape change, from discocyte (on left) to spheroechinocyte (on right) in the human erythrocyte, following enrichment with Ca^{2+} in the presence of ionophore A23187. Scanning electronmicrograph (work of M. Michalska).

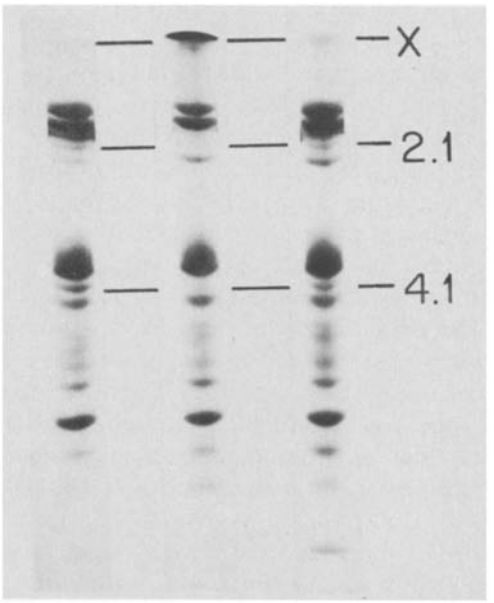
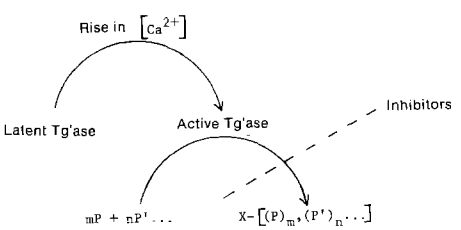


Fig. 18. Cross-linking of membrane proteins in Ca^{2+}-enriched human erythrocytes can be prevented by adding small molecular weight substrates or inhibitors for transglutaminase. Right-hand gel: the presence of 20 mM aminoacetonitrile during incubation of the cells (50% hematocrit, 37 °C, 3 hr, 10 μM ionophore) with 1.5 mM Ca^{2+}, prevents loss of bands 2.1 and 4.1, as well as the formation of membrane protein polymer (X). Center gel: cells withou aminoacetonitrile, but with Ca^{2+}. Left-hand gel: cells with 1.5 mM Mg^{2+} instead of Ca^{2+}.

A prominent feature of the membrane profile of Ca^{2+}-treated cells, as revealed by electrophoretic analysis, is the appearance of high molecular weight polymers (Fig. 18) which can be shown to contain appreciable amounts of γ-glutamyl-ϵ-lysine cross-links. This effect of Ca^{2+} may be prevented if either histamine, cystamine, aminoacetonitrile or a non-competitive inhibitor of transglutaminase is included in the incubation medium



prior to and during Ca^{2+}-loading (11, 29, 96, 112).

The electrophoretic analysis is somewhat complicated by the fact that, in addition to the transglutaminase-catalyzed cross-linking process:
a Ca^{2+}-triggered degradation of at least two trans-membrane proteins, i.e., band 3 and glycophorin, takes place (41, 113–115). Pepstatin was shown to inhibit the proteolytic phenomenon rather selectively.

If the Ca^{2+}-loading of the cells was carried out in the presence of ^{14}C-histamine, covalent labelling in the spectrin and band 3 protein regions was observed. Immunological examinations revealed that, in addition to spectrin and band 3, ankyrin (band 2.1), band 4.1 and hemoglobin were also part of the γ-glutamyl-ε-lysine cross-linked polymeric cluster (41; also Bjerrum et al., unpublished results). It appears that the treatment of intact erythrocytes with Ca^{2+} activates an *in situ* covalent fusion of nearest neighbors at the membrane cytoskeletal interface (Fig. 19)[14].

The fusion between the cytoskeletal matrix and membrane proteins by γ-glutamyl-ε-lysine bonds, giving rise to large immobile structures as in the human erythrocyte, might be a general paradigm for cells undergoing terminal differentiation in the process of aging and dying[15]. Transglutaminase-catalyzed cross-linking was shown to play an essential role in the process of cornification in keratinocytes (30, 119). Another system currently under study in our laboratory relates to lens transglutaminase (120–123), with obvious possible overtones for cataract formation[16]. After demonstrating a Ca^{2+}-dependent transglutaminase activity in lens, isolating the enzyme and identifying some of its intrinsic protein substrates, attention was focused on a model system (125). Rabbit lenses were bathed in Ca^{2+}-containing buffer, then solubilized by the addition of urea-dithiothreitol-sodium dodecylsulfate and electrophoresed in 10% polyacrylamide gels, which allowed resolution of protein components below 100 000 chain weight. In comparison to controls where EDTA or Mg^{2+} replaced Ca^{2+}, two unrelated major differences were evident: a new band appeared at about 55 000 MW, and a band disappeared from the 62 000 MW region (Fig. 20). Since the appearance of the 55 000 weight material could be selectively inhibited by histamine, and since this amine would interfere with the γ-glutamyl-ε-lysine type of cross-linking of proteins by the intrinsic transglutaminase, formation of the 55 000 MW protein in the Ca^{2+}-enriched lens is thought to be the result of transglutaminase action. On the other hand, the disappearance of the 62 000 MW protein was attributed to proteolysis, because it could be selectively prevented by leupeptin, an inhibitor of trypsin-like enzymes and of Ca^{2+}-dependent intracellular proteases. The parallel occurrence of a cross-linking and a proteolytic event reminds one of the phenomenon observed in Ca^{2+}-enriched human erythrocytes (114, 115).

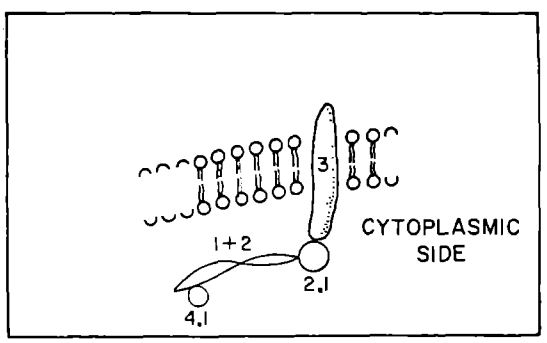


Fig. 19. In intact human erythrocytes the disappearance of monomeric forms of 1 and 2, 2.1, 3 and 4.1 membrane proteins, occurring when intracellular Ca^{2+} is high, can be inhibited by added amines (i.e. by alternate transglutaminase substrates). Radioactive amines label 1 and 2, and 3, indicating that γ-glutamine side chains of these proteins would participate in cross-bridge formation in the absence of added amines.

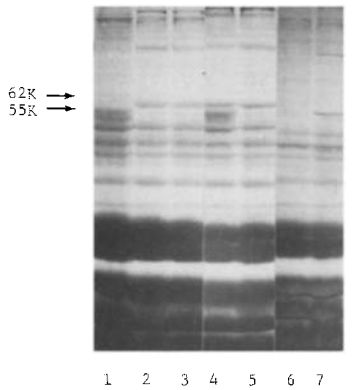
[14] Catalase activity was also found to be associated with the polymer (116).

Transglutaminase and cell activation. The thrombin-stimulated human platelet and the fertilized sea urchin egg

There could be situations where, following specific stimulation, Ca^{2+}-ions could either enter the

[15] Possibility of transglutaminase participation in the formation of paired helical filaments found in the brains of Alzheimer patients is now under investigation (117, 118).

[16] In addition to γ-glutamyl-ε-lysine bridges (121), the formation of protein bound γ-glutamyl-putrescine was also reported (124) in cataract proteins.



Lane	$CaCl_2$	$MgCl_2$	EDTA	Leupeptin	Histamine
1	+	-	-	-	-
2	-	-	+	-	-
3	-	+	-	-	-
4	+	-	-	+	-
5	-	-	+	+	-
6	+	-	-	-	+
7	-	-	+	-	+

Fig. 20. Cross-linking and proteolysis in Ca^{2+}-enriched rabbit lenses (125). Whole, frozen rabbit lenses were incubated in 500 μl of 50 mM Tris-HCl, 100 mM NaCl, pH 7.5 for 1 hr at 37 °C with the specified inhibitors (1 mM leupeptin or 63 mM histamine) followed by the addition of 100 μl of either $CaCl_2$ (8.3 mM, final concentration), $MgCl_2$ (8.3 mM) or EDTA (1.7 mM) for an additional 3 hr incubation at 37 °C. Following the incubation, the lenses were solubilized in 4 ml of 0.02 M Na_xPO_4, 9 M urea, 40 mM dithiothreitol, 2% sodium dodecylsulfate. Electrophoresis on 10% SDS-polyacrylamide was carried out on 20 μl aliquots of sample using the Laemmli system.

cell from the outside or could become liberated from intra-cellular storage sites in high enough concentration to cause activation of transglutaminase. The phytohemagglutinin-treated lymphocyte might have been an example of this type but, as it was mentioned before, putrescine incorporation in this case was shown to follow a different (hypusine) pathway (62, 64). Considerable effort was focused in recent years on the possible involvement of transglutaminase in receptor-mediated endocytosis with regard to the internalization of ligands such as the epidermal growth factor, α_2-macroglobulin and insulin (126–131; see also chapter by Davies). Activation of transglutaminase in Aplysia nerve may be significant in the axonal transport of proteins (132, 133).

In our own laboratory, two cell activation systems are currently under study. One relates to the formation of γ-glutamyl-ϵ-lysine cross-linked proteins in human platelets following thrombin stimulation and the prevention of this transglutaminase-catalyzed cross-linking process by alternate substrates and inhibitors of the enzyme (134).

The other system is the fertilized egg of the sea urchin *(Arbacia punctulata* or *lixula).* When the eggs are pre-incubated in ^{3}H-putrescine, there is a significant enhancement in the covalent incorporation of the isotope into proteins soon after fertilization (61)[17]. Methylglyoxalbis(guanylhydrazone), which is an inhibitor of S-adenosylmethionine decarboxylase as well as diamine oxidase, had no effect on the incorporation. Emetine, an inhibitor of protein synthesis, also had no effect. Dansylcadaverine, however, was inhibitory. Following acid hydrolysis, the label could be recovered in the form of putrescine and proteolytic degradation of labelled proteins gave rise to γ-glutamylputrescine (Cariello, Wilson and Lorand, unpublished results).

Concluding remarks

It is now clear that Ca^{2+}-dependent transamidases of the endo-γ-glutamine:ϵ-lysine transferase type play important roles in a number of biological systems. We have particularly good understanding about the modes of operation of these enzymes when the cross-linking of protein substrates by γ-ϵ peptide bonds leads to the formation of identifiable polymeric structures in the extra-cellular environment (e.g. as in clot formation). However, the intracellular covalent fusion of membrane and cytoplasmic components by similar bonds is also a rather well established phenomenon in Ca^{2+}-enriched cells, such as human erythrocytes and keratinocytes, serving as a general paradigm for structural changes accompanying cell aging.

Another post-translational role for these enzymes is to modify γ-glutamine residues of specific

[17] Washing the eggs before fertilization and removing them from the isotope-containing medium made no difference. Thus, the observed effect could not be due to increased permeability of the fertilized egg to putrescine.

protein substrates upon cell activation by the incorporation of biological amines (e.g. putrescine). Formation of protein-bound γ-glutamylputrescine by such reaction was shown to occur during the process of egg fertilization in the sea urchin (61) and has been suggested to accompany axonal transport in Aplysia neuron (132, 133). Mouse peritoneal macrophages cultured in mouse serum accumulate very high levels of transglutaminase (1–2% of cellular proteins) due to accelerated synthesis (Davies, this volume).

The covalent bonding of putrescine and spermidine to proteins in the cyst coat of the protozoan *Colpoda steinii* (135) may or may not be due to transglutaminase action. The origin of polyamine (primarily putrescine)-conjugated peptide material in human plasma (136) and of free γ-glutamyl-putrescine in the mammalian brain (137) also awaits elucidation. More than seventy γ-glutamyl derivatives of amino acids and amines have been isolated from plants, many of them from mushrooms (138). Could some of these represent proteolytic breakdown products of cell proteins which became derivatized with the amines in a transglutaminase-catalyzed earlier metabolic step? Or, did they simply arise by conventional biosynthetic reactions requiring either activation by ATP or the participation of more typical γ-glutamyltransferase? Current evidence seems to favor the latter two pathways (138).

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