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Crosslinking and labeling of membrane proteins by transglutaminase-catalyzed reactions

(membrane structure/fluorescent probes)

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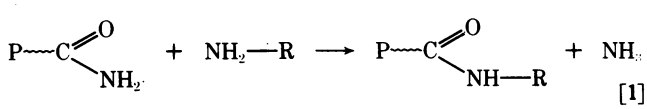
ABSTRACT **Transglutaminase enzymes catalyze the formation of ε(γ-glutamyl)lysyl crosslinks, or the substitution of a variety of primary amines for the amide function of protein-bound glutaminyl residues. These enzymes should therefore be useful in crosslinking the proteins of membranes and in attaching a variety of chemical probes and labels to these proteins. This usefulness is demonstrated in experiments with the enzyme liver transglutaminase and the membranes of mouse erythrocytes and of rabbit skeletal muscle sarcoplasmic reticulum.**

Transglutaminases are enzymes that have been used in investigations of soluble proteins for many years (1, 2). They catalyze Ca^{++}-dependent acyl transfer reactions at carboxamide groups of protein-bound glutaminyl residues, usually with a primary amine as substrate:



[1]

If R-NH_2 is a suitable exogenously added amine, the protein P can be labeled with appropriate R groups specifically at glutaminyl residues. In the absence of an exogenous amine, ε-NH_2 groups of appropriate protein-bound lysyl residues can serve as substrates, thereby generating intra- or intermolecular ε(γ-glutamyl)lysyl crosslinks. This capacity to mediate the attachment of specific labels to proteins, or to introduce crosslinks between them, should make transglutaminases very useful in studies of membrane structure, to which they have not yet been systematically applied. In this paper, we illustrate this usefulness in crosslinking and labeling experiments using guinea pig liver transglutaminase with mouse erythrocyte membranes and with the sarcoplasmic reticulum of rabbit skeletal muscle. We have recently reported related studies with the membrane-containing bacteriophage PM-2 (3).

MATERIALS AND METHODS

Guinea pig liver transglutaminase was prepared by the method of Connellan *et al.* (4), and gave a single band upon electrophoresis in 1% sodium dodecyl sulfate/5.6% polyacrylamide gels. Enzyme activity was determined by the colorimetric hydroxamate procedure (5).

Mouse blood was freshly drawn and used immediately. The erythrocytes were washed four times by centrifugation to remove the buffy coat and serum proteins. Erythrocyte ghosts were prepared according to Dodge *et al.* (6), and were made permeable by freezing and thawing twice. *Crosslinking studies* were carried out by incubating intact erythrocytes at 2.5 mg of membrane protein per ml, in 0.15 M NaCl/7 mM phosphate buffer (pH 7.4), or ghosts at 1.6 mg of protein per ml in 7 mM phosphate buffer (pH 7.4) with 10 mM $CaCl_2$, 2.0 mM cysteine, and the transglutaminase at a series of concentrations from 0.2 μM to 40 μM. Incubation was generally for 1 hr at 37°. Controls were performed with either the enzyme or the $CaCl_2$ omitted. After incubation of the intact erythrocytes, the cells were thoroughly washed, and then the ghosts were prepared from them by the method of Dodge *et al.* (6). Reactions with the ghosts were terminated by solubilization in 2% sodium dodecyl sulfate containing 10 mM EDTA, 1% mercaptoethanol, and 4 mM phenylmethylsulfonyl fluoride, and were then dialyzed overnight against 10 mM Tris-acetate buffer (pH 7.5) containing 1 mM EDTA, 0.1% mercaptoethanol, and 1% sodium dodecyl sulfate. The samples were then heated for 3 min at 100°, and were subjected to electrophoresis on 7.5%, 5.6%, or 3.25% polyacrylamide gels (7). The gels were stained for protein with Coomassie blue, fast green, or procion blue; or for carbohydrate with periodic acid-Schiff reagent. The extent of crosslinking of a particular component was measured by the decrease in area of the particular band on densitometer tracings of a series of gels containing the same amount of total protein.

Labeling experiments were performed with washed intact erythrocytes in a 7 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl; or with ghosts in 7 mM phosphate buffer (pH 7.4); both containing 10 mM $CaCl_2$, 20 mM cysteine, and the transglutaminase at either 10 or 1 μM. The amines used were either [^{3}H]tyramine at a concentration of 5 mM (specific activities of either 1 Ci/mmol or 150 mCi/mmol, New England Nuclear) or dansyl cadaverine (Cyclo Chemical) at 5 mM.

Sarcoplasmic reticulum was prepared from rabbit white skeletal muscle by the method of Martonosi (8). Labeling experiments at a protein concentration of 10 mg/ml were carried out with several concentrations of dansyl cadaverine and 10 μM transglutaminase in a buffer containing 0.1 M KCl, 5 mM histidine (pH 7.3), 10 mM $CaCl_2$, and 10 mM dithiothreitol.

The extent of labeling with [^{3}H]tyramine was determined by slicing the gels into 1-mm sections and measuring the radioactivity in a Beckmann LS-200 Liquid Scintillation System after solution of the sample in 0.2 ml of 30% hydrogen peroxide at 85° and the addition of scintillation fluid. With the fluorescent dansyl cadaverine-labeled samples, the gels were photographed with UV illumination under controlled conditions, and the negatives were scanned in a Joyce-Loebl MK IIIB recording microdensitometer.

Further details will be published elsewhere (A. Dutton and S. J. Singer, to be published).

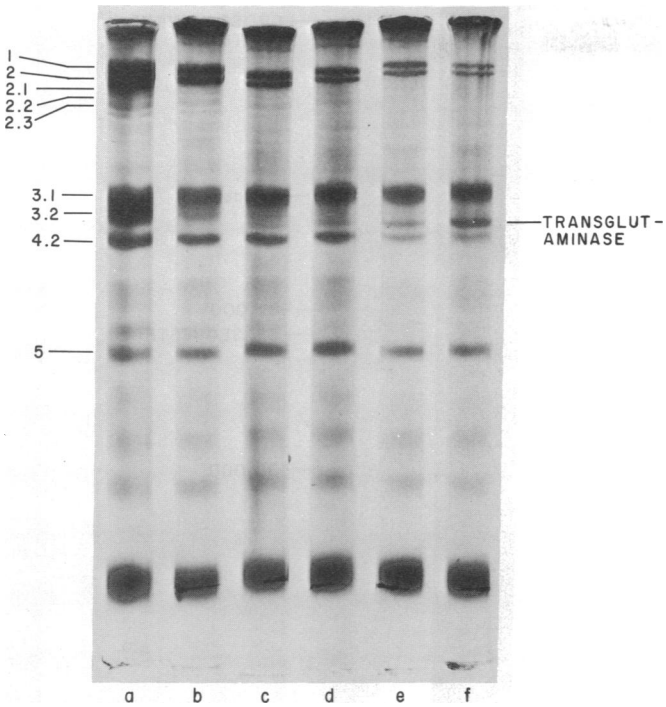


FIG. 1. Gel electrophoresis patterns demonstrating the increasing extent of crosslinking of the proteins of mouse erythrocyte membranes with increasing concentrations of transglutaminase. Gel a, control without enzyme. Gels b–f, after incubation with 1.0, 2.5, 5.0, 10, and 20 μM transglutaminase, respectively. Electrophoresis was in 0.1% sodium dodecyl sulfate/5.6% polyacrylamide gels, and the proteins were stained with Coomassie blue. The band designations at the left follow those in ref. 7.

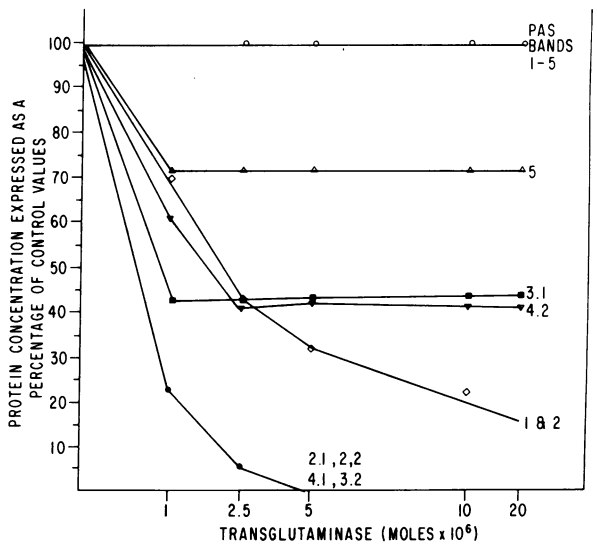


FIG. 2. Quantitative analysis of the data of Fig. 1 showing the disappearance, due to crosslinking by transglutaminase, of the protein bands in the gels as a function of the concentration of transglutaminase used.

RESULTS

Mouse Erythrocytes: Crosslinking Experiments. No significant crosslinking of the polypeptides of intact erythrocytes or of resealed ghosts was observed up to concentrations of 40 μM transglutaminase. The maximum extent of crosslinking and labeling was obtained in ghost preparations that had been frozen and thawed several times before use. In Fig. 1 are shown polyacrylamide gel patterns of the proteins of the disrupted ghosts treated under the same conditions but with increasing concentrations of the transglutaminase. The extent to which the components in different bands became crosslinked is plotted in Fig. 2, and was remarkably different. Certain bands (2.1 and 3.2) were essentially completely crosslinked at low enzyme concentrations; certain bands [bands 1 and 2, or spectrin (9)] showed continually increasing extent of crosslinking as the enzyme concentration was increased; certain bands (3.1 and 4.2) showed extensive crosslinking (about 40–50%) at low enzyme concentration, and no further crosslinking as the enzyme concentration increased; and certain bands (band 5 and periodic acid-Schiff staining bands) showed little or no crosslinking under these conditions. Component 6 was present in too small amounts to measure adequately. No significant amounts of discrete bands of higher molecular weight than component 1 were observed in 3.25% gels, even with experiments with low enzyme concentrations and short incubation times. Instead, as crosslinking increased, increasing amounts of protein remained at the top of the gel.

Mouse Erythrocytes: Labeling Experiments. No more than 1% of the amount of label that was bound to permeable ghosts was attached to intact cells with either [^{3}H]tyramine or dansyl cadaverine and 10 or 1 μM enzyme. This low level of labeling was most probably due to contamination of the intact cells with a small amount of ghosts. With permeable ghosts, extensive labeling occurred with both amines, band 3 acquiring the largest amount of both labels (Fig. 3). However, significant differences were observed in the extent of labeling of the other bands with the two amines. The distribution of label was also dependent on the concentration of the enzyme that was used (Table 1). Some crosslinking also occurred in these labeling experiments, more with tyramine than with dansyl cadaverine, and decreasing with increasing concentration of the added amine.

Transglutaminase became bound to the ghosts during incubation and could not be removed by washing. At 10 μM enzyme, this was seen by a new band appearing in the gel

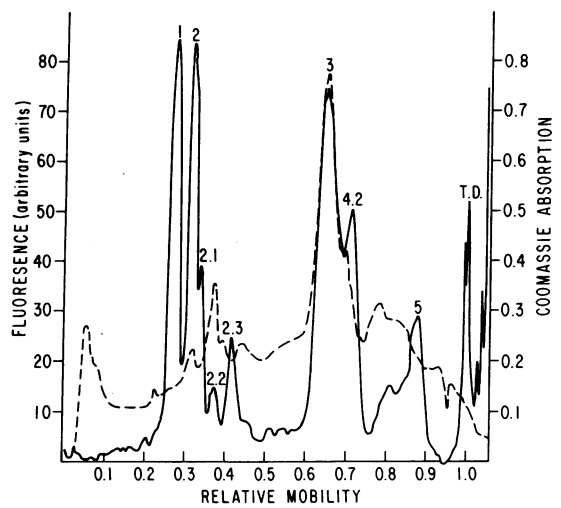


FIG. 3. Gel electrophoretic analysis of the proteins of mouse erythrocyte membranes after transglutaminase-catalyzed labeling of the membranes with dansyl cadaverine (*Materials and Methods*). The solid curve represents Coomassie blue staining of total protein, the dashed line the fluorescence in the gel due to the attached dansyl cadaverine groups. Electrophoresis was carried out in a 1% sodium dodecyl sulfate/3.25% polyacrylamide gel.

Table 1. Transglutaminase-catalyzed incorporation of amines into the major membrane proteins of the permeable mouse erythrocyte ghost upon incubation with 1 μM enzyme and 5 mM amine.

Membrane protein band	% Incorporation[a]		Specific activity[b]	
	Tyramine	Dansyl cadaverine	Tyramine	Dansyl cadaverine
Crosslinked protein[c]	30	10	—	—
1	2.9	0	1.3	0
2	3.5	3.5	1.8	1.6
2.1	2.0	3	—	—
2.2	2.0	10	—	5.0
2.3	3.0	0	—	0
3.1 3.2	35	44	5.0	3.0
4.1–4.2	2.5	10	1.0	2.5
4.4–4.5	5.0	16	—	—
5	0	0	0	0

[a] Calculated from the amount of label in each band divided by the total amount of label in all the protein bands.
[b] Specific activity is expressed as moles of label per mole of protein. It was determined from measurements of radioactivity (tyramine) or fluorescence (dansyl cadaverine) for the labels; and from measurements by densitometry of protein concentration in the Coomassie-stained bands.
[c] Protein found at top of the gel.

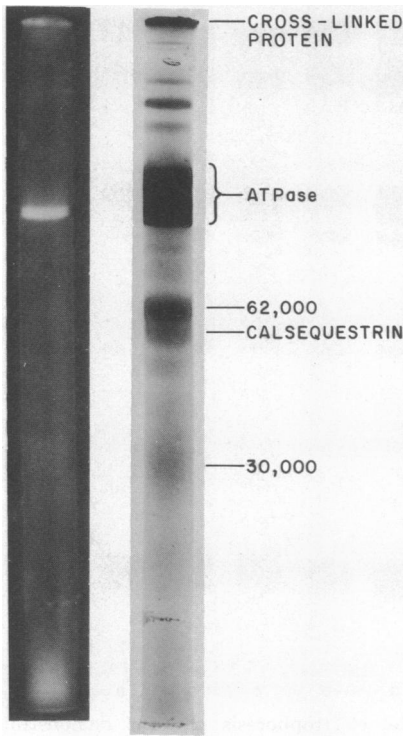


FIG. 4. Gel electrophoresis patterns of the proteins of sarcoplasmic reticulum membranes after the transglutaminase-catalyzed labeling of the membranes with dansyl cadaverine (*Materials and Methods*). The gel on the left was first photographed under ultraviolet light, and then was stained with Coomassie blue to give the pattern on the right. Electrophoresis was carried out in a 0.1% sodium dodecyl sulfate/5.6% polyacrylamide gel.

electrophoresis patterns with a mobility between bands 3.2 and 4.2 (molecular weight 85,000) characteristic of the free enzyme (Fig. 1).

Sarcoplasmic Reticulum: Crosslinking and Labeling Experiments. Under the conditions previously described, only a small amount of crosslinking of the proteins in sarcoplasmic reticulum membranes was produced by the transglutaminase. This was not examined systematically, however, since our primary interest was in covalently labeling the membrane protein. With 1 mM dansyl cadaverine and 10 μM transglutaminase, appreciable labeling was produced (Fig. 4). The major part of that label was attached to the noncrosslinked Ca^{++}-ATPase (55%), while most of the remainder was in crosslinked material at the top of the gel. Calsequestrin was not labeled to a significant extent. The Ca^{++}-ATPase had approximately 1 mole of dansyl group attached per mole of protein, and the label appeared to be concentrated in the front of the band (Fig. 4). Under these conditions, there was hardly any effect on the Ca^{++} transport (10) by the modified vesicles, as will be reported extensively elsewhere.

DISCUSSION

The primary purpose of this paper is to illustrate the usefulness of the pure enzyme liver transglutaminase in membrane studies; a detailed analysis of our results will therefore not be attempted here. In addition to the data obtained with mouse erythrocyte membranes, we have examined human erythrocytes with closely similar results to be reported elsewhere. Liver transglutaminase catalyzes either the crosslinking of the proteins of intact membranes, or if suitable amines are added, the covalent labeling of these proteins. While there are available at present a number of chemical methods for crosslinking membrane proteins, and several chemical and enzymatic methods for labeling them (for review, see ref. 11), the use of transglutaminase offers several demonstrated or potential advantages over many of these.

(i) The reaction conditions are very mild, and the total extent of modification is relatively small, so that little or no denaturation of the membrane proteins is expected. Thus, for example, the Ca^{++} transport rate of the Ca^{++}-ATPase in intact sarcoplasmic reticulum vesicles was not significantly changed by the attachment of a mole of dansyl cadaverine groups per mole of enzyme.

(ii) The specificity of the modifications is very high. In the first place, only protein-bound glutaminyl residues are the acceptor sites of the reactions involved. Thus, no modification of amino phospholipids or amino sugars of the membrane occurs, as can be the case with small molecule labeling or crosslinking reagents. Second, even among the proteins, the specificity of the reaction may be considerable. The extent to which a particular glutaminyl residue is labeled, for example, is known to depend upon the accessibility and the local environment of the residue on the protein surface, and upon the structure of the amine substrate (2). These specificity features of the labeling reactions are illustrated by the results in Fig. 3 and Table 1, with fragmented ghosts of mouse erythrocytes. The labeling of the membrane proteins at the cytoplasmic surface by dansyl cadaverine and tyramine is highly nonrandom. Band 3 is most highly labeled, and bands 1 and 2 (spectrin) are only slightly labeled, yet spectrin as a peripheral protein should be readily accessible to the enzyme. The lower the concentration of transglutaminase, the more highly specific is the labeling (Table 1); presumably, those glutaminyl residues that are most reactive to the enzyme are then preferentially labeled. Furthermore, the dis-

tribution of label is somewhat different for the two amine substrates.

Another important aspect of the specificity of transglutaminase-catalyzed reactions is their membrane sidedness. Most small molecule crosslinking and labeling agents in current use penetrate membranes. No dissociable small molecule intermediate is involved in the transglutaminase enzyme mechanism, as is the case with lactoperoxidase-iodine labeling (12). If a membrane is impermeable to the enzyme itself (molecular weight 85,000), it will be modified exclusively at that surface exposed to the enzyme. Thus, intact mouse (and human) erythrocytes were not labeled to an extent that was significant compared to the fragmented ghosts. However, labeling of minor membrane proteins exposed at the outer surface of the membrane of the intact erythrocyte (such as the Rh antigen of the human cell) might be detected if a radioactive amine of appropriately large specific activity was used. In a similar connection, the highly selective labeling of the Ca^{++}-ATPase in sarcoplasmic reticulum (Fig. 4) may reflect the labeling of only the proteins that are exposed at the outer surface of the vesicle, but this has to be further investigated.

Specificity is also a feature of the crosslinking reactions. It might be expected that only in those cases where a lysyl ϵ-NH_3^+ group and a glutaminyl amide residue are in proper stereochemical juxtaposition in the intact membrane will an intermolecular crosslink be formed, and that those few crosslinks that are formed would form at different rates. These factors can account for the very different extents of crosslinking observed with the different proteins of mouse erythrocyte membranes (Fig. 2). It is intriguing that with certain bands (bands 3 and 4) rapid crosslinking of about half of the protein occurs at a low enzyme concentration, but no further crosslinking is found if the enzyme concentration is increased. Since it is unlikely that a structural reorganization of the membrane is involved, these biphasic results suggest a heterogeneity of the proteins in each of these bands, with one or more proteins within the band for chemical or structural reasons being readily crosslinked, and one or more proteins not.

(iii) The diversity of the labeling reactions is considerable. In principle, any of a wide variety of unhindered primary amines can function as a substrate in exchange reactions catalyzed by transglutaminase, although the rates of the reactions vary considerably depending upon the amine (2). The importance of this variety of amine substrates is that many different kinds of labels, such as radioactive, fluorescent, or haptenic groups, or spin labels, can be covalently attached specifically to proteins of the membrane. In a previously reported study (3), [^{14}C]glycine ethyl ester was used as a substrate. In this paper, [^{3}H]tyramine and dansyl cadaverine have been used, and we have also successfully labeled erythrocyte ghosts with [^{14}C]glycine ethyl ester, [^{14}C]histamine, [^{14}C]noradrenaline, and [^{3}H]putrescine. A very highly radioactive labeling reagent could be made by radioiodinating tyramine. The fluorescent properties of dansyl cadaverine (13) should make it a useful probe of the dynamics of membrane proteins. We (E. D. Rees, A Dutton, and S. J. Singer, unpublished results) have recently transglutaminase-labeled sarcoplasmic reticulum membranes with 2,4-dinitrophenyl cadaverine, to which antibodies directed to the 2,4-dinitrophenyl hapten can be bound. Appropriate spin-label amines should be effective substrates as well.

Another significant feature of these labeling reactions is their extent. One or more moles of label can be covalently bound per mole of protein (Table 1) and in such cases, therefore, every molecule of a particular membrane protein is labeled. This is clearly important for biophysical and biochemical studies of the modified membranes. For reasons of specificity discussed above, it is likely that only a few, and possibly even only one, exposed glutaminyl resides on a given membrane protein become labeled.

In the course of labeling reactions with exogenously added amines, competing crosslinking reactions catalyzed by the enzyme may occur, particularly when poorer amine substrates are used. If such crosslinking were undesirable, they could probably be largely eliminated by first amidinating the ϵ-NH_2 groups of the lysyl residues of the membrane proteins with ethylacetimidate (14, 15) [which is unlikely to affect significantly the structural or biochemical properties of the membrane (15, 16)] and then carrying out the labeling reaction.

From these experiments and their discussion, it should be clear that transglutaminase-catalyzed crosslinking and labeling reactions of the proteins of intact membranes should prove to be highly useful for studying the molecular properties and functions of membranes.

We are indebted to Prof. Russell F. Doolittle for suggesting the use of transglutaminase-catalyzed reactions to us. Studies were supported by USPHS Grant GM-15971.

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