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COUPLING OF AMINES TO AND CROSS-LINKING OF ENDOGENOUS CYTOSOL OR MEMBRANE PROTEINS BY HEPATIC TRANSGLUTAMINASE

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Abstract—Endogenous protein acceptors for rabbit transglutaminase were demonstrated in the cytosol fraction of liver homogenates by the coupling of labeled compounds containing primary amino groups, such as putrescine, and by the apparent cross-linking of proteins, as indicated by a low mobility band observed in gel electrophoresis in 0.1% sodium dodecyl sulfate. When Ca^{2+} was omitted or when iodoacetamide was added to either of these reactions, no coupling or cross-linking occurred. Cross-linking of proteins of isolated rabbit liver plasma membranes could be demonstrated in the presence of purified rabbit liver transglutaminase, Ca^{2+}, and a reducing agent. A marked diminution of at least two peaks corresponding to proteins of high molecular weight (> 150,000 daltons) was observed in densitometric scans of gels of proteins from membranes or cytosol cross-linked by the enzyme. It is proposed that these proteins participated in the cross-linking reaction. Since cross-linking was inhibited only weakly by relatively high concentrations of various amines, it is probable that a physiological role of hepatic transglutaminase is the catalysis of $\epsilon(\gamma$-glutamyl) lysine bonds between cellular proteins, a process that might be controlled by the amount of intracellular Ca^{2+} available to the enzyme.

Transglutaminases are a family of enzymes which, in the presence of Ca^{2+}, catalyze the exchange of the amide group of protein glutaminyl residues for any of a wide variety of primary amines [1]. Numerous proteins are able to serve as acceptors in this coupling reaction [2, 3], and it appears that amine incorporation is probably more affected by conformation than by the sequence of amino acids [4].

Plasma transglutaminase, also called Factor XIII, is a proenzyme which requires a protease, such as thrombin, for activation [5]. The activated plasma enzyme catalyzes the cross-linking of fibrin monomers by forming the $\epsilon(\gamma$-glutamyl) lysine isopeptide bond [6], and thereby participates in blood coagulation by stabilizing fibrin clots. In addition to their use in the study of cross-linking sites of fibrin [7–9], transglutaminases have been used to couple amines [10, 11] and proteins [12] to membranes. However, the biological role of other transglutaminases, particularly those found in the cytosol fraction of homogenates of most organs [13], is still unknown although some speculations have been made. A specific transglutaminase identified in hair follicles [14] is apparently involved in the formation of the $\epsilon(\gamma$-glutamyl) lysine bonds, which have been shown to be present in hair [15]. It has been proposed that this same isopeptide bond probably occurs in the nonfollicular epidermis [16], where transglutaminase also has been found [17, 18].

Coupling of amines to cellular proteins or cross-linking of these proteins might be involved in the physiological role for intracellular transglutaminases. The purpose of this study was to identify possible endogenous substrates or products of these reactions and, more specifically, to determine evidence for the formation of intermolecular or fibrin-like cross-links of cellular proteins. To investigate this, a "homologous" enzyme–substrate system was used which consisted of the purified enzyme from rabbit liver and cytosol or plasma membrane fractions of the same source.

MATERIALS AND METHODS

Materials

[Carbonyl-^{14}C]isonicotinic acid hydrazide (isoniazid) and [^{14}C]tyramine (*p*-hydroxyphenyl[2-^{14}C]ethylamine HCl) were purchased from Amersham/Searle (Arlington Heights, IL); [glycine-1-^{14}C]glycine ethyl ester HCl and [1,4-^{14}C]putrescine · 2HCl were purchased from New England Nuclear (Boston, MA). Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine, and 2-mercaptoethanol were purchased from the Eastman Kodak Co. (Rochester, NY) and ammonium persulfate from the Fisher Scientific Co. (Pittsburgh, PA). Sodium dodecyl sulfate, iodoacetamide, rabbit muscle phosphorylase *a*, soybean trypsin inhibitor, horse heart cytochrome *c* (type VI), and GuHCL‡ were obtained from the Sigma Chemical Co. (St. Louis, MO); fatty acid-free bovine serum albumin from Pentex Laboratories (Kankanee,

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‡ Abbreviations used are: GuHCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetra-acetic acid; and EDTA, ethylenediamine tetra-acetic acid.

IL), Blue Dextran 2000 and Sephadex G-150 (superfine) from Pharmacia (Piscataway, NJ); and Bio-Gel A-50m (2% agarose) from Bio-Rad Laboratories (Richmond, CA). Molecular weight markers for SDS-PAGE (mol. wt range 53,000–265,000) were purchased from Gallard–Schlesinger (Carle-Place, NY). Purified egg ovalbumin was a gift from Dr. R. Hill of Duke University, NC. Other commonly used chemicals were reagent grade whenever possible.

Methods

Isolation of the enzyme. Rabbit transglutaminase was purified from liver cytosol fraction by precipitation with acetic acid at pH 5.0, $(NH_4)_2SO_4$ fractionation and by DEAE-Sephadex and Sephadex G-100 column chromatography. Enzyme activity was measured by hydroxylamine incorporation into *N*-carbobenzoxyglutaminylglycine [19]. The molecular weight of the enzyme was between 83,000 and 87,000 as estimated by gel filtration, SDS-PAGE and amino acid composition. The details of the purification procedure and other properties of the rabbit enzyme were described previously [20].

Preparation of cytosol. All procedures used for the isolation of subcellular fractions were carried out at 4°.

The liver of a New Zealand white rabbit (2.5 to 3.0 kg) was perfused free of blood *in situ* with ice-cold 0.25 M sucrose. A 20-g piece of the liver was homogenized in 60 ml of 0.25 M sucrose with a Potter–Elvehjem homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 150,000 *g* for 90 min; the pellet was discarded and the supernatant fraction was recentrifuged at 130,000 *g* for 60 min.

In order to obtain the cytosol fraction from lung homogenates, lungs were removed from rabbits, as described by Orton *et al.* [21], washed free of blood by perfusing the pulmonary circulation with 150 ml of 0.9% NaCl, and then the cytosol was prepared with 0.25 M sucrose as described above. Protein was measured by the biuret method [22]. Except where mentioned otherwise, fresh cytosol was used.

Preparation of liver plasma membranes. The perfused liver was cut into small pieces and 40 g was homogenized (eight slow strokes) in a 400-ml ice-cold solution of 1 mM sodium borate buffer, pH 7.5, with a Dounce glass-to-glass homogenizer. The homogenate was stirred gently for 20 min and then centrifuged at 750 *g* for 10 min. The pellet, consisting mostly of nuclei, was discarded and the supernatant fraction was recentrifuged. The subsequent steps of the isolation procedure, including the sucrose density gradient centrifugation, were done according to the method of Dorling and Le Page [23]. The purity of the membrane fractions was monitored by electron microscopy. Plasma membranes with preserved desmosomes were mostly nonvesiculated and only minor contamination by endoplasmic reticulum was seen.

Coupling of amines to cytosol proteins. The reaction between amines and cytosol was carried out in 0.1 M Tris–HCl, pH 8.0, containing 5 mM of the labeled amine (0.02 μCi/μmole), 20 mg of cytosol protein, and 10 mM $CaCl_2$ in a final volume of 1 ml. Following incubation at 37° for 30 min, 4 ml of 10% trichloroacetic acid (TCA) was added to each tube and the samples were centrifuged. The precipitate was washed four times with 4-ml volumes of 5% TCA and then

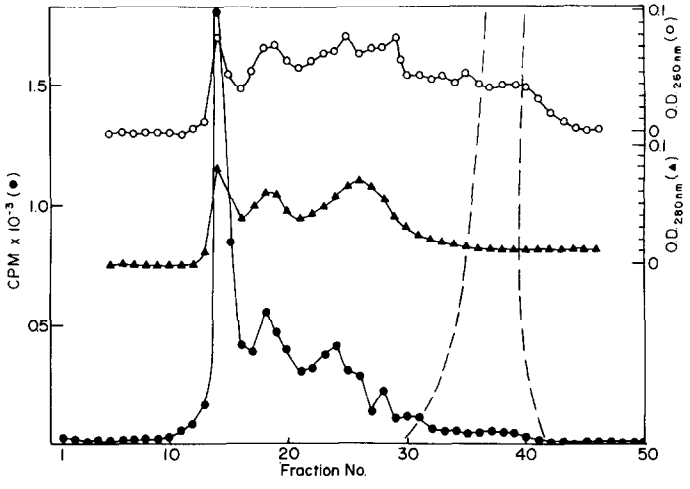

Fig. 1. Gel filtration of rabbit liver cytosol containing [^{14}C]putrescine-coupled protein. The coupling reaction consisted of 20 mg of liver cytosol, 10 mM $CaCl_2$, 5 mM putrescine · 2HCl (sp. act. 0.2 μCi/μmole; total radioactivity added was equivalent to approximately 1 μCi) in 0.1 M Tris–HCl, pH 8.0, in 1.0 ml. The solution was shaken at 37° for 1 hr and then 2 ml of chilled "stopping" solution containing 50 mM putrescine and 10 mM iodoacetamide in 0.1 M Tris–HCl, pH 7.5, was added to the reaction tube. The contents were transferred to dialysis tubing along with the rinses (3 × 1 ml) of the reaction tube made with the "stopping" solution. Dialysis was carried out at 5° against one liter of 0.05 M Tris–HCl, pH 7.5, containing 1 mM EDTA and 1 mM putrescine and changed hourly for 6 hr until negligible radioactivity was measured in the dialysis medium. A 5-ml aliquot of the putrescine-coupled cytosol complex was withdrawn and applied to a Sephadex G-150 column (2.5 × 90 cm) equilibrated with 0.1 M Tris–HCl, pH 7.5, using ascending flow (4 ml/hr). Each eluted fraction contained 12 ml. For the determination of radioactivity 1 ml was withdrawn from each fraction and mixed with 10 ml Aquasol and counted in a Packard Tri-Carb liquid scintillation spectrometer. The dashed line represents the radioactivity given by the separate application of 0.1 μCi [^{14}C]putrescine.

Table 1. Formation of [^{14}C]putrescine–protein adduct under various conditions

	Concn (mM)	Incorporation of [^{14}C]putrescine (cpm)	% Inhibition
Complete system *		5460	
No Ca^{2+}		80	99
Iodoacetamide	1	3588	34
	5	120	98
Spermine	1	4776	13
	5	3000	45
Isoniazid	1	5390	1
	5	4076	25
Hydroxylamine	1	4786	12
	5	2366	57

* Except where otherwise indicated, each sample contained 5 mM [^{14}C]putrescine (sp. act. 0.02 μCi/μmole), 10 mM CaCl, in 0.1 M Tris–HCl buffer, pH 8.0. Conditions are described in detail in Methods. Results are the means of three trials made in duplicate.

dissolved in 1.0 ml of 0.2 N NaOH. A 0.5-ml aliquot was removed and mixed with an equal volume of water in a counting vial, 10 ml Aquasol (New England Nuclear) was added and the vials were counted in a Packard Tri-Carb liquid scintillation spectrometer.

Cross-linking studies. Incubation for cross-linking studies was performed at 37° for 1 hr in a 0.2 to 1 ml reaction mixture containing 10 mM $CaCl_2$ and 0.1 M Tris–HCl, pH 7.5. Whenever purified rabbit liver transglutaminase was used, 5 mM cysteine was added as a reducing agent to the incubation mixture to insure enzyme stability. The concentration of protein in the reaction mixture was 10–15 mg/ml when unfractioned cytosol was used. After incubation and before electrophoresis, the cytosol samples were dialyzed (3 × 1 liter) at 4° for 4 hr with 0.05 M Tris–HCl containing 1 mM EGTA. In cross-linking studies using plasma membranes, the reaction mixture was centrifuged after incubation and the pellet washed with 0.1 M Tris–HCl, pH 7.5, before electrophoresis.

Gel electrophoresis. SDS-PAGE of the cytosol samples was performed as described by Weber and Osborn [24]. All the samples were soluble in 1% SDS-mercaptoethanol, as no pellets were obtained by centrifugation at 150,000 *g* for 2 hr. Usually 70–80 μg of cytosol protein was applied to each disc gel. For membrane proteins, the method of electrophoresis was modified according to that described by Skerrow and Matoltsy [25]. The protein bands in acrylamide gels were stained with Coomassie blue and then scanned at a wavelength of 550 nm using a Gilford 400 S spectrophotometer with a linear transport accessory.

Protein. Unless otherwise indicated, protein in sucrose-free solutions was determined by the method of Lowry *et al.* [26].

RESULTS

Coupling of amines to liver cytosol proteins

Using the coupling conditions described under Methods, each of the labeled compounds (0.1 μCi) with primary amino groups were incorporated into TCA-insoluble proteins when incubated with fresh liver cytosol in the presence of Ca^{2+}. Putrescine (5.5 × 10^3 cpm) and glycine ethyl ester (4.8 × 10^3 cpm) were incorporated to a greater extent than the aromatic amines isoniazid (2.4 × 10^3 cpm) and tyramine (1.4 × 10^3 cpm) at pH 8.0. The incorporation of putrescine was negligible in the absence of added Ca^{2+} or when 5 mM iodoacetamide was included in the reaction mixture, as shown in Table 1. Spermine, isoniazid and hydroxylamine, all compounds shown previously to be coupled to proteins or glutamine peptide derivatives with transglutaminase [1, 27, 28], also inhibited the incorporation of putrescine. The addition of a reducing agent, glutathione or cysteine, was not required and did not increase the coupling of amines when the cytosol fraction was used in the reactions. The rate of incorporation increased proportionally with the amount of cytosol protein and could be readily increased by the addition of purified hepatic transglutaminase. Amine incorporation into a constant amount of cytosol was directly proportional to the quantity of enzyme added. No amine incorporation could be demonstrated in the absence of cytosol.

Cesium chloride equilibrium density centrifugation of [^{14}C]putrescine-coupled cytosol (see the legend in Fig. 1) yielded no radioactivity isopycnic with regions of DNA or RNA (data not shown); radioactivity was associated with the protein band at the top or low density region ($\rho < 1.6$) of the gradient. Ninety-one to ninety-eight per cent of the radioactivity of [^{14}C]putrescine-labeled cytosol could be precipitated by the addition of 10% (w/v) trichloroacetic acid providing the sample was dialyzed previously against Tris buffer containing the unlabeled amine. The dialyzed [^{14}C]putrescine–cytosol sample applied to a column of Sephadex G-150 yielded radioactivity in all the eluted protein fractions with the major peak observed in the void (V_0) region (Fig. 1). Negligible radioactivity was observed in the elution volume of putrescine.

Cross-linking studies

When the liver cytosol fraction was incubated in the presence of Ca^{2+} and subsequently analyzed by SDS-PAGE, band C (Fig. 2) formed near the origin or at the cathode region of the gel. When Ca^{2+} was omitted from the reaction, the band was not observed. The intensity of band C was relatively high in comparison to other peptides and increased during an incubation period of 2 hr. Under the denaturing conditions of the SDS-PAGE system, incubation of protein in buffer containing SDS-mercaptoethanol results in cleavage of noncovalent and disulfide bonds of most proteins [29,

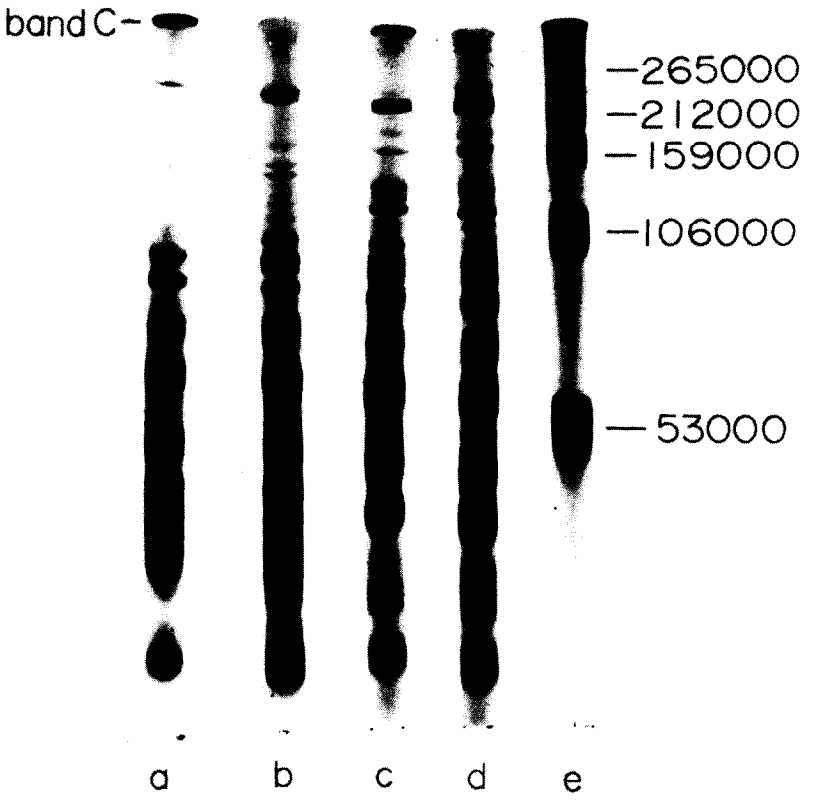

Fig. 2. SDS-polyacrylamide gel electrophoresis of rabbit liver and lung cytosol following the incubation in the presence of Ca^{2+}. Cytosol (10–15 mg protein/ml) was incubated in the presence of 10 mM $CaCl_2$ at 37° for 1 hr in 0.1 M Tris–HCl, pH 7.5, in 1.0 ml. SDS-PAGE of the samples was performed according to the method of Weber and Osborn [24]. Gels *a* and *c* represent liver and lung cytosol, respectively, when 5 mM iodoacetamide was added after the incubation. Band C is visible at the top of the gels. Electrophoresis of liver, *b*, and lung cytosol, *d*, is also shown following the inclusion of 5 mM iodoacetamide during the incubation. A molecular weight marker mixture (range 53,000–365,000 daltons) was used as a reference in gel *e*. Oligomers of the markers with molecular weight larger than 265,000 daltons account for the heavily stained upper portion of gel *e*.

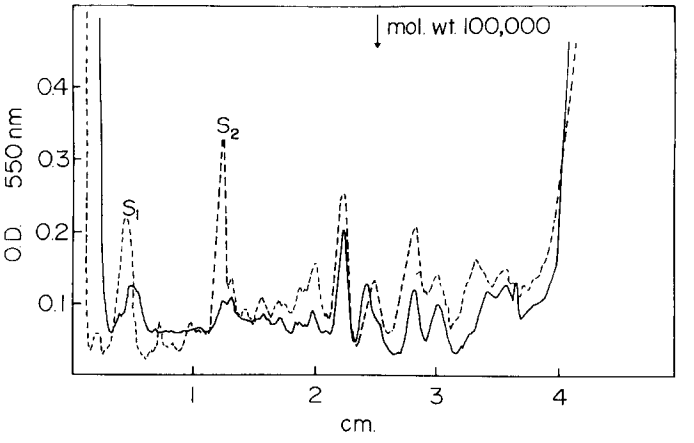

Fig. 3. Densitometric scanning profiles of SDS-polyacrylamide gels of liver cytosol following incubation in the presence and absence of Ca^{2+}. For the details of incubation and SDS-PAGE see Methods. The 9-cm gels, stained with Coomassie brilliant blue R-250, were scanned at 550 nm, as described in Methods. The interface between the gel and the solution in the chamber produced a narrow peak of high absorbance at the origin or cathode region. Band C (see Fig. 2) caused widening of this peak. Peaks S_1 and S_2 decreased markedly in height following the incubation in the presence of Ca^{2+} (solid line). The broken line represents cytosol incubated in the absence of Ca^{2+}.

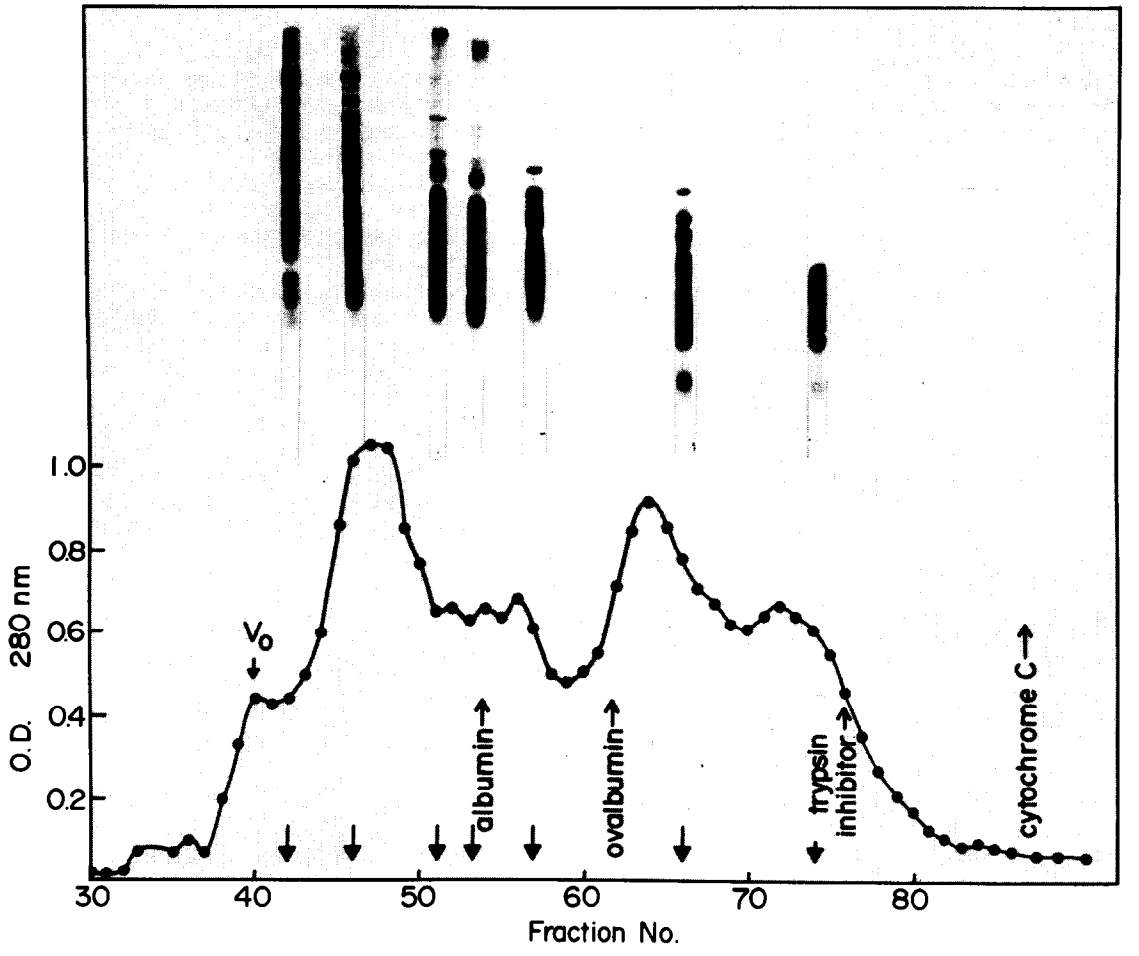

Fig. 4. Gel filtration of rabbit liver cytosol, and SDS-polyacrylamide gels of fractions following incubation in the presence of Ca^{2+}. Liver cytosol was fractioned by gel filtration on a Sephadex G-150 column (1.5 × 60 cm) in 0.05 M Tris–HCl, pH 7.5, buffer containing sodium azide 0.02% using descending flow. The flow rate was 3 ml/hr and the fraction volume 0.8 ml. Blue dextran 2000 was used as a marker for the void volume (V_0). Albumin (69,000 daltons), ovalbumin (43,000 daltons), soybean trypsin inhibitor (22,500 daltons) and cytochrome *c* (11,500 daltons) were used as standards for molecular weights. Selected fractions were adjusted to 0.5 mg protein in 1 ml of 0.1 M Tris–HCl, pH 7.5, and incubated in the presence of 10 mM Ca^{2+} at 37° for 1 hr; SDS-PAGE was performed as described in Methods. The fractions corresponding to the gels are marked with arrows. Band C is visible only in the gel of fraction 51.

30]. Peptides cross-linked by transglutaminase should reveal bands of lower mobility or greater molecular weight by SDS-PAGE, since $\epsilon(\gamma$-glutamyl) lysine bonds are not cleaved in this system. Therefore, band C represents the cross-linking of peptide chains previously separable by SDS-mercaptoethanol.

As shown in Fig. 2, band C was not formed when 5 mM iodoacetamide was included in the reaction mixture. When 5 mM glycine ethyl ester or putrescine was included in the reaction, the formation of band C was essentially abolished. After the incubation period, the addition of iodoacetamide or Ca^{2+} to those samples which did not contain these chemicals had no influence on the experimental electrophoretic profiles. Likewise, when samples previously incubated with Ca^{2+} were dialyzed against a solution of 0.1 mM EGTA, their electrophoretic profiles were not changed.

To ascertain that band C could be formed in a system free of fibrinogen, which has subunits of molecular weight 73,000, 60,000 and 53,000 daltons by SDS-PAGE [31], we examined the cross-linking of endogenous proteins of cytosol prepared from perfused lung, an organ apparently free of this precursor protein. Band C was clearly visible on SDS-PAGE of lung cytosol samples incubated with Ca^{2+} but was not intensely stained when compared to the band produced by liver cytosol (Fig. 2). This could be due to low transglutaminase activity since cytosol from this organ coupled [^{14}C]putrescine into its endogenous acceptors at a much lower rate than that observed with liver cytosol. The lung cytosol also showed relatively low activity in coupling hydroxylamine to *N*-carbobenzoxy-glutaminylglycine (unpublished observations).

In densitometric scans of the stained SDS-PAGE gels, only minor changes were seen in addition to the formation of band C (Fig. 3). The area of each of the two small peaks, peaks S_1 and S_2, with apparent molecular weights greater than 150,000 daltons, was reduced markedly in samples incubated with Ca^{2+}.

When different fractions of cytosol proteins,

obtained by gel filtration (Sephadex G-150) were incubated in the presence of Ca^{2+}, band C was formed only with those fractions which eluted slightly ahead of albumin (Fig. 4). This region in the elution profile corresponds to rabbit liver transglutaminase, which has a molecular weight of approximately 85,000 daltons, as shown in previous studies [20]. When these fractions were lyophilized, resuspended in 0.2 ml of 0.1 M Tris–HCl, pH 7.5, and then incubated in the presence of Ca^{2+} and cysteine, no band C material could be detected by SDS-PAGE. Freeze-drying is likely to destroy the activity of the rabbit enzyme (T. Abe and R. P. DiAugustine, unpublished observations). When purified rabbit liver transglutaminase (1–4 μM) was added to and incubated with the lyophilized fractions (2.5 mg protein/ml) previously shown to yield band C, the formation of the band was again evident by SDS-PAGE. Under these same catalytic conditions, purified rabbit liver transglutaminase *per se* did not form band C or any other new bands. Birckbichler *et al.* [32], however, reported that, in the absence of exogenous substrates, purified pig liver transglutaminase was converted to polymeric material.

When rabbit liver plasma membranes (0.15 mg protein/ml) were incubated with purified rabbit liver transglutaminase (1.8 μM) in the presence of Ca^{2+} and cysteine, there was significant formation of a high molecular weight peptide when analysis was made by SDS-PAGE (Fig. 5). We referred also to this peptide as band C.* The reaction with plasma membranes was both Ca^{2+} and cysteine dependent and inhibited by 5 mM iodoacetamide. In densitometric gel scans of peptides derived from membranes reacted with transglutaminase and co-factors, two peaks (M_1 and M_2), each greater than 150,000 daltons, were reduced significantly in height (Fig. 6). Washing the membrane pellet after the reaction with 0.1 M Tris buffer, as described in Methods, reduced the intensity of the band corresponding to transglutaminase without otherwise changing the SDS-PAGE profile.

DISCUSSION

In this study we were able to demonstrate that cell cytosol prepared from rabbit liver homogenate could, in the presence of Ca^{2+}, couple different amines to endogenous cytosol protein acceptors, and also cross-link protein(s) to form band C, a very large molecular weight complex (>250,000 daltons), as indicated by SDS-PAGE. Since the coupling of amines and the formation of band C required Ca^{2+} and could be inhibited completely by iodoacetamide (Fig. 2), it is likely that these reactions were catalyzed by transglutaminase [1, 19]. Further evidence for transglutaminase catalyzing the formation of band C was provided when fresh cytosol was fractionated by gel filtration; the capacity to form band C was essentially in those protein fractions eluting in the region of transglutaminase. The fact that band C in our cytosol experiments was stained less intensively when putrescine or glycine ethyl ester was present in the reaction mixture suggests that these amines were probably coupled to glutamine residues that otherwise would have participated in cross-links catalyzed by transglutaminase. This is in accord with the finding that the cross-linking of fibrin or fibrinogen by transglutaminase is inhibited by high concentrations of glycine ethyl ester [14].

In densitometric tracings (Fig. 3) of gels of cytosol samples, two proteins with high molecular weights (>150,000 daltons), observed as the peaks S_1 and S_2, decreased markedly in height after cell cytosol was incubated in the presence of Ca^{2+}. These two peaks could represent proteins which participated in the formation of band C. However, we cannot ascertain whether a homologous or heterogeneous polymer(s) was formed. The experiments of Mosher [33] support the idea that transglutaminase can catalyze heterogeneous polymers.

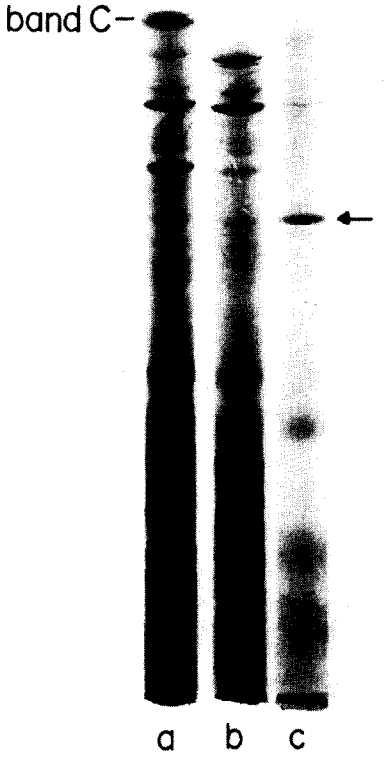

Fig. 5. SDS-polyacrylamide gel electrophoresis of rabbit liver plasma membranes following the incubation with purified rabbit liver transglutaminase. Liver plasma membranes (0.15 mg protein/ml) were incubated with 1.8 μM transglutaminase, 10 mM Ca^{2+} and 5 mM cysteine in 0.2 ml of 0.1 M Tris–HCl, pH 7.5, at 37° for 1 hr. After the incubation the membranes were washed in 0.1 M Tris–HCl, pH 7.5, as described in Methods. SDS-PAGE was performed by the method of Skerrow and Matoltsy [25]. Seventy μg protein was applied to each 5% acrylamide disc gel. Plasma membranes were incubated with the enzyme, Ca^{2+} and cysteine (a); 5 mM iodoacetamide was included in the reaction mixture (b); purified rabbit liver transglutaminase (about 85,000 daltons) is indicated by an arrow (c).

*The high molecular weight peptide formed from plasma membranes was labeled also as band C because it had essentially an identical mobility by SDS-PAGE with the band C peptide formed in cross-linking experiments with the cytosol fraction. Although a common nomenclature has been used on this basis, it should not be inferred that such band C peptides have an identical structure or originated from the same peptide subunits.

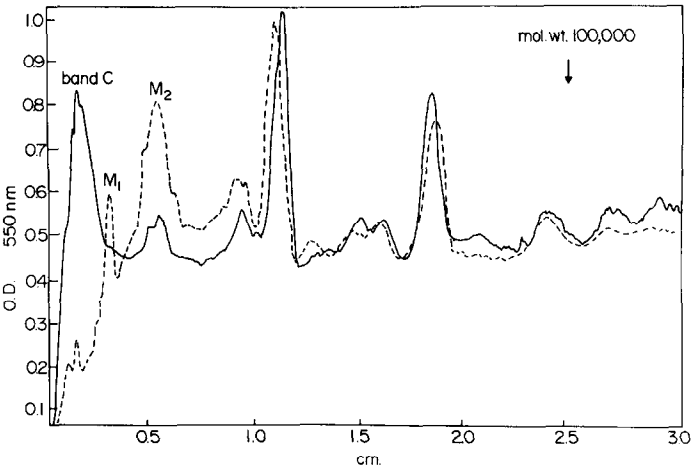

Fig. 6. Densitometric scanning of SDS-polyacrylamide gels of rabbit liver plasma membranes incubated with purified rabbit hepatic transglutaminase and cysteine in the presence and absence of Ca^{2+}. The gels stained with Coomassie brilliant blue R-250 were scanned at a wavelength of 550 nm, as described in Methods. The profile with a solid line represents the incubation in the presence of Ca^{2+}; the broken line represents the same reaction made in the absence of Ca^{2+}. A peak corresponding to band C is observed. Peaks M_1 and M_2 decreased in height following the incubation in the presence of Ca^{2+}.

Plasma membranes provided another system for examining the catalytic role of transglutaminase in the formation of band C. This was demonstrated by SDS-PAGE only when the complete system consisting of purified rabbit liver transglutaminase, cysteine and calcium were incubated together with membranes (Fig. 5). This strongly supports the idea that the formation of band C was catalyzed by transglutaminase. It was shown previously that labeling of amines and cross-linking of several proteins of erythrocyte ghost membranes [34, 35] or sarcoplasmic reticulum [34] can be catalyzed by hepatic transglutaminase.

Birckbichler *et al.* [36] were able to show the presence of $\epsilon(\gamma$-glutamyl) lysine bonds in mammalian cell membrane proteins. Accordingly, rabbit hepatic plasma membrane proteins corresponding to peaks M_1 and M_2, which in our densitometric data decreased in height when band C was formed (Fig. 6), might also contain these isodipeptide bonds and still be capable of forming additional intermolecular $\epsilon(\gamma$-glutamyl) lysine bonds to yield band C peptide. These cross-links could account for at least some of the very large polypeptides (>100,000 daltons) observed in membranes of various cells [37–39]. Although experiments demonstrating transglutaminase-catalyzed coupling of amines to membrane proteins were not made in the present study, other investigators have reported that large membrane proteins, approximately 150,000 daltons or greater, are preferential substrates in this reaction [40, 41].

The cross-linking of proteins in cytosol or membranes was only weakly inhibited by a relatively high concentration of amines. This might support the supposition that the "preferred" role of intracellular transglutaminase is similar to that of the plasma enzyme, i.e. cross-linking of proteins or peptides rather than coupling of small physiological amines, such as putrescine, to proteins. Investigations of the metabolism and disposition of putrescine and other polyamines in eucaryotes have not provided any evidence for their covalent incorporation into proteins [42, 43].

The concentration of Ca^{2+} in serum (10^{-3} M) is high enough to permit cross-linking of fibrin monomers. However, a low concentration of hepatic intracellular Ca^{2+} available to transglutaminase might preclude enzyme activity in this organ. The concentration of Ca^{2+} in the cell cytosol is maintained at 10^{-6}–10^{-7} M by complex homeostatic mechanisms associated with mitochondria and other membranal systems including the plasma membrane and the endoplasmic reticulum [44]. Ca^{2+} is known to have an important role as an intracellular messenger, and specialized responses evoked by specific stimuli are associated with movement of Ca^{2+} into the cell [44]. For instance, endocrine and exocrine secretion can be induced directly by increasing intracellular Ca^{2+} [45]. It is possible that such a rise or redistribution of intracellular Ca^{2+} could also activate transglutaminase to cross-link cellular proteins. This becomes more plausible in light of the recent findings of Siefring *et al.* [46] who showed that the accumulation of Ca^{2+} in intact human erythrocytes caused membrane protein polymers to form that were larger than spectrin and contained numerous $\epsilon(\gamma$-glutamyl) lysine cross-links.

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