MODIFICATION OF HUMAN ERYTHROCYTE GHOSTS WITH TRANSGLUTAMINASE

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Summary: Guinea pig liver transglutaminase was shown to catalyze the incorporation of dansylcadaverine and putrescine into two major protein fractions of human erythrocyte ghosts. As judged by sodium dodecylsulfate gel electrophoresis under reducing conditions, one of these is a high molecular weight polymer which may contain spectrin. The other corresponds to band 3, an 88,000 dalton polypeptide. Amine substrates of transglutaminase were synthesized with specific properties to further explore this useful enzymatic technique of covalently labelling proteins in erythrocyte ghosts and in other biological membranes.

There is a family of transamidating enzymes which catalyze nucleophilic displacement reactions specifically involving the γ-amides of some glutamine residues in proteins. From the physiological point of view, several of these enzymes are already known to be responsible for the selective post-translational cross-linking of native proteins by producing intermolecular γ-glutamyl-ε-lysine bridges. Cross-linking of fibrin during blood coagulation (1) or the formation of copulation plug in seminal fluid (2) are outstanding examples. Such enzymes, best designated as endo-γ-glutamine:ε-lysine transferases, have been isolated from sources as diverse as blood plasma (3), platelets (4), placenta (5), liver (6, 7), muscle (8, 9), red blood cells (9), hair follicle (10, 11), coagulating gland (12), sea urchin eggs (13). Though considerable differences of substrate specificities and catalytic properties are discernable within the group, they all share the ability of promoting the covalent incorporation of a variety of synthetic primary

amine derivatives (H_2NR) at neutrality into a number of proteins ($PCONH_2$) i.e., $PCONH_2 + H_2NR \longrightarrow PCONHR + NH_3$.

The enzymes display a high degree of specificity towards the R group of the synthetic amine and, interestingly, the best substrates among these -- exemplified by dansylcadaverine (14-16) -- comprise side chains analogous to ε-lysine residues in proteins. In biological systems in which cross-linking by such enzymes occurs, the presence of synthetic amines inhibits the formation of protein-to-protein bridges (17).

In general, transamidases might also be useful for modifying proteins in cell membranes. The present paper describes our preliminary results with isolated human erythrocyte ghosts using guinea pig liver transglutaminase, perhaps the least specific of this group of enzymes. ^{14}C-Putrescine and dansylcadaverine were selected as synthetic substrates for labelling. Previously, this technique has been used for the covalent modification of proteins of the hyaline layer of sea urchin embryos (13), mouse erythrocyte ghosts (18), rabbit sarcoplasmic reticulum (18) and fibroblast membrane (19).

MATERIALS AND METHODS

Human red blood cell ghosts were prepared from freshly outdated bank blood in 5 mM sodium phosphate buffer, pH 8, by the procedure of Fairbanks et al. (20) and used promptly.

Transglutaminase was isolated from guinea pig livers (7) and was stored as a frozen solution containing 25 mg protein per ml of 50 mM Tris chloride buffer of pH 7.5, containing 1 mM of EDTA.

^{14}C-Putrescine (60 mCi per mmole) was purchased from Amersham-Searle; dansylcadaverine (14) was a gift from AB Kabi, Stockholm. These compounds as well as dithiothreitol (Pierce Chemical Co.), calcium chloride and EDTA

were dissolved in 50 mM Tris chloride buffer of pH 7.5.

Reaction mixtures in cellulose nitrate tubes (Spinco No. 303369) typically comprised 100 µl of packed ghosts, 20 µl of transglutaminase, 10 µl of 0-5.6 mM ^{14}C-putrescine or of 0-5 mM dansylcadaverine, 10 µl of 0.2 M dithiothreitol and, finally, 20 µl of 40 mM calcium chloride. In controls, 50 µl of 1 mM EDTA was added in lieu of enzyme, dithiothreitol and calcium chloride. The reactions were allowed to proceed at 37^{o} for 3 hr at which time 1.5 ml cold 5 mM sodium phosphate buffer (pH 8) was admixed and the ghosts sedimented. The sedimented ghosts were resuspended in buffer and centrifuged in order to remove unreacted amines. The sediments were dissolved by adding 150 µl of water and 50 µl of a mixture of 5% sodium dodecylsulfate; 50% sucrose; 50 mM Tris chloride (pH 8), 5 mM EDTA and 200 mM of dithiothreitol (20). Aliquots of 50 µl were taken for electrophoresis on 5% polyacrylamide gels (100 mA per gel; 100 min) according to the protocol of Fairbanks et al. (20) as modified by Steck and Yu (21). Bands which incorporated dansylcadaverine could be readily visualized under a UV handlamp (17). Positions of fluorescence were marked with ink and the gels were stained for proteins by Coomassie blue R (20). The gels pertaining to the experiments with ^{14}C-putrescine were stained with Coomassie blue, photographed and scanned at 540 mµ (Beckman Spectrophotometer Model 24), sliced transversely and treated with hydrogen peroxide for the assay of radioactivity (22) in a Packard Tri-Carb liquid scintillation counter (Model 3385). Occasionally, aliquots of the detergent solutions of the ghosts treated with ^{14}C-putrescine and dansylcadaverine were mixed prior to electrophoresis so as to be able to obtain doubly labelled gel bands.

RESULTS AND DISCUSSION

Guinea pig liver transglutaminase is a calcium dependent enzyme (6). As

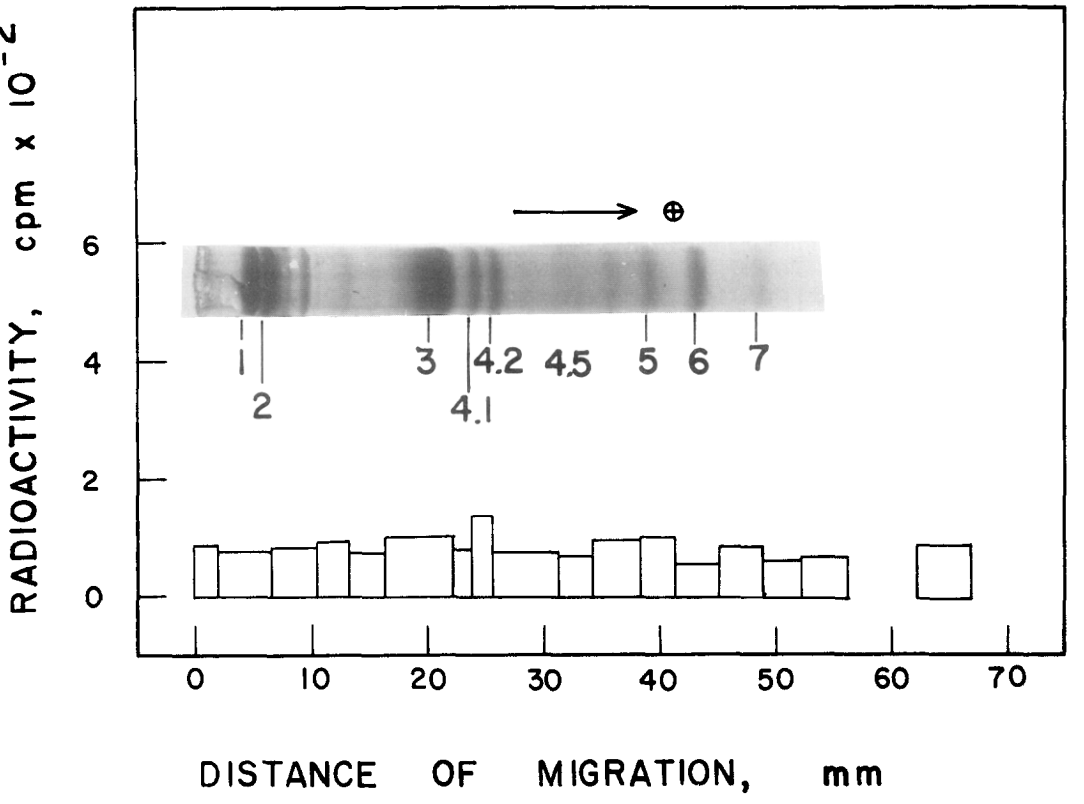

Fig. 1. Electrophoretic profiles of human erythrocyte ghosts after exposure to transglutaminase and ^{14}C-putrescine in the absence of calcium ions. Open bars denote radioactivity found in gel slices. The photographic insert shows the protein pattern after staining with Coomassie blue; numbering of bands corresponds to that given in the literature (23).

such, red cell ghosts treated with transglutaminase in the presence of an excess of EDTA showed an electrophoretic pattern (Fig. 1) indistinguishable from that of untreated ghosts (20). Similarly, treatment in EDTA with this enzyme plus dansylcadaverine or ^{14}C-putrescine produced no visible fluorescence in the gels and no appreciable incorporation of ^{14}C into any of the protein bands (Fig. 1). Thus, non-enzymatic uptake of these amine substrates (for example, by Schiff-base formation) does not occur to any appreciable extent with red cell membranes.

In the presence of calcium ions, transglutaminase brought about the in-

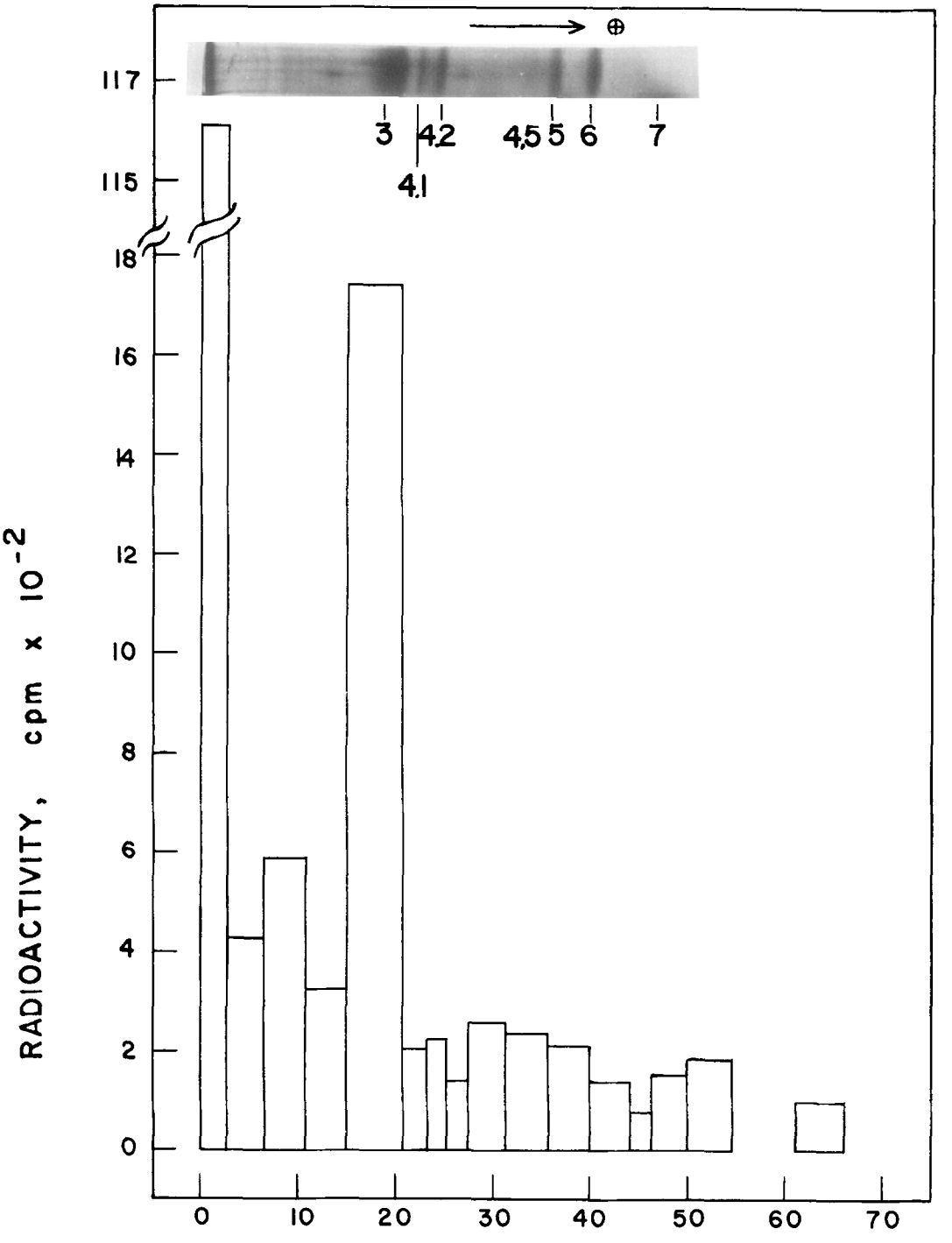

Fig. 2. Electrophoretic profiles of human erythrocyte ghosts after reaction with transglutaminase and ^{14}C-putrescine in the presence of calcium ions. Presentation as in Fig. 1.

corporation of dansylcadaverine and ^{14}C-putrescine into two major protein fractions. Intense labelling was found in material remaining at the top of the gel as seen by its fluorescence (not shown) and radioactivity (Fig. 2). Apparently transglutaminase caused both cross-linking and labelling of certain proteins. This situation is analogous to what happens when fibrin is enzymatically cross-linked in the presence of synthetic amines (17).

The high molecular weight material remaining on top of gels after transglutaminase treatment may contain spectrin (designated as bands 1 and 2 in Fig. 1), since these are missing from the electrophoretic profile. These polypeptides of about 240,000 and 215,000 daltons are thought to be associated in a dense reticulum at the cytoplasmic surface of the plasma membrane (23) and may be particularly susceptible to covalent cross-linking by a transamidase which catalyzes the formation of γ-glutamyl-ϵ-lysine crossbridges. It should be noted that bands 1 and 2 are readily cross-linked by a variety of reagents (23-25). Evidently, the concentrations of dansylcadaverine or ^{14}C-putrescine employed were not sufficient to inhibit this process appreciably at the monomeric state.

The second, less intensely labelled region on the gels corresponds to band 3, an 88,000 dalton polypeptide, believed to be involved in facilitated diffusion (26, 27). While this protein can be selectively disulfide cross-linked to form covalent dimers both in the membrane and in solution (24, 25, 28), no dimeric form was seen either in this system (Fig. 2) or in that of Dutton and Singer with mouse erythrocyte ghosts (18).

The selective modification of membrane proteins with transglutaminase in situ should provide a useful tool for labeling and cross-linking studies. In addition to the compounds containing a fluorescent reporter group [e.g. dansylcadaverine (29)] or an isotope label (e.g. ^{14}C-putrescine), we tested a number

of synthetic amines as transglutaminase substrates and, for obvious reasons, the following seem to be of special interest in membrane research: (a) nitroxide spin-labelled N-(5'-Aminopentyl)-2, 2, 5, 5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxamide; (b) di- and tri-iodinated compounds, such as N^{α}-Acetyl-3, 5-diiodo-L-tyrosine-N-(5'-aminopentyl)amide (or N^{α}-acetyl-3, 5-diiodotyrosyl-cadaverine); N-(5'-Aminopentyl)-5-acetamido-2, 4, 6-triiodo-N-methylisophthalam amide (or Iothalamoylcadaverine); N-(5'-Aminopentyl)-3-acetamido-2, 4, 6-triiodobenzamide and N-(5'-Aminopentyl)-2, 3, 5-triiodobenzamide (or 2, 3, 5-triiodobenzoylcadaverine); (c) amines with a nitrobenzene haptenic group against which antibodies are readily available: N-(5-Aminopentyl)-2, 4-dinitro-aniline (or 2, 4-dinitrophenylcadaverine); N^{ε}-Aminocaproyl-p-nitroanilide; N-(5-Aminopentyl)-2, 4-dinitrobenzenesulfonamide (or 2, 4-dinitrobenzene-sulfonylcadaverine). Details of methods of synthesis and evaluation (P. Stenberg et al., unpublished data) will be the subject of a separate paper.

Finally, the extreme susceptibility of the spectrin polypeptides in ghosts to transglutaminase merits attention. Since red cells are known to contain a transglutaminase, the question arises as to whether its function may somehow pertain to regulating the physical state of the spectrin skeleton, just as trans-amidation between fibrin molecules stiffens the clot structure (30, 31). There is some evidence that the spectrin reticulum on the cytoplasmic side provides the framework for the anchoring and positioning of transmembrane proteins and thus may actually influence the outside interactions of the cell (cf. 23). An enzyme system catalyzing the reversible cross-linking of spectrin molecules might have some physiological relevance. Since formation of γ-glutamyl-ε lysine bridges between spectrin molecules by amide exchange could proceed with little change in free energy, the possibility of transglutaminase playing such a dynamic role is worth considering.

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