KERATIN CROSS-LINKING AND EPIDERMAL TRANSGLUTAMINASE

A Review with Observations on the Histochemical and Immunohistochemical Localization of the Enzyme

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Enzymes known as transglutaminases mediate cross-linking of polypeptide chains by \( \epsilon-(\gamma\text{-glutamyl}) \) lysine bonds. Such bonds stabilize structural proteins of many tissues; transglutaminases specific for these tissues have been identified.

A calcium- and sulfhydryl-dependent transglutaminase with a molecular weight of 55,000 has been purified from bovine snout epidermis and used to elicit a specific antiserum to the enzyme. Sites of epidermal transglutaminase activity have been localized in the cytoplasm of upper malpighian and granular cells by two complementary methods. When thin-tissue sections were incubated with a fluorescent lysine analog (dansyl cadaverine) and calcium, tissue acceptor sites became fluorescent. Localization was confirmed by fluorescein-conjugated antibody labeling of the enzyme in situ.

These observations indicate that epidermal transglutaminase cross-links epidermal proteins during the final stages of keratinization.

Until the 1960s, the only known type of cross-linking between protein molecules was the disulfide bond. Since then, however, several other types of stabilizing bonds in structural biopolymers have been described. For example, collagen is cross-linked by aldehyde bonds, and the final step in elastin synthesis is the introduction of interpeptide desmosine bonds. Fibrin, the structural protein of wound healing, is highly cross-bonded also. In this case, the gamma amide of glutamine and the transforming monomeric fibrin into a tight lattice of high tensile strength. This last type of bond, the “gamma glutamyl bond,” and the enzymes responsible for its formation are the subject of the present discussion.

THE \( \epsilon-(\gamma\text{-GLUTAMYL}) \) LYSINE BOND IN KERATIN

In 1970, Asquith and associates [1] identified the \( \epsilon-(\gamma\text{-glutamyl}) \) lysine dipeptide in enzymic digests of wool keratin. This dipeptide is destroyed by acid hydrolysis but can be recovered intact when enzymes are used to digest proteins. In the same year, Harding and Rogers [2] confirmed these observations. Furthermore, they discovered that citrulline-rich proteins of the medulla and inner root sheath of wool follicles contained even more of these cross-links than cortical proteins [3,4]. Baden and Goldsmith [5] subsequently tested enzymic hydrolysates of epidermal stratum corneum for \( \gamma\text{-glutamyl bonds}. Using an indirect technique developed by Pisano et al [6], they cyanoethylated the epsilon amino groups of lysine residues in the epidermal proteins of cow snout. Lysines involved in covalent cross-links would escape cyanoethylation and would be detectable as free lysine after acid hydrolysis. About 8% of the lysine residues of proteins found in bovine stratum corneum failed to cyanoethylate; almost the same results were obtained with human stratum corneum. According to their interpretation, the unblocked lysines were involved in covalent bonds, probably \( \gamma\text{-glutamyl bonds}. These workers, however, have been unable to isolate the \( \epsilon-(\gamma\text{-glutamyl}) \) lysine dipeptide directly from stratum corneum [7].

TRANSGLUTAMINASES AND THE \( \gamma\text{-GLUTAMYL BOND} \)

Factor XIII (plasma transglutaminase) performs a singular role in coagulation. Circulating as a zymogen, it becomes activated by thrombin, which simultaneously cleaves terminal peptides from fibrinogen molecules. In the presence of calcium, the activated enzyme then catalyzes the formation of \( \gamma\text{-glutamyl bonds} between fibrin monomers. Persons deficient in Factor XIII exhibit a bleeding diathesis and may also have poor wound healing [8,9]. Women with the disorder cannot bear a living child without replacement therapy [8].

It is now known that a whole family of enzymes related to Factor XIII (see [10,11] for review) are transglutaminases; that is, they all cross-link fibrin and form \( \gamma\text{-glutamyl bonds} between lysine or

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lysine substitutes (e.g., cadaverine, putrescine, selected amine esters) and peptide-bound glutamine. Like Factor XIII, they are calcium dependent and require free sulfhydryl groups for activity. However, they differ biochemically and immunologically from the enzyme which was first described (Tab.). Some have been found only in specific tissues, others are more widely distributed.

In 1971, soon after γ-glutamyl bonds had been identified in hair proteins, Harding and Rogers [12] detected transglutaminase activity in wool root homogenates. They used [14C]lysine and casein as substrates, in addition to routine fibrin cross-linking assays. Later in the same year, Chung and Folk [13] substantially purified hair follicle transglutaminase from guinea-pig follicles and established its distinctiveness from the well-known liver (tissue) transglutaminase. The new enzyme had a much lower molecular weight than Factor XIII and did not appear to have a zymogen form in tissue.

**EPIDERMAL TRANSGLUTAMINASE**

Since transglutaminase activity was so easy to detect in follicular tissue, we questioned whether such activity could be found in nonfollicular epidermis as well. Selecting the hairless epidermis of the bovine snout with its wide keratinizing zone as a model, we planned a study of transglutaminases in epidermal keratinization. The first phase of this plan—the isolation, purification, and characterization of bovine epidermal transglutaminase (ET)—has been completed. After briefly summarizing this information, we will describe recent biochemical findings with the enzyme, its antibody, and its substrate.

Bovine ET is water soluble. It passes easily from tissue into buffered aqueous solutions, where it can be detected with standard fibrin cross-linking [14] or dansyl cadaverine–casein [15] assays. After partial purification by ion exchange chromatography and Pevikon electrophoresis, the enzyme was contaminated by only two other proteins. Since these were of significantly different molecular weights, they could be separated from the enzyme by Sephadex G-200 gel filtration (Fig. 1). When compared with standard proteins filtered through the same gel apparatus, the enzyme emerged at a volume corresponding to a molecular weight of 55,000. Sedimentation velocity of the protein was 4.45s, characteristic of a protein of 50,000 to 60,000 molecular weight. After sedimentation equilibrium analysis of the purified substance, the final molecular weight was determined to be 55,800 ± 200. In SDS gel electrophoresis, the enzyme migrated as a single band. A graph of its relative mobility compared with standard proteins is shown in Figure 2. The apparently higher molecular weight in SDS is thought to represent anomalous behavior of the enzyme in the denaturing media.

ET is calcium dependent and inhibited by chelating agents such as EDTA (ethylenediamine tetraacetate) and EGTA (ethylene glycol bis[β-amino ethyl ether] N,N′-tetraacetic acid). It is also inactivated by the sulfhydryl blocking reagents iodoacetamide and p-chloromercuribenzoate. The enzyme operates best at alkaline pH (8.8) and has a K_m of 2 x 10^{-4} for the dansyl cadaverine substrate. It is stable at 4°C for several weeks in the purified state.

Other authors have worked with ET from several species and, in general, their findings are similar to ours [16]. Baden and Goldsmith have identified transglutaminase activity in homogenates of turtle, frog, rat, cow, and human epidermis. We have also studied newborn rat epidermis and human epidermis and find that ET from these tissues

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**Table. The transglutaminases**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Composition</th>
<th>Molecular weight</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Factor XIII</td>
<td>Plasma</td>
<td>Subunits: a,b, Zymogen form (thrombin-activated)</td>
<td>a: 81,000 ± 4000, b: 81,000 ± 4000</td>
<td>Fibrin</td>
</tr>
<tr>
<td>Platelet Factor XIII</td>
<td>Megakaryocytes, platelets, placenta</td>
<td>Subunits: a, Zymogen form (thrombin-activated)</td>
<td>81,000 ± 4000</td>
<td>Fibrin</td>
</tr>
<tr>
<td>Tissue (liver) transglutaminase</td>
<td>Muscle, kidney, liver</td>
<td>No subunits</td>
<td>76,000-90,000</td>
<td>N.D.*</td>
</tr>
<tr>
<td>Vesiculase</td>
<td>Anterior prostate gland (guinea pig)</td>
<td>No subunits</td>
<td>N.D.</td>
<td>Basic coagulable protein from seminal vesicle</td>
</tr>
<tr>
<td>Hair follicle transglutaminase</td>
<td>Hair follicle</td>
<td>No subunits</td>
<td>54,000-55,000</td>
<td>Citrulline-rich proteins of medulla, inner root sheath, possibly cortical protein</td>
</tr>
<tr>
<td>Epidermal transglutaminase</td>
<td>Epidermis</td>
<td>No subunits</td>
<td>55,800 ± 200</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* N.D. = not determined.
the epidermal enzyme but failed to inhibit Factor XIII. The antiserum did not, however, block the incorporation of dansyl cadaverine into casein; on the contrary, that reaction was enhanced. Such enhancement of activity by antibodies has been described for other enzymes, particularly when small substrates are involved in the assay [17]. Incorporation of dansyl cadaverine into casein by Factor XIII was not enhanced by anti-ET.

LOCALIZATION OF EPIDERMAL TRANSGLUTAMINASE IN SKIN

Direct histochemical stains, in which a labeled substrate is applied to a fresh or fixed tissue slice, have been used for many years to identify and localize tissue-bound enzymes. We have developed such a method for ET, using a fluorescent dansyl cadaverine substrate similar to that used in in vitro experiments.

A solution of dansyl cadaverine in Tris-buffered saline, pH 7.5, containing 10 mM CaCl₂ was directly applied to fresh-frozen 5-μ sections of cow snout and allowed to react with the tissue for 30 min. Free dansyl cadaverine was then removed by 2 washes with Tris-buffered saline over a 60-min period. Control sections were incubated in calcium and buffer constituents only. The resulting sections were mounted in 1:10 buffered glycerol and viewed under a Zeiss ultraviolet microscope with a BG-3 exciter and 500-nm barrier filter. Since the fluorescent techniques applied here are qualitative, only clear-cut positive and negative findings were recorded. Specificity of the fluorescent staining was determined by studying the development of fluorescence with and without

![Fig. 1. Step III, purification of bovine epidermal transglutaminase. Enzyme activity is symmetric about a peak of protein eluting at a molecular weight of 55,000 on Sephadex G-200.](Image)

![Fig. 2. Sodium dodecyl sulfate acrylamide gel electrophoresis of purified bovine epidermal transglutaminase. The relative mobility of the enzyme is plotted against the mobility of standard proteins in the same medium. Chromatographically behaves much like the bovine enzyme (unpublished observations).](Image)

IMMUNOLOGIC STUDIES WITH EPIDERMAL TRANSGLUTAMINASE

Purified ET was immunogenic: specific antisera were easily elicited in goats. These antisera yielded a line of identity when diffused in agar against crude or highly purified preparations of ET. By contrast (Fig. 3), no precipitin line was seen when anti-ET was diffused against either bovine or human Factor XIII, an indication that these two enzymes are immunologically as well as biochemically distinct. ET did not precipitate in agar with either rabbit antiserum to whole bovine serum proteins, rabbit antiser to the subunit a or b chains of human Factor XIII, or goat antihuman Factor XIII prepared in our laboratories from highly purified human Factor XIII.

In vitro experiments with anti-ET confirmed that the antiserum inhibited fibrin cross-linking by

![Fig. 3. Ouchterlony diffusion pattern of epidermal transglutaminase, Factor XIII, and their antisera. Well I contains anti-Factor XIII; well 2 contains anti-transglutaminase. Wells A and B contain Factor XIII of bovine and human origin respectively; epidermal transglutaminase is present in C and D.](Image)
calcium and such chelating agents as EDTA or EGTA and PCMB (parachloromercuribenzoate), a sulphydryl blocker. In addition, other dansylated amino acids such as dansylglutamine, which were determined beforehand to lack substrate properties, were substituted for the dansyl cadaverine moiety in the reaction. Finally, sections were preincubated in buffer solution for 30 min before application of the dansyl cadaverine substrate to see whether any components of the reaction could be solubilized (e.g., the enzyme, which is known to be highly water soluble) and thus prevent the development of fluorescence.

Direct application of dansyl cadaverine substrate to tissue slices resulted in the appearance of brilliant fluorescence over the malpighian and granular layers. The fluorescence was patchy and cytoplasmic with a granular pattern over the mid-malpighian layer but increased in intensity and extent until virtually the entire cytoplasm of every cell was filled in the granular layers. Minimal fluorescence was present in the lower layers. Some membrane fluorescence was also seen in the lower and mid-malpighian layers; the significance of this finding has yet to be determined. Figure 4A demonstrates the type of fluorescence seen in upper malpighian and granular layers with dansyl cadaverine staining. Figure 4B is a high-power view of the junction between the granular and horny layers, which demonstrates the absence of fluorescence in horny layer.

When calcium was omitted from the dansyl cadaverine substrate, very little fluorescence developed. No fluorescence was seen when the EDTA or EGTA concentration exceeded the calcium concentration. The addition of 2 mM PCMB to the dansyl cadaverine substrate solution inhibited the development of fluorescence completely, as did the substitution of dansyl glutamine for dansyl cadaverine in the same molar concentrations.

Tissue slices preincubated in buffer before the application of dansyl cadaverine substrate did not develop fluorescence. By contrast, postincubation after dansyl cadaverine staining for periods up to 24 hr did not change the original fluorescence developed.

Thus we have demonstrated that direct applica-

![Fig. 4. A: The pattern of dansyl cadaverine incorporation into tissue slices demonstrates increased intensity and extent of fluorescence as the granular layer is approached. B: High-power view of junction between granular and horny layers, same section as A.](image)

![Fig. 5. A: Immunofluorescent staining of snout epidermis with fluorescein-linked goat antiserum against epidermal transglutaminase. B: Control section of cow snout epidermis stained with goat antiserum against Factor XIII.](image)
tion of a fluorescent transglutaminase substrate to thin-tissue sections results in an intense fluorescence over upper epidermal cell layers. The fluorescence is specific for substrates of the enzyme and is absent if inhibitors of the enzyme are added to the substrate solution or if calcium is eliminated. It is also absent in tissues preincubated in buffer solutions or in tissues incubated with fluorescent dansylated amino acids known not to act as substrates for the enzyme.

Since this histochemical assay requires both ET and an acceptor molecule (peptide-bound glutamine), it remained to be determined whether studies by another method, e.g., fluorescein-linked antiserum to the ET, would confirm the dansyl cadaverine studies.

**ANTIBODY LOCALIZATION OF EPIDERMAL TRANSGLUTAMINASE**

Antiserum to ET was linked to fluorescein by standard procedures [18]. The labeled antiserum had a fluorescein:protein ratio of 2.5 and formed a single fluorescent precipitin line when diffused in agar against purified ET.

For comparison, preimmunization goat serum and antiserum to whole Factor XIII were similarly linked to fluorescein. When diffused in agar, the latter gave a fluorescent precipitin line against highly purified Factor XIII; preimmunization serum gave no precipitin line with either ET or Factor XIII.

As an additional test of the specificity of the antiserum, aliquots of anti-ET were incubated at 4°C for 60 min with preparations of partially purified ET in order to absorb fluorescent anti-ET activity from the antiserum and thus abolish tissue fluorescence. The amount of ET to be added was estimated from Ouchterlony diffusion experiments in which dilutions of ET were diffused against anti-ET. Controls for this experiment contained buffer rather than ET as diluent.

Fresh epidermal tissue from the cow snout was snap-frozen in liquid nitrogen and sectioned immediately into 4 to 5-μ sections. These were transported directly from the cryostat to a freeze-dry apparatus which maintained the tissue at -80°C for 3 days under a vacuum while water was slowly withdrawn from the tissue. Freeze-dried sections were then stored at -20°C in vacuum tubes until used.

Sections to be stained were transferred to a dry chamber saturated with paraformaldehyde vapor. Vapor fixation was allowed to proceed for 60 min. Each section was then individually laid on a drop of serum for 30 min and subsequently washed with several changes of Tris-buffered saline for 60 min. After this, the sections were mounted in buffered glycerol and viewed under a Zeiss ultraviolet photomicroscope.

The pattern of fluorescence in the experimental sections incubated with anti-ET was similar to that seen in staining with dansyl cadaverine substrate. Patchy fine granular fluorescence was first seen in the mid-malpighian layer and increased in intensity and distribution in the upper malpighian and granular layers to involve the entire cytoplasm (Fig. 5A). No nuclear or membrane staining was seen, and basal and immediate suprabasal cells did not stain. No subcellular concentration of the fluorescence to the cell membrane or to areas of keratohyaline granules was apparent. Sections stained with preimmunization serum or with anti-XIII (Fig. 5B) were uniformly negative. Sections stained with anti-ET which had been absorbed with ET showed minimal or no fluorescence.

**DISCUSSION**

Epidermal transglutaminase has been purified from cow snout epidermis, characterized, and compared with Factor XIII, a closely related plasma enzyme which may contaminate homogenates of epidermis. The enzyme is similar to a cross-linking enzyme isolated from guinea-pig hair follicle homogenates by other authors. Hair follicle transglutaminase (HFT) and epidermal transglutaminases share several properties. They have similar molecular weights (ET, 55,800; HFT, 55,000) and show comparable mobility in several media (Sephadex A50, Pevikon) (unpublished experiments). Neither has a zymogen form in tissue. However, we have no evidence yet that ET, unlike HFT, has subunits. Results obtained with HFT indicate that the molecule may be composed of two subunits, each weighing 27,000 daltons [13].

What can we say about the specific epidermal substrate for ET? The enzyme is localized to areas of the epidermis where keratinization, rather than division and differentiation, is taking place. Evidence for this is found in both histochemical studies with labeled donor substrate, which identify the acceptor substrate (but only in areas where the enzyme is also present), and in direct antibody labeling of the enzyme. The immunochemical and histochemical methods confirm each other and each test withstands rigorous specificity controls.

From our knowledge of other transglutaminases and their substrates, we can briefly speculate about the epidermal substrate. First, the transglutaminase substrates so far identified have a structural role (vaginal plug, fibrin clot, inner root sheath proteins) and are synthesized as soluble monomers and compartmentalized from the cross-linking enzyme until needed. During coagulation, a double control exists; both the enzyme (a zymogen) and fibrinogen must undergo limited proteolysis by thrombin before propagated cross-linking supervenes. In the case of vesiculase, the guinea-pig prostatic enzyme, enzyme and substrate are synthesized in separate organs and come together only when cross-linking is scheduled to occur.

Another general property of transglutaminase substrates seems to be that they function for a limited time. The fibrin clot acts as a seal and grillwork for the ingrowth of other tissue components and is then solubilized by plasmin. The vaginal plug of the guinea pig also seals the vagina.
and facilitates insemination before it disintegrates. So, too, an unknown mechanism causes the inner root sheath of the hair to disappear in the infundibulum of the follicle after its presumed function of shaping the developing hair shaft is completed.

Epidermal keratin appears to fall into the same category. After the long, complicated process of manufacturing this tough resistant substance, it is simply sloughed into the environment.

With the tools now available, we should be able to identify the natural substrates of the epidermal and hair follicle transglutaminases. One of them, a specific fibril of the inner root sheath, has already been isolated [19].

As we continue to learn more about the biochemical and physiologic role of the transglutaminases and their substrates in epidermis and its appendages, we should be able to apply this information to the study of human diseases of keratinization and hair fiber formation.

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REFERENCES