The Journal of Biological Chemistry
Vol. 275, No. 4, Issue of January 28, pp. 2636–2646, 2000
Printed in U.S.A.

Cholesterol 3-Sulfate Interferes with Cornified Envelope Assembly by Diverting Transglutaminase 1 Activity from the Formation of Cross-links and Esters to the Hydrolysis of Glutamine*

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The loss of transglutaminase 1 enzyme (TGase 1) activity causes lamellar ichthyosis. Recessive X-linked ichthyosis (XI) results from accumulation of excess cholesterol 3-sulfate (CSO4) in the epidermis, but the pathomechanism how elevated epidermal CSO4 causes ichthyosis is largely unknown. Here we provide evidence that XI is also a consequence of TGase 1 dysfunction. TGase 1 is a key component of barrier formation in keratinocytes: it participates in the cross-linking of cell envelope (CE) structural proteins, and also forms the lipid bound envelope by esterification of long chain ω-hydroxyceramides onto CE proteins. Using involucrin and an epidermal ω-hydroxyceramide analog as substrates, kinetic analyses revealed that at membrane concentrations above 4 mol %, CSO4 caused a marked and dose-dependent inhibitory effect on isopeptide and ester bond formation. Sequencing of tryptic peptides from TGase 1-reacted involucrin showed a large increase in deamidation of substrate glutamines. We hypothesize that supraphysiological levels of CSO4 in keratinocyte membranes distort the structure of TGase 1 and facilitate the access of water into its active site causing hydrolysis of substrate glutamine residues. Our findings provide further evidence for the pivotal role of the TGase 1 enzyme in CE formation.

Assembly of an effective epidermal barrier structure is an essential adaptation to terrestrial life. In mammals the outermost bulwark of this barrier is the cornified layer of the epidermis, composed of flattened corneocytes mortared together by orderly lipid laminae. During terminal differentiation, individual corneocytes acquire a specialized cell peripheral structure termed the cornified cell envelope (CE),1 which is responsible for maintenance of mechanical and chemical protection and indirectly contributes to water permeability barrier (see Refs. 1 and 2, for reviews). The CE is composed of two parts. The ~10 nm thick protein envelope is formed by covalent cross-linking of several structural proteins by sulfhydril oxidases and transglutaminases (TGases). This highly insoluble protein meshwork is coated by the lipid envelope, a ~5 nm thick layer of ω-hydroxyceramides with uniquely long (C26–C36) fatty acyl moieties (3). These are covalently attached by ester bonds through their ω-hydroxyl group to selected glutamines to envoplakin, periplakin, and involucrin components of the protein envelope (4).

Terminal differentiation of keratinocytes is accompanied by vigorous lipid metabolism and synthesis of keratinization-specific lipids in the granular layer. Newly synthesized lipids are temporarily stored in cytoplasmic lamellar bodies, in which they are arranged as stacks of tetralaminar sheets. The lamellar body lipids consist largely of free fatty acids, (glucosyl)ceramides, cholesterol, and its acyl or sulfate esters (3). In the uppermost granular layer the lamellar bodies fuse with the cell membrane, and release their contents which assume broad, multilamellar lipid sheets between corneocytes. This process approximately coincides with the initiation of assembly of both the protein envelope and lipid envelope of the CE (5). It is thought that the ester-linked long chain ω-hydroxyceramides comprising the lipid bound envelope interdigitate with the interstitial lipid layers and might function in a Velcro-like fashion by fixing the protein envelope to surrounding lipid structures, and vice versa. In this way, the lipid bound envelope contributes to the maintenance of an orderly array of lipid layers during normal wear and tear and mechanical stress of the epidermis.

Genetic errors of CE and skin barrier formation can manifest as ichthyosiform symptoms. Some of these diseases have been distinguished on the basis of abnormal metabolism of stratum corneum lipids (6, 7). Some congenital ichthyoses reveal abnormal deposition of apolar or polar lipids or cholesterol in the intercorneocyte lipid layers, and thereby appear to disrupt the normal lipid layerings and composition required for effective epidermal barrier function (8). These include recessive X-linked ichthyosis (XI) which is caused by an accumulation of excess cholesterol 3-sulfate (CSO4) owing to arylsulfatase C/cholesterol sulfatase enzyme defects (9).

CSO4 is a ubiquitous cholesterol metabolite, the amount of which is determined by the relative activity of cholesterol sulfotransferase and cholesterol sulfatase enzymes (10). CSO4 gradually accumulates during epidermal keratinocyte differentiation, peaking normally at levels of 4–5% of total lipids in the upper stratum granulosum and it is hydrolyzed in the cornified layer, so that normal corneocyte scales contain less than 1% CSO4 of total lipids (11, 12). In XI the lack of its breakdown results in an elevated CSO4 content in the basal and spinous layers, peaking at >10% (by weight) of total lipids in the stratum corneum (13).

However, it is not yet clear how excessive epidermal CSO4 diminishes barrier function in the epidermis, and whether the mild increase of epidermal (water) permeability alone is sufficient to account for the severe symptoms of the disease. Several...
published reports have addressed the alterations of physical properties of corneocyte lipids from excess CSO4 (14–16). It has been shown that CSO4 can cause phase separation of cholesterol-fatty acid layers (14) and that the XI phenotype can be ameliorated by topical cholesterol treatment (17). Thus it was suggested that XI arises due to a defect of intercorneocyte lipid layer formation. In a conceptually related argument, CSO4 was shown to interfere with spontaneous sheet formation of epidermal lipids in vivo, perhaps due to the strong charge of its sulfate moiety conferring detergent properties to CSO4. Thus it was theorized that CSO4 affects epidermal barrier function both by deranging skin lipid layers and by replacing cholesterol in the lipid sheets (16). In another study, it was proposed that since CSO4 has trypsin and chymotrypsin inhibitory properties in vitro, it might thereby affect breakdown of desmosomes, thus causing retention hyperkeratosis and abnormal scaling (18).

Finally, more recently, it was demonstrated that CSO4 can induce TGase 1 expression in cultured keratinocytes (19), but the connection between excess TGase expression and disease etiology remains unclear. CSO4 in keratinocyte membranes was shown to activate the protein kinase C isoforms ε, ζ, and η, presumably by direct allosteric effects on their tertiary structures. As these membrane-bound enzymes are involved in the signaling pathways of keratinocyte differentiation (20, 21), the connection between excess TGase expression and disease etiology remains unclear. CSO4 can induce TGase 1 expression in cultured keratinocytes (19), but the connection between excess TGase expression and disease etiology remains unclear. CSO4 in keratinocyte membranes was shown to activate the protein kinase C isoforms ε, ζ, and η, presumably by direct allosteric effects on their tertiary structures. As these membrane-bound enzymes are involved in the signaling pathways of keratinocyte differentiation (20, 21), the connection between excess TGase expression and disease etiology remains unclear. CSO4 can induce TGase 1 expression in cultured keratinocytes (19), but the connection between excess TGase expression and disease etiology remains unclear. CSO4 in keratinocyte membranes was shown to activate the protein kinase C isoforms ε, ζ, and η, presumably by direct allosteric effects on their tertiary structures. As these membrane-bound enzymes are involved in the signaling pathways of keratinocyte differentiation (20, 21), CSO4 could induce TGase 1 expression in this way (19).

Mammalian TGases (glutamyl-amine aminotransferases, EC 2.3.2.13) constitute an evolutionarily related family of Ca2+-dependent enzymes (22). The catalytic mechanism of TGases involves the release of ammonia from the reactive glutamine by an ester bond (27). Lastly, water can also enter the active site of TGases to attack the acyl-enzyme intermediate, which leads to a net deamidation of a reactive glutamine to a glutamic acid residue (28, 29).

Seven members of the TGase family have been identified in the human genome so far, of which four (TGases 1, 2, 3, and X) are expressed in the epidermis (30, 31), although to date only TGases 1 and 3 have verified roles in CE assembly (32, 33). TGase 1 is expressed as a 106-kDa monomeric protein, which is constitutively N-myristoylated and S-palmitoylated on its amino-terminal 10-kDa domain, thereby directing the enzyme to plasma membranes (34–36). Membrane-bound TGase 1 enzyme is essential for both the assembly of the protein envelope by cross-linking CE structural proteins located in the intimate vicinity of the cellular membrane, and the esterification of the ω-hydroxyceramides to proteins, primarily involucrin (27). Genetic defects of TGase 1 cause the often devastating disease lamellar ichthyosis (37, 38). The homozygous TGase 1 knockout mice show defective CE assembly and die from dehydration a few hours after birth (39).

Involucrin is ubiquitously expressed in stratified squamous epithelia, suggesting it is commonly involved in CE formation (40, 41). Mammalian involucrins evolved by tandem duplications of glutamine and glutamic acid-rich sequences spanning between the evolutionary relatively conserved amino-terminal (“head”) and carboxyl-terminal (“tail”) domains (42). Recent in vivo observations indicate that the CE formation may be initi-
whether the applied concentrations of CSO 4 disrupted or aggregated difluoride membranes following SDS-PAGE on 4–20% gradient gels and analyzed by autoradiography after transfer onto polyvinylidene

tion mixtures were diluted with SDS-PAGE sample buffer (45), boiled, and we found no changes in light scattering for CSO4 concentrations below buffer (without isotope) and examined by light scattering at 310 nm. As ingredients in various combinations were diluted 10-fold in reaction

groove conditions to eukaryote plasma membranes. Using this model system we have demonstrated that involucrin is absorbed to membranes containing physiological levels of phosphatidylserine at Ca2+ concentrations in the range typically seen in keratinocytes.

Applying our SLV experimental system for modeling the earliest stages of CE assembly, we demonstrate here that supraphysiological levels of CSO4 severely interfere with involucrin cross-linking and α-hydroxyceramide esterification by TGase 1. Our data reveal new insights into the pathophysiology of XI disease.

**MATERIALS AND METHODS**

**Production of Recombinant TGase 1 and Human Involucrins—**Full-length human TGase 1 and involucrin proteins were expressed and purified exactly as described (44). A Δ2N mutant form of human involucrin was made from the pET11a expression plasmid by use of the GCACATGACTGCTGTAACGGCGACTGCTGACGAAGATG primer and its reverse strand using the QuickChange (Stratagene) kit, and further processed identically to the wild type. Occasionally, involucrin expression was induced in a LB broth containing 100 nmol (0.5 mCi/liter) of L-[35S]cysteine and 100 nmol (0.5 mCi/liter) of L-[35S]methionine (both from Amersham Pharmacia Biotech).

**Preparation of SLV—**The following mixtures were made in chloroform/methanol (2:1): 55 mol % dimyristoyl phosphatidylcholine, 15 mol % dipalmitoyl phosphatidylserine, 0–10 mol % CSO4, cholesterol up to 99 mol % (all from Sigma), and 1 mol % of the synthetic ceramide analog N-(16-hydroxyhexadecyl)oxypalmitoylphosphorylsphingosine (lipid Z) (27). The solvent was evacuated, and the lipids were taken up in aqueous buffer and dispersed by sonication as before (44). The prepared SLV suspension was equipped with 0.94 pmol (0.1 μg) of TGase 1 and its membrane binding was facilitated by incubating at 37 °C for 15 min prior to adding substrates.

**Cross-linking of Involucrin by TGase 1—**SLV (200 μl, 2 μmol of lipid) formulated with 0–10 mol % CSO4 were loaded with TGase 1 above as described (44) and washed three times with acetone/triethylamine/acetic acid (90:5:5) (46) to remove the SDS and noncovalently bound lipids. After further washing with acetone, the pellet was dried under vacuum and redisolved in 50 mM Tris-HCl (pH 7.5). Quantitation of the N+-glutamyllysine isopeptide cross-link was done by amino acid analysis following exhaustive proteolytic fragmentation of the products by the nonspecific protease Pronase and a mixture of carboxypeptidases (47). Determination of Kinetic Parameters of 14C-Putrescine Incorporation by TGase 1—Vmax, Kcat values were determined exactly as described (44).

**Isolation and Quantitation of Lipid Z Esterification of Involucrin**—There are four different outcomes of TGase catalysis of reactive Glu residues in the present experimental system, and are as follows: deamidated (that is, a Glu residue is formed); ε-ester-linked to the synthetic ceramide analog lipid Z; and isopeptide cross-linked, either to the N+-amino group of an involucrin Lys residue or, where added, to the dianine substrate putrescine forming γ-glutamylputrescine (EP). Finally some substrate glutamine residues are recovered in unmodified form. The following procedures were designed to separately identify and quantitate each of these five end products. Samples of TGase 1-reacted involucrin were freed from SLV lipids as above. The protein was then digested with 2% (by weight) modified trypsin (Roche Molecular Biochemicals). The grossly different chromatographic properties of N+-Lys82 cross-linked and lipid Z-linked tryptic involucrin peptides allowed their separation and quantitation by amino acid analysis following acid hydrolysis. In the samples reacted with lipid Z, first the digest was passed through a C18 HPLC column under strongly desorbing solvent conditions as described (27), where only the lipopeptides are retarded and all other non-lipid-containing peptides are recovered in the column flow-through (4). The amount of
the five lipid Z-ester linked peptides (see Fig. 5A) was determined by amino acid analysis. The peptide pool recovered from the C18 column flow-through was further separated by C18 HPLC chromatography as described (44). Here peptides involved in cross-link formation were recovered as distinct peaks (P1 and P2 of Fig. 2B) when cross-linked to another involucrin peptide. Sequences and cross-linking sites of these peptides were determined by peptide sequencing as before (48). To eliminate interference of overlapping (non-cross-linked) tryptic peptides of involucrin, the absolute molar amount of cross-linked residues was calculated from the Thr content, since Thr was absent from neighboring contaminating peptide peaks and was equimolar with the N-γ-glutamyl)lysine isopeptide present in the cross-linked peptide (Table I).

However, the peptides harboring unmodified, deamidated or putrescine-linked glutamine residues were not resolvable by HPLC but instead were analyzed by peptide sequencing. After Edman degradation, the phenylthiohydantoin-derivatized residues each appeared as a distinct peak in the sequencer’s HPLC profile (see Fig. 6). The ratio of deamidation and putrescine cross-linking was determined from the cross-linking chromatograms corresponding to each expected Gln residue as four of the reactive Gln residues were preceded by an unreactive Gln, the ratio of the unmodified and deamidated Gln/Glu residues was corrected for carryover from the previous sequencing cycle of Gln, using the formula,

\[
Q/E = \frac{[Q_n - Q_{-1} \cdot (1 - Q_{-1}/Q_n)] \cdot [1 + E_{-1} + E_{+1}]}{[E_n - E_{-1} \cdot (1 - E_{-1}/E_n)] \cdot [1 - E_{-1}/(Q_{-1} + E_{-1})]}
\]

(Eq. 1)

where \(X_n\) denotes the amount of the amino acid released from the sequencing cycle corresponding to the reactive residue position, and \(X_{-1}\) or \(X_{+1}\) denote the yield of the same amino acid in the previous or consecutive sequencing cycle. Similarly, the amount of EP was corrected for incomplete cleavage and carryover by the formula,

\[
EP = (EP_1)^2 \cdot (EP_1/EP_{+1})
\]

(Eq. 2)

Where EP denotes the amount of γ-glutamylputrescine in the first cycle of its appearance and EP_{+1} is that from the next cycle. Molar absorption of EP was taken equal to that of Lys at the detection wavelength of the Porton 3000 sequencer (268 nm). Thus based on the directly measured absolute amounts of Gln residues occupied by the N-γ-glutamyl)lysine cross-link and the lipid Z ester, we could calculate from the sequencing chromatograms the fate of the remainder of the 600 pmol of the Gln residues of involucrin that was unreacted, deamidated, and in control experiments, putrescine-linked. The data represent the means of three or more independent measurements.

RESULTS AND DISCUSSION

Involucrin is Cross-linked to Itself by TGase 1 on SLV—Wild type 35S-involucrin was reacted with TGase 1 on the surface of SLV for 2 h in the absence of exogenous glutamyl acceptor substrates. The protein was cross-linked into dimers, trimers, tetramers, and higher oligomers, as evidenced by autoradiography of protein blots after separation by SDS-PAGE (Fig. 1A). Some of the protein showed faster electrophoretic mobility than the monomer, indicative of intramolecular cross-link formation (49). Oligomers larger than tetramers were not separated by the gels used, but remained at the interface of the separation gel. Inclusion of 1 mol % lipid Z into the SLV membranes did not eliminate involucrin cross-linking by TGase 1 (Fig. 1B), but the addition of 20 mM putrescine as a competitive inhibitor of protein bound lysine e- amino groups (Fig. 1C) or omission of Ca2+ (not shown) caused a virtually complete inhibition of oligomer formation. These data indicate that involucrin is a complete substrate for the TGase 1 enzyme bound to SLV, in that it provides both donor Gln and acceptor Lys residues, and confirm a similar conclusion for the reaction of crude TGase 1 with involucrin in solution assays (49).

TGase 1 Forms Intramolecular Cross-links between Lys62 and Gln496 or Gln133 Residues of Involucrin—In order to identify the residues involved in the oligomerization by cross-linking of involucrin, following TGase 1 reaction on SLV and fragmentation by trypsin, peptides were separated by C18 reverse-phase HPLC, and the elution profile of obtained peptide peaks was compared with that of the un-cross-linked protein (44) (Fig. 2A and B). The cross-linking by TGase 1 caused the appearance of two novel peaks (P1 and P2 on Fig. 2B), and concomitant reduction of the relative intensity of three peaks compared with the initial profile. Protein sequencing revealed that the novel peaks resulted from cross-linking formation of two Gln donor peptides with a single Lys (Table I) and that the residues involved in N-γ-glutamyl)lysine cross-linking were Gln496 or Gln133 with Lys62, respectively. No other novel potentially cross-linked peaks with yields >0.005 mol/mol of involucrin were found. Given the large numbers of Gln and Lys residues in involucrin (50), this remarkable degree of specificity leading to both head-to-tail and head-to-head oligomerization provides further evidence for the importance of the membrane surface in directing TGase 1 specificity. Identical results were obtained if 1 mol % lipid Z incorporated into SLV was given at 20 mM concentrations. However, if 1 mM putrescine was used instead (2), the inhibition of activity was apparent at 5 or higher mol % membrane CSO4. The phenomenon is indicative of competitive inhibition.

FIG. 4. Activity of membrane-bound TGase 1 at different SLV CSO4 concentrations measured by [35S]putrescine incorporation. A, radioactive putrescine incorporation into involucrin (circles) and succinylated casein (triangles) substrates showed no significant (p < 0.05) dependence from membrane CSO4 levels under standard assay conditions, when [35S]putrescine was given at 20 mM concentration. However, if 1 mM putrescine was used instead (B), the inhibition of activity was apparent at 5 or higher mol % membrane CSO4. The phenomenon is indicative of competitive inhibition.
but cross-links between Lys$^{62}$ and Gln$^{196}$ or Gln$^{133}$ were commonly observed in CEs formed in cultured keratinocytes (51). Likewise, although Gln$^{133}$ is not conserved in prosimians (42), cross-linked peptides involving it were found in cultured keratinocyte CEs. As these keratinocytes undergo only a limited degree of barrier formation, we can conclude that cross-linking though Lys$^{62}$ or Gln$^{133}$ should represent early stages of CE assembly, and that the numerous other Lys and Gln residues identified in our in vivo studies must be utilized in later stages (51).

**K62N Mutant Involucrin Is Not Oligomerized by SLV-Bound TGase 1**—As a control for the cross-linking through the sole Lys$^{62}$ residue, we made a K62N mutant form of involucrin. This completely eliminated the ability of involucrin to serve as complete substrate for TGase 1 on SLV, as autoradiography after SDS-PAGE showed no detectable inter- or intrachain cross-linked involucrin products (Fig. 1D). Likewise, the HPLC profile of tryptic peptides of the involucrin mutant showed no sign of the TGase 1-mediated changes which were observed with the wild type protein (although as expected, peak 9 disappeared since a trypsin cleavage site was lost as a consequence of the mutation, and another new peak appeared (data not shown)).

**Effect of CSO$_4$ Content in SLV on Involucrin Cross-linking by TGase 1**—The effect of CSO$_4$ on the membrane-dependent cross-linking of involucrin was examined by formulating the carrier SLV with 0–10 mol % CSO$_4$ in 2% increments. CSO$_4$ concentrations above 12% began to destabilize SLV assembly and therefore were not used. Analysis of the electrophoretic mobility of TGase 1-treated $^{35}$S-involucrin revealed a CSO$_4$ concentration-dependent inhibition of cross-linking, which was apparent at 6 mol % and almost completely eliminated the bands of inter- or intramolecularly cross-linked involucrin at 10% (Fig. 3A). Assaying the amounts of N$^\gamma$-(γ-glutamyl)lysine isopeptide cross-link showed a significant decline of cross-link amount at 6 mol % CSO$_4$ ($p < 0.001$) and which was reduced to $<10$% at 10 mol % CSO$_4$ (Fig. 3B).

TGase 1 activity was assayed by using a large excess of $[^{14}$C]putrescine as the amine substrate with both involucrin and the standard TGase assay substrate succinylated casein. Incorporation of the labeled amine was not significantly affected at any concentration below 8 mol % CSO$_4$ and only a slight decrease ($p < 0.1$) of activity was noted at 10 mol % (Fig. 4A). Kinetic parameters of $[^{14}$C]putrescine incorporation into involucrin and the standard substrate succinylated casein, which does not adsorb to SLV under these conditions (44) were measured (Table II), and indicated that up to 10 mol % CSO$_4$ there was no statistically significant ($p < 0.1$) change in the apparent $V_{\text{max}}$ of TGase 1. However, when 1 mM putrescine was used, SLV CSO$_4$ content caused a dose-dependent decrease of the reaction rate. The assay of kinetic parameters revealed only an insignificant effect of CSO$_4$ on $V_{\text{max}}$ with both protein substrates. However, for the substrate putrescine, membrane CSO$_4$ caused a dose-dependent increase of $K_M(\text{app})$ and thus reduced the catalytic efficiency ($K_M/V_{\text{max}}$) values, a phenomenon characteristic of competitive inhibition.

**Effect of SLV Content of CSO$_4$ on ω-Hydroxyacylceramide Estearification by TGase 1**—In addition, we explored the effects of CSO$_4$ on TGase 1-mediated ω-hydroxyacylceramide attachment to involucrin by esterification. One mol % of the artificial ω-hydroxyacylceramide analog lipid Z was incorporated into the SLV and reacted with involucrin by TGase 1 as before (27). Isolation of peptide-linked ceramides was done by $C_4$ HPLC separation of tryptic peptides of involucrin under strongly desorbing solvent conditions, where only the lipopeptide adducts are retarded and free peptides elute with the column flow-through (4). Amounts of recovered peptide-lipid Z adducts showed a visible decline of peak areas in SLV containing $>4$ mol % CSO$_4$ content (Fig. 5A). Quantitative analysis of these peaks by amino acid analysis after acid hydrolysis indicated a significant decrease of summed lipopeptide formation at 4 mol % ($p < 0.001$) SLV CSO$_4$ content, a 10-fold reduction at 8 mol %, and $>30$-fold less with 10 mol % CSO$_4$ (Fig. 5B).

Taken together, the inhibition of isopeptide cross-linking and ester formation imply that CSO$_4$ serves as a competitive inhibitor of TGase 1 reactions. However, since CSO$_4$ is clearly not a substrate for TGases, it is possible that instead it interferes with the reaction by limiting access to the active site for substrates or by conformational alteration of the enzyme. In order.

### Table II

| CSO$_4$ Content | Putrescine | Succinylated Casein | Involucrin |
|-----------------|-----------|---------------------|------------|
| 0%              | 3.2 ± 0.2 | 2.4 ± 0.4           | 2.7 ± 0.3  |
| 4%              | 3.4 ± 0.3 | 3.6                 | 2.9        |
| 6%              | 1.6 ± 0.2 | 1.7                 | 2.0        |
| 8%              | 0.6 ± 0.2 | 0.64                | 0.37       |

### Table II: Apparent kinetic parameters for putrescine incorporation into involucrin and succinylated casein by 0.94 pmol membrane bound TGase 1 at different membrane CSO$_4$ concentrations

All SLV were formulated with 55% phosphatidylcholine and 15% phosphatidylserine. $K_M(\text{app})$, $V_{\text{max}}$, and $K_M/V_{\text{max}}$ data are that of putrescine incorporation. Values with 0% CSO$_4$ are the same as published before (30).
CSO4. Lipid Z esterified onto involucrin was quantitated by isolating lipopeptides selectively retained on the column from the tryptic digest of lipid Z in SLV. As controls, we also used 20 mM putrescine as a competitive inhibitor, in which case the EP derivative is expected (44). Even so, there was a small but significant increase in deamidation rate at the highest levels of CSO4 tested (Fig. 7C). However, all was lost to deamidation by 6% membrane CSO4. For Gln107, Gln118, Gln133, and Gln133, 20–30% was used for ester formation in the absence of CSO4, and again, an increasing percentage of deamidation eradicating this reaction product by 8–10 mol % CSO4 (Fig. 7H for Gln122, other data not shown). Finally, addition of 20 mM putrescine to the above system led to near complete modification of Gln122 to EP and efficiently suppressed the cross-linking and deamidation by TGase 1.

When wild type involucrin was reacted with TGase 1 on SLV formulated without CSO4 in the absence of any other glutamyl-acceptor substrate, 51% of the Gln496 residue was modified, of which most was engaged in cross-link formation, but steadily increased the amount of deamidation, so that in SLV with 10% CSO4, 53% was deamidated and only 8% was used for cross-link formation. Similar deamidation rates were seen for the Gln133 residue which also participated in cross-link formation. For Gln107, Gln118, and Gln122 which did not engage in Nε-(γ-glutamyl)lysine cross-link formation with Lys62, most was likewise deamidated (Fig. 7G, other data not shown). Next, we used SLV containing 1 mol % lipid Z and where applicable, lipid Z-attached peptides were extracted and where applicable, lipid Z-attached peptides were extracted. The figure shows superimposed elution chromatograms of residue Gln107 recovered from peptide peak 22 (see Fig. 2A) of involucrin reacted with TGase 1 in the presence of 20 mM putrescine on SLV made with 0 (black line) or 8 mol % (red line) membrane CSO4. Peak areas were utilized to calculate the ratio of different Gln modifications by TGase 1.

We resolved these issues in detail, we analyzed the fate(s) of each reactive Gln residue using different substrate conditions.

Analysis of TGase 1-mediated Modifications of Reactive Gln Residues in Involucrin with Increasing SLV CSO4 Content—Five Gln residues (Gln107, Gln118, Gln122, Gln133, and Gln496) of involucrin serve as substrates for membrane-bound TGase 1 (27, 44). Each of these Gln residues can undergo either hydrolysis or ester bond formation or transglutaminase (27, 44). Each of these Gln residues can undergo either hydrolysis or ester bond formation or transglutaminase (44). Peaks of cross-linked peptides (Fig. 2B) were also collected and quantified. The peaks harboring TGase 1-reactive glutamines embraced a mixture of glutamine-derived moieties, that were either unmodified, deamidated to glutamic acid, or eventually modified to EP. These products were collected in the same fraction, and sequenced as a mixture. The phenylthiohydantoin-derivatives of Gln, Glu, and EP from TGase 1 substrate residues Gln107, Gln118, Gln122, Gln133, and Gln496 were measured in the appropriate sequencing cycles (Fig. 6) and peak area derived absolute molar amount values were corrected for carryover from previous cycles, spontaneous deamidation, and coupling yield by pristine algebra. Data for all five glutamines are summarized in Table III.

When wild type involucrin was reacted with TGase 1 on SLV formulated without CSO4 in the absence of any other glutamyl-acceptor substrate, peak areas were utilized to calculate the ratio of different Gln modifications by TGase 1.

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When wild type involucrin was reacted with TGase 1 on SLV formulated without CSO4 in the absence of any other glutamyl-acceptor substrate, peak areas were utilized to calculate the ratio of different Gln modifications by TGase 1.
### Table III

Distribution of products from substate glutamine residues of involucrin after reacting with 0.94 pmol of TGase 1 on SLV formulated with different CSO4 content

Values were calculated from amino acid analysis after acid hydrolysis data combined with Gln:Glu (\(\gamma\)-glutamylputrescine) ratios from sequencing yields. Numbers represent mean of three determinations rounded to whole percentage. NA, not available.

| Substrates                          | CSO 4 Residue | Glutamine mol % | Glutamate mol % | \(\gamma\)-Glutamylputrescine % | \(\alpha\gamma\)-Glycyllysine % | \(\gamma\)-Glutamyl lipid Z % |
|-------------------------------------|---------------|-----------------|-----------------|-------------------------------|-------------------------------|-----------------------------|
| Only involucrin (wild type)         | 0             | 496             | 49              | NA                            | 46                            | NA                          |
|                                     | 133           | 73              | 3               | NA                            | 24                            | NA                          |
|                                     | 122           | 68              | 32              | NA                            | 0                             | NA                          |
|                                     | 118           | 82              | 18              | NA                            | 0                             | NA                          |
|                                     | 107           | 72              | 28              | NA                            | 0                             | NA                          |
| 4                                  | 496           | 47              | 10              | NA                            | 43                            | NA                          |
|                                     | 133           | 73              | 7               | NA                            | 20                            | NA                          |
|                                     | 122           | 65              | 35              | NA                            | 0                             | NA                          |
|                                     | 118           | 79              | 21              | NA                            | 0                             | NA                          |
|                                     | 107           | 69              | 31              | NA                            | 0                             | NA                          |
| 6                                  | 496           | 46              | 28              | NA                            | 26                            | NA                          |
|                                     | 133           | 69              | 21              | NA                            | 10                            | NA                          |
|                                     | 122           | 62              | 38              | NA                            | 0                             | NA                          |
|                                     | 118           | 75              | 25              | NA                            | 0                             | NA                          |
|                                     | 107           | 65              | 35              | NA                            | 0                             | NA                          |
| 8                                  | 496           | 45              | 44              | NA                            | 11                            | NA                          |
|                                     | 133           | 66              | 29              | NA                            | 5                             | NA                          |
|                                     | 122           | 57              | 43              | NA                            | 0                             | NA                          |
|                                     | 118           | 73              | 27              | NA                            | 0                             | NA                          |
|                                     | 107           | 63              | 37              | NA                            | 0                             | NA                          |
| 10                                 | 496           | 37              | 58              | NA                            | 5                             | NA                          |
|                                     | 133           | 63              | 35              | NA                            | 2                             | NA                          |
|                                     | 122           | 54              | 46              | NA                            | 0                             | NA                          |
|                                     | 118           | 70              | 30              | NA                            | 0                             | NA                          |
|                                     | 107           | 59              | 41              | NA                            | 0                             | NA                          |
| Involucrin (wild type) + 20 mM putrescine | 0             | 496             | 20              | 79                            | 0                             | NA                          |
|                                     | 133           | 34              | 0               | 66                            | 0                             | NA                          |
|                                     | 122           | 31              | 1               | 69                            | 0                             | NA                          |
|                                     | 118           | 39              | 0               | 61                            | 0                             | NA                          |
|                                     | 107           | 72              | 0               | 38                            | 0                             | NA                          |
| 4                                  | 496           | 21              | 7               | 77                            | 0                             | NA                          |
|                                     | 133           | 33              | 1               | 66                            | 0                             | NA                          |
|                                     | 122           | 30              | 3               | 67                            | 0                             | NA                          |
|                                     | 118           | 38              | 1               | 61                            | 0                             | NA                          |
|                                     | 107           | 37              | 2               | 61                            | 0                             | NA                          |
| 6                                  | 496           | 19              | 5               | 76                            | 0                             | NA                          |
|                                     | 133           | 33              | 3               | 66                            | 0                             | NA                          |
|                                     | 122           | 39              | 5               | 66                            | 0                             | NA                          |
|                                     | 118           | 37              | 3               | 60                            | 0                             | NA                          |
|                                     | 107           | 36              | 3               | 61                            | 0                             | NA                          |
| 8                                  | 496           | 18              | 9               | 73                            | 0                             | NA                          |
|                                     | 133           | 31              | 7               | 62                            | 0                             | NA                          |
|                                     | 122           | 28              | 7               | 65                            | 0                             | NA                          |
|                                     | 118           | 36              | 6               | 58                            | 0                             | NA                          |
|                                     | 107           | 35              | 9               | 56                            | 0                             | NA                          |
| 10                                 | 496           | 17              | 13              | 70                            | 0                             | NA                          |
|                                     | 133           | 30              | 11              | 59                            | 0                             | NA                          |
|                                     | 122           | 28              | 10              | 61                            | 0                             | NA                          |
|                                     | 118           | 35              | 11              | 54                            | 0                             | NA                          |
|                                     | 107           | 34              | 12              | 54                            | 0                             | NA                          |
| Involucrin (wild type) + 1 mol % lipid Z | 0             | 496             | 51              | 7                             | 37                            | 5                           |
|                                     | 133           | 73              | 3               | NA                            | 16                            | 3                           |
|                                     | 122           | 69              | 6               | NA                            | 0                             | 25                          |
|                                     | 118           | 83              | 3               | NA                            | 0                             | 14                          |
|                                     | 107           | 71              | 5               | NA                            | 0                             | 24                          |
| 4                                  | 496           | 50              | 15              | NA                            | 32                            | 3                           |
|                                     | 133           | 72              | 10              | NA                            | 13                            | 5                           |
|                                     | 122           | 67              | 17              | NA                            | 0                             | 16                          |
|                                     | 118           | 82              | 8               | NA                            | 0                             | 10                          |
|                                     | 107           | 69              | 16              | NA                            | 0                             | 15                          |
| 6                                  | 496           | 47              | 35              | NA                            | 17                            | 0                           |
|                                     | 133           | 69              | 21              | NA                            | 7                             | 3                           |
|                                     | 122           | 60              | 36              | NA                            | 0                             | 4                           |
|                                     | 118           | 79              | 16              | NA                            | 0                             | 5                           |
|                                     | 107           | 68              | 23              | NA                            | 0                             | 9                           |
| 8                                  | 496           | 48              | 47              | NA                            | 7                             | 0                           |
|                                     | 133           | 66              | 31              | NA                            | 3                             | 0                           |
|                                     | 122           | 60              | 36              | NA                            | 0                             | 4                           |
|                                     | 118           | 76              | 22              | NA                            | 0                             | 2                           |
|                                     | 107           | 63              | 33              | NA                            | 0                             | 4                           |
| 10                                 | 496           | 37              | 61              | NA                            | 2                             | 0                           |
|                                     | 133           | 67              | 32              | NA                            | 1                             | 0                           |
| Substrates                  | CSO | Residue | Glutamine | Glutamate | γ-Glutamylputrescine | ε-Glutamyllysine | γ-Glutamyl lipid Z |
|----------------------------|-----|---------|-----------|-----------|----------------------|------------------|-------------------|
| Only involucrin (K62N mutant) | 0   | 496     | 19        | 81        | NA                   | 0                | NA                |
|                            | 133 | 32      | 68        | NA        | 0                    | NA               | NA                |
|                            | 122 | 32      | 68        | NA        | 0                    | NA               | NA                |
|                            | 118 | 38      | 62        | NA        | 0                    | NA               | NA                |
|                            | 107 | 32      | 68        | NA        | 0                    | NA               | NA                |
| 4                          | 496 | 18      | 82        | NA        | 0                    | NA               | NA                |
|                            | 133 | 30      | 70        | NA        | 0                    | NA               | NA                |
|                            | 122 | 29      | 71        | NA        | 0                    | NA               | NA                |
|                            | 118 | 37      | 63        | NA        | 0                    | NA               | NA                |
|                            | 107 | 29      | 71        | NA        | 0                    | NA               | NA                |
| 6                          | 496 | 16      | 84        | NA        | 0                    | NA               | NA                |
|                            | 133 | 28      | 72        | NA        | 0                    | NA               | NA                |
|                            | 122 | 26      | 74        | NA        | 0                    | NA               | NA                |
|                            | 118 | 37      | 63        | NA        | 0                    | NA               | NA                |
|                            | 107 | 27      | 73        | NA        | 0                    | NA               | NA                |
| 8                          | 496 | 13      | 87        | NA        | 0                    | NA               | NA                |
|                            | 133 | 26      | 74        | NA        | 0                    | NA               | NA                |
|                            | 122 | 24      | 76        | NA        | 0                    | NA               | NA                |
|                            | 118 | 35      | 65        | NA        | 0                    | NA               | NA                |
|                            | 107 | 25      | 75        | NA        | 0                    | NA               | NA                |
| 10                         | 496 | 11      | 89        | NA        | 0                    | NA               | NA                |
|                            | 133 | 24      | 76        | NA        | 0                    | NA               | NA                |
|                            | 122 | 23      | 77        | NA        | 0                    | NA               | NA                |
|                            | 118 | 33      | 67        | NA        | 0                    | NA               | NA                |
|                            | 107 | 22      | 78        | NA        | 0                    | NA               | NA                |
| Involucrin (K62N mutant) + 20 mM putrescine | 0   | 496     | 13        | 0         | 87                   | 0                | NA                |
|                            | 133 | 28      | 1         | 71        | 0                    | NA               | NA                |
|                            | 122 | 29      | 0         | 71        | 0                    | NA               | NA                |
|                            | 118 | 36      | 1         | 63        | 0                    | NA               | NA                |
|                            | 107 | 30      | 1         | 69        | 0                    | NA               | NA                |
| 4                          | 496 | 14      | 1         | 85        | 0                    | NA               | NA                |
|                            | 133 | 27      | 1         | 72        | 0                    | NA               | NA                |
|                            | 122 | 28      | 2         | 70        | 0                    | NA               | NA                |
|                            | 118 | 35      | 3         | 62        | 0                    | NA               | NA                |
|                            | 107 | 30      | 2         | 68        | 0                    | NA               | NA                |
| 6                          | 496 | 12      | 4         | 84        | 0                    | NA               | NA                |
|                            | 133 | 27      | 4         | 69        | 0                    | NA               | NA                |
|                            | 122 | 27      | 5         | 68        | 0                    | NA               | NA                |
|                            | 118 | 34      | 6         | 60        | 0                    | NA               | NA                |
|                            | 107 | 29      | 7         | 64        | 0                    | NA               | NA                |
| 8                          | 496 | 11      | 11        | 88        | 0                    | NA               | NA                |
|                            | 133 | 26      | 9         | 65        | 0                    | NA               | NA                |
|                            | 122 | 26      | 9         | 65        | 0                    | NA               | NA                |
|                            | 118 | 34      | 8         | 58        | 0                    | NA               | NA                |
|                            | 107 | 27      | 11        | 62        | 0                    | NA               | NA                |
| 10                         | 496 | 9       | 16        | 75        | 0                    | NA               | NA                |
|                            | 133 | 25      | 15        | 60        | 0                    | NA               | NA                |
|                            | 122 | 26      | 14        | 60        | 0                    | NA               | NA                |
|                            | 118 | 33      | 15        | 52        | 0                    | NA               | NA                |
|                            | 107 | 26      | 15        | 59        | 0                    | NA               | NA                |
| Involucrin (K62N mutant) + 1 mol % lipid Z | 0   | 496     | 25        | 62        | NA                   | 0                | 13                |
|                            | 133 | 33      | 60        | NA        | 0                    | 7                | 0                 |
|                            | 122 | 33      | 54        | NA        | 0                    | 13               | 0                 |
|                            | 118 | 39      | 46        | NA        | 0                    | 15               | 0                 |
|                            | 107 | 33      | 51        | NA        | 0                    | 16               | 0                 |
| 4                          | 496 | 24      | 74        | NA        | 0                    | 2                | 0                 |
|                            | 133 | 32      | 65        | NA        | 0                    | 3                | 0                 |
|                            | 122 | 31      | 63        | NA        | 0                    | 6                | 0                 |
|                            | 118 | 37      | 54        | NA        | 0                    | 9                | 0                 |
|                            | 107 | 30      | 63        | NA        | 0                    | 7                | 0                 |
| 6                          | 496 | 22      | 78        | NA        | 0                    | 0                | 0                 |
|                            | 133 | 31      | 69        | NA        | 0                    | 0                | 0                 |
|                            | 122 | 29      | 70        | NA        | 0                    | 1                | 0                 |
|                            | 118 | 36      | 62        | NA        | 0                    | 2                | 0                 |
|                            | 107 | 29      | 70        | NA        | 0                    | 1                | 0                 |
| 8                          | 496 | 21      | 79        | NA        | 0                    | 0                | 0                 |
|                            | 133 | 29      | 71        | NA        | 0                    | 0                | 0                 |
|                            | 122 | 28      | 72        | NA        | 0                    | 0                | 0                 |
|                            | 118 | 34      | 66        | NA        | 0                    | 0                | 0                 |
|                            | 107 | 27      | 63        | NA        | 0                    | 0                | 0                 |
| 10                         | 496 | 17      | 83        | NA        | 0                    | 0                | 0                 |
|                            | 133 | 28      | 72        | NA        | 0                    | 0                | 0                 |
|                            | 122 | 26      | 74        | NA        | 0                    | 0                | 0                 |
|                            | 118 | 33      | 67        | NA        | 0                    | 0                | 0                 |
|                            | 107 | 24      | 76        | NA        | 0                    | 0                | 0                 |
|                            | 122 | 58      | 42        | NA        | 0                    | 0                | 0                 |
|                            | 118 | 76      | 22        | NA        | 0                    | 0                | 0                 |
|                            | 107 | 61      | 39        | NA        | 0                    | 0                | 0                 |
reactive Gln residues (Fig. 7I; Table III).

When the K62N mutant form of involucrin was reacted with TGase 1 in the absence of any other glutamyl-acceptor substrate, water was used as the acyl acceptor resulting in near complete (80–90%) deamidation of the five reactive Gln residues, the degree of which was not significantly increased with higher CSO4 levels (Fig. 7, D and J; Table III). In this case, about 10% of the reactivity of each Gln residue was used for esterification of lipid Z in the absence of CSO4, but this was lost to deamidation at ≥6 mol % CSO4 (Fig. 7, E and K). Again, the inclusion of 20 mM putrescine into the reactions resulted in extensive EP formation as expected, although with increasing amounts of CSO4, the ratio between EP formation and deamidation was reduced significantly and was essentially the same as with the wild type involucrin (compare Fig. 7, F and L with C and I).

These data indicate the glutamyl donor and acceptor substrate preferences of involucrin cross-linking by membrane bound TGase 1. Of these, only Gln496, Gln118, and Lys62 can be used for N'-γ-glutamyllysine cross-link formation, but all the five reactive glutamines form isopeptide bonds and are esterified with the ceramide-alcohol lipid Z. We propose that steric factors might hinder the intimate juxtaposition of Lys62 to the acyl-enzyme complex when involucrin reacts with TGase 1 on its Gln107, Gln118, or Gln122.

We also noted that cross-linking through Lys62 decreased the overall degree of reactivity of the neighboring reactive Gln residues, in comparison to the uncross-linkable mutant (Fig. 7, compare panels B and E). This might be a consequence of the inhibition of substrate diffusion on the SLV surface following the formation of larger involucrin oligomers, and/or the TGase 1 itself by “stockading” the enzyme by its own products, a mechanism impossible with the K62A mutant protein. Finally, cross-linking through Lys62 increased the yield of especially Gln107, Gln118, Gln122, and Gln133 for esterification (Fig. 7, as examples, compare panels H and K). This might be a consequence of diminished likelihood for TGase 1-mediated hydrolysis of ester bonds via reversal of once preformed ester linkages to the acyl-enzyme intermediate, possibly by the same stockading mechanism. We have noted previously that isopeptide formation is energetically favored over ester formation, since the latter can be converted to an isopeptide bond by an amine.

**Fig. 7.** The fates of TGase 1 reactive residues Gln496 and Gln122 of involucrin with different substrates at increasing membrane CSO4 levels. The degrees of modifications of each Gln residue were quantitated as determined in Figs. 5A and 6. Shown here are the data for the most reactive Gln496 residue (A–F), which participates in both isopeptide and ester bond formation, and for Gln122 (G–L) which is involved only in ester formation under physiologically relevant conditions. The applied substrates and color codes are noted for each panel.
cholesterol in the lipid sheets (16). More recently, it was dem-
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