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*

(Received for publication, June 8, 1999, and in revised form, October 27, 1999)

Zoltán Nemes‡, Máté Demény§, Lyuben N. Marekov‡, László Fésüs§, and Peter M. Steinert‡¶

From the ‡Laboratory of Skin Biology, NIAMSD, National Institutes of Health, Bethesda, Maryland 20892-2752 and the §Department of Biochemistry and Molecular Biology, University Medical School of Debrecen, Debrecen H-4012, Hungary

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 (CSO_4) in the epidermis but the pathomechanism how elevated epidermal CSO_4 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 %, CSO_4 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 CSO_4 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 sulfhydryl 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 (C_{28}-C_{36}) 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 (CSO_4) owing to arylsulfatase C/cholesterol sulfatase enzyme defects (9).

CSO_4 is a ubiquitous cholesterol metabolite, the amount of which is determined by the relative activity of cholesterol sulfotransferase and cholesterol sulfatase enzymes (10). CSO_4 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% CSO_4 of total lipids (11, 12). In XI the lack of its breakdown results in an elevated CSO_4 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 CSO_4 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

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Bldg. 6, Rm. 425, NIAMS, NIH, Bethesda, MD 20892-2752. Tel.: 301-496-1578; Fax: 301-402-2886; E-mail: pemast@helix.nih.gov.

[1] The abbreviations used are: CE, cell envelope; CSO_4, cholesterol sulfate; EP, γ-glutamylputrescine; HPLC, high performance liquid chromatography; lipid Z, *N*-[16-(16-hydroxyhexadecyl)oxypalmitoyl] sphingosine; XI, X-linked ichthyosis; SLV, synthetic lipid vesicles; TGase, transglutaminase; PAGE, polyacrylamide gel electrophoresis.

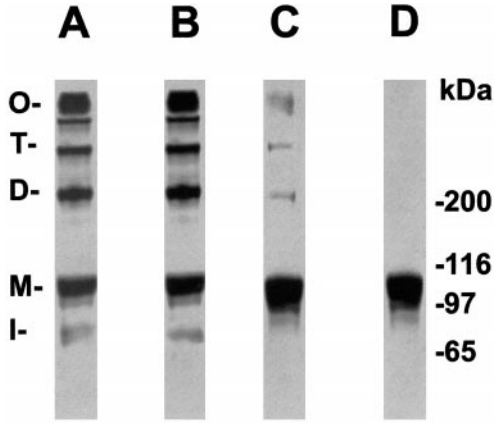

FIG. 1. **Cross-linking of involucrin by TGase 1 on SLV.** Recombinant human ^{35}S-involucrin was incubated with TGase 1 attached to SLV containing 15% phosphatidylserine (and no CSO_4) in the presence of Ca^{2+} for 60 min. 20-μg aliquots of involucrin products were separated by SDS-PAGE, transferred onto membranes, and detected by autoradiography. *A,* involucrin formed intrachain cross-links (*I*), dimers (*D*), trimers (*T*), and unresolved higher oligomers (*O*). *B,* cross-linking of involucrin was not impeded by the inclusion of 1 mol % lipid Z ceramide substrate; *C,* oligomerization was inhibited by 20 mM putrescine. *D,* in the case of the K62N mutant form of involucrin, no cross-linking occurred.

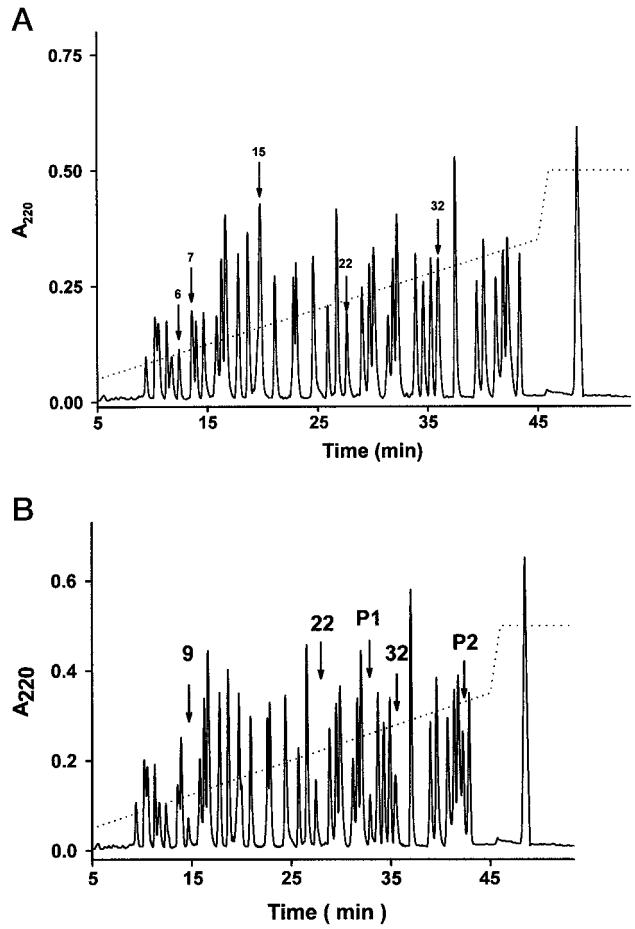

FIG. 2. **Identification of cross-linking sites in involucrin.** C_{18} HPLC profiles of tryptic involucrin peptides were compared before (*A*) and after (*B*) reaction with TGase 1 bound to SLV containing no CSO_4. Peaks harboring the 5 Gln residues reactive with SLV-bound TGase 1 are indicated with *arrows* on *panel A*. For clarity, the same peak numbering system was retained as before (44). TGase 1-dependent appearance of the novel peaks P_1 and P_2 is noted. The sequences of these peaks are shown in Table I.

published reports have addressed the alterations of physical properties of corneocyte lipids from excess CSO_4 (14–16). It has been shown that CSO_4 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, CSO_4 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 CSO_4. Thus it was theorized that CSO_4 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 CSO_4 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 CSO_4 can induce TGase 1 expression in cultured keratinocytes (19), but the connection between excess TGase expression and disease etiology remains unclear. CSO_4 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), CSO_4 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 Ca^{2+}-dependent enzymes (22). The catalytic mechanism of TGases involves the release of ammonia from the reactive glutamine residues, and the residual glutamyl moieties form an acyl-enzyme thioester, a labile intermediate susceptible to nucleophilic attack by primary amines, notably ϵ-amino groups from protein bound lysines (forming N^{ϵ}-(γ-glutamyl)lysine isopeptide cross-links), or polyamines (resulting in N,N'-bis(γ-glutamyl)polyamine cross-links (23, 24). However, the thioester intermediate can also be transferred to primary alcohols (25, 26). We have shown that in the epidermis, the terminal (ω) hydroxyl group of ω-hydroxyceramides is an effective substrate for membrane-bound TGase 1, and this route links these lipids to protein-bound glutamines 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-

TABLE I
Amino acid sequences of peptide peaks from HPLC separation of tryptic involucrin peptides (Fig. 2B) affected by cross-linking by TGase 1 on SLV

Peak	Sequence	Sequence position in involucrin
Peaks showing decreased area		
9	HMTAVK	63–68
22	LLDQQLDQELVK	129–140
32	QEAQLELPEQQVGQPK	486–501
Peaks appearing after cross-linking		
P1	QEEK^{62} HMTAVK	59–63
	:	
	LLDQQ^{133}LDQELVK	129–140
P2	QEEK^{62} HMTAVK	59–63
	:	
	QEAQLELPEQQ^{496}VGQPK	486–501

ated by the deposition of a monomolecular layer of involucrin on the inner keratinocyte membrane (41, 43). In a previous paper (44) we described an *in vitro* model system for characterizing the function of TGase 1 on the surface of synthetic lipid vesicles (SLV) of composition similar to eukaryote plasma membranes. Using this model system we have demonstrated that involucrin is absorbed to membranes containing physiological levels of phosphatidylserine at Ca^{2+} 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 CSO_4 severely interfere with involucrin cross-linking and ω-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 K62N mutant form of human involucrin was made from the pET11a expression plasmid use of the GCACATGACTGCTGTAACGGGACTGCCTGAGCAAGAATG 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-[^{35}S]cysteine and 100 nmol (0.5 mCi/liter) of L-[^{35}S]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 % CSO_4, cholesterol up to 99 mol % (all from Sigma), and 1 mol % of the synthetic ceramide analog *N*-[16-(16-hydroxyhexadecyl)oxypalmitoyl]sphingosine (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 % CSO_4 were loaded with TGase 1 as above and 600 pmol (40 μg) of involucrin in the presence of 1 mM $CaCl_2$. They were immediately incubated for 2 h in either the absence or presence of 20 mM putrescine with 100 nCi of [^{14}C]putrescine (NEN Life Science Products Inc., Boston, MA, 110 Ci/mmol). The reactions were stopped by the addition of EDTA to 10 mM. In control experiments we assessed whether the applied concentrations of CSO_4 disrupted or aggregated the SLV. SLV confectioned with 0–15 mol % CSO_4 and the above ingredients in various combinations were diluted 10-fold in reaction buffer (without isotope) and examined by light scattering at 310 nm. As we found no changes in light scattering for CSO_4 concentrations below 12 mol %, we routinely used SLV containing ≤10 mol % CSO_4.

Analysis of Cross-linking of Involucrin by TGase 1—The above reaction mixtures were diluted with SDS-PAGE sample buffer (45), boiled, and analyzed by autoradiography after transfer onto polyvinylidene difluoride membranes following SDS-PAGE on 4–20% gradient gels

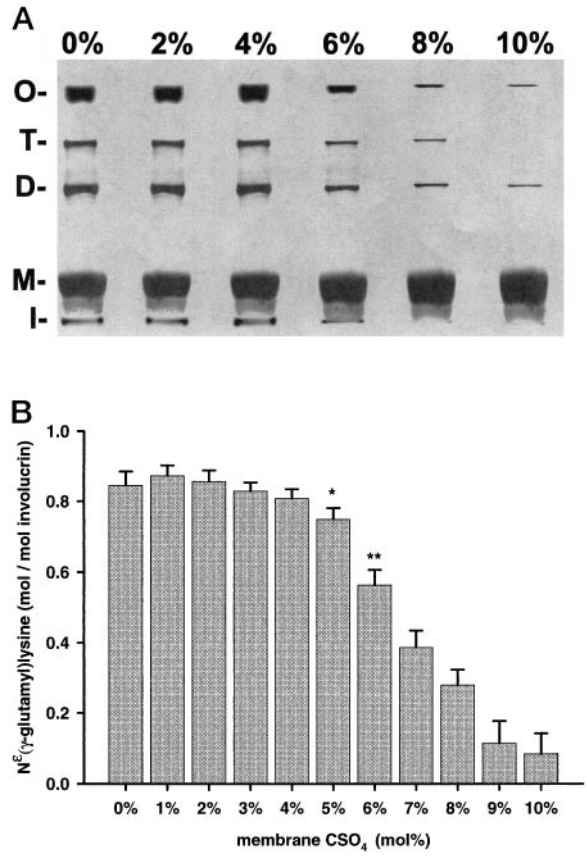

FIG. 3. **Increasing amounts of CSO_4 inhibits of involucrin cross-linking.** Formation of cross-linked oligomers from involucrin was analyzed at different SLV membrane CSO_4 concentrations as in Fig. 1. Reduction of cross-linked products is apparent above 6 mol % CSO_4.

(Novex). In some experiments, 0.1 ml of 20% SDS was added to the samples and the mixture was vortexed. This mixture was precipitated 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 redissolved in 50 mM Tris-HCl (pH 7.5). Quantitation of the N^{ϵ}-(γ-glutamyl) lysine 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 ^{14}C-Putrescine Incorporation by TGase 1—V_{max}, K_m, and k_{cat} values were determined exactly as described (44).

Isolation and Quantitation of Lipid Z Esterification of Involucrin Reactive Glutamines by TGase 1—The tryptic peptides of involucrin reacted with TGase 1 on SLV formulated with 1% lipid Z for 2 h were recovered and quantitated exactly as described (27).

Analysis of Cross-linking and Deamidation of TGase 1 Reactive Glutamine Residues in Involucrin—There are four different outcomes of TGase catalysis of reactive Gln residues in the present experimental system, and are as follows: deamidated (that is, a Glu residue is formed); ester-linked to the synthetic ceramide analog lipid Z; and isopeptide cross-linked, either to the N^{ϵ}-amino group of an involucrin Lys residue or, where added, to the diamine 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^{ϵ}-Lys^{62} 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 C_4 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. 5*A*) was determined by amino acid analysis. The peptide pool recovered from the C_4 column flow-through was further separated by C_{18} HPLC chromatography as described (44). Here peptides involved in cross-link formation were recovered as distinct peaks (P1 and P2 of Fig. 2*B*) 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-$^{\epsilon}$(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 relative intensity of PITC/phenylthiohydantoin-derivatized Gln, Glu, and EP peaks from the sequencing cycles 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,

$$Q/E = \frac{[Q_n - Q_{n-1} \cdot (1 - Q_{n+1}/Q_n)][1 + E_{n-1} + E_{n-1})]}{[E_n - E_{n-1} \cdot (1 - E_{n+1}/E_n)][1 - E_{n-1}/(Q_{n-1} + E_{n-1})]} \quad \text{(Eq. 1)}$$

where X_n denotes the amount of the amino acid released from the sequencing cycle corresponding to the reactive residue position, and X_{n-1}, or X_{n+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,

$$EP - (EP_n)^2 \cdot (EP_n/(EP_n - EP_{n+1}) \quad \text{(Eq. 2)}$$

Where EP_n denotes the amount of γ-glutamylputrescine in the first cycle of its appearance and EP_{n+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^{ϵ}-(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 ^{35}S-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. 1*A*). 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. 1*B*), but the addition of 20 mM putrescine as a competitive inhibitor of protein bound lysine ϵ-amino groups (Fig. 1*C*) or omission of Ca^{2+} (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 Intermolecular Cross-links between Lys^{62} and Gln^{496} or Gln^{133} 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 C_{18} reverse-phase HPLC, and the elution profile of obtained peptide peaks was

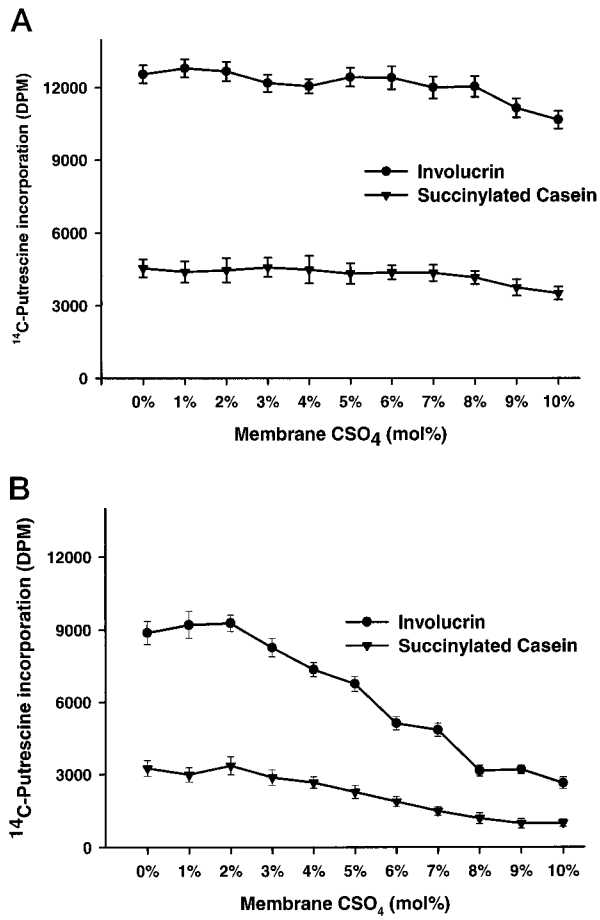

FIG. 4. **Activity of membrane-bound TGase 1 at different SLV CSO_4 concentrations measured by [^{14}C]putrescine incorporation.** *A*, radioactive putrescine incorporation into involucrin (*circles*) and succinylated casein (*triangles*) substrates showed no significant ($p < 0.05$) dependence from membrane CSO_4 levels under standard assay conditions, when [^{14}C]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 CSO_4. The phenomenon is indicative of competitive inhibition.

compared with that of the uncross-linked protein (44) (Fig. 2, *A* and *B*). The cross-linking by TGase 1 caused the appearance of two novel peaks (P1 and P2 on Fig. 2*B*), 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-link formation of two Gln donor peptides with a single Lys (Table I) and that the residues involved in N^{ϵ}-(γ-glutamyl)lysine cross-linking were Gln^{496} or Gln^{133} with Lys^{62}, 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 offered as an alternative glutaminyl acceptor substrate (data not shown), in agreement with previous findings that isopeptide bonds formed by amine substrates are energetically more favored products of TGase catalysis that ester bonds (27).

Interestingly, Lys^{62} has been precisely conserved in all mammalian involucrins (42), inferring that oligomerization through this residue is an ancient aspect of CE formation. However, cross-links involving Lys^{62} were not found in CEs recovered from mature foreskin epidermal stratum corneum tissue (41),

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. $K_{M(app)}$ values pertain to the protein, unless otherwise stated. V_{max} and K_{cat} data are that of putrescine incorporation. Values with 0% CSO_4 are the same as published before (30).

	V_{max}	$K_{cat(putrescine)}$	$K_{M(app)}$	K_{cat}/K_M
	pmol min^{-1}	*min^{-1}*	*μM*	*min^{-1} nM*
0% CSO_4				
Putrescine			400 ± 50	
Succinylated casein	3.2 ± 0.2	3.4	145 ± 18	23 ± 5
Involucrin	2.4 ± 0.4	2.55	2.9 ± 0.4	880 ± 90
4% CSO_4				
Putrescine			530 ± 65	
Succinylated casein	3.4 ± 0.3	3.6	125 ± 16	28 ± 4
Involucrin	2.7 ± 0.3	2.9	2.8 ± 0.3	1030 ± 120
6% CSO_4				
Putrescine			715 ± 95	
Succinylated casein	1.6 ± 0.2	1.7	161 ± 21	10 ± 3
Involucrin	1.9 ± 0.3	2.0	3.9 ± 0.5	520 ± 105
8% CSO_4				
Putrescine			920 ± 130	
Succinylated casein	0.6 ± 0.2	0.64	193 ± 18	4 ± 1.3
Involucrin	0.35 ± 0.1	0.37	5.2 ± 0.6	70 ± 25

but cross-links between Lys^{62} and Gln^{496} 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. 1*D*). 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. 3*A*). Assaying the amounts of N^{ϵ}-(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. 3*B*).

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. 4*A*). 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_{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_{max} with both protein substrates. However, for the substrate putrescine, membrane CSO_4 caused a dose-dependent increase of $K_{m(app)}$ and thus reduced the catalytic efficiency (K_{cat}/K_M) values, a phenomenon characteristic of competitive inhibition.

Effect of SLV Content of CSO_4 on ω-Hydroxyceramide Esterification by TGase 1—In addition, we explored the effects of CSO_4 on TGase 1-mediated ω-hydroxyceramide attachment to involucrin by esterification. One mol % of the artificial ω-hydroxyceramide 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. 5*A*). 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. 5*B*).

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

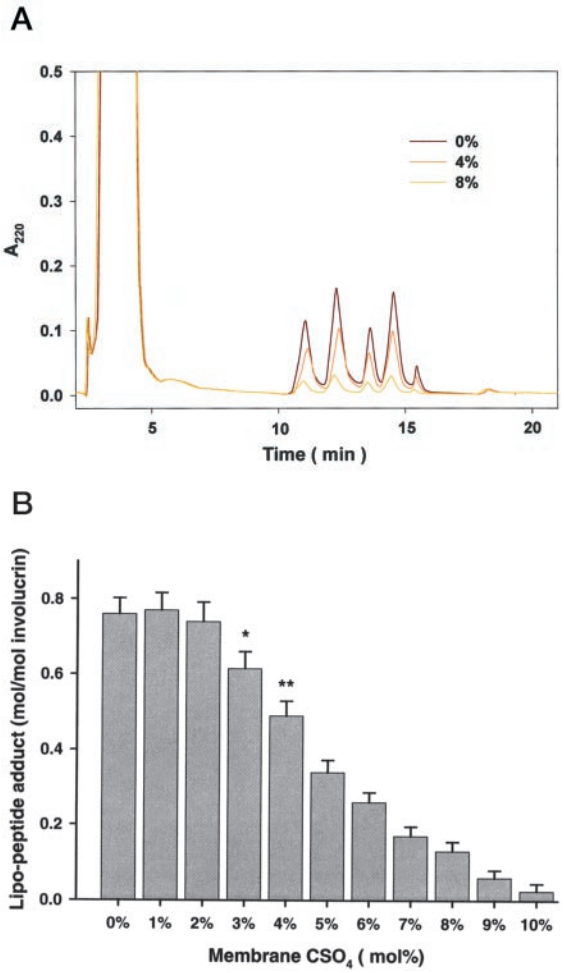

FIG. 5. **Increasing amounts of CSO_4 inhibits of involucrin esterification.** Involucrin was reacted with TGase 1 on SLV confectioned with 1 mol % of the ω-hydroxyceramide analog lipid Z and 0–10 mol % CSO_4. Lipid Z esterified onto involucrin was quantitated by isolating tryptic lipopeptides by C_4 HPLC (27). *A,* reduction of lipopeptide peaks with increasing CSO_4 is shown by superimposing the chromatograms of lipopeptides selectively retained on the column from the tryptic digest of 60 μg of involucrin labeled with lipid Z by TGase 1 on SLV with 0 (*red line),* 4 (*orange line*), and 8 (*yellow line*) mol % CSO_4. Quantitation of these reactions (*B*) revealed a significant decline of involucrin esterification by lipid Z at 3 (*, $p < 0.1$) or 4 (**, $p < 0.01$) mol % SLV CSO_4 content.

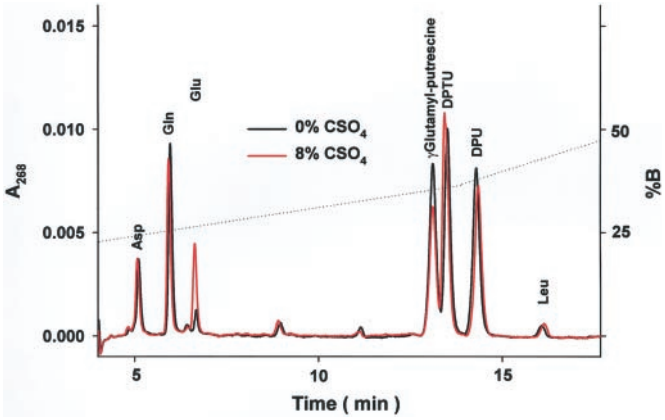

FIG. 6. **An assay for the rate of deamidation and putrescine cross-linking of reactive Gln residues by protein sequencing.** The figure shows superimposed elution chromatograms of residue Gln^{133} recovered from peptide peak 22 (see Fig. 2*A*) of involucrin reacted with TGase 1 in the presence of 20 mM putrescine on SLV made with 0 (*black line*) or 8 mol % (*red line*) membrane CSO_4. Peak areas were utilized to calculate the ratio of different Gln modifications by TGase 1.

to resolve 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 CSO_4 Content—Five Gln residues (Gln^{107}, Gln^{118}, Gln^{122}, Gln^{133}, and Gln^{496}) 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 transglutamination as one of four fates of each reactive Gln residue, we formulated SLV with different membrane CSO_4 contents, and used the following different substrate conditions: wild type involucrin or its K62N mutant were reacted either with only itself, or with 1 mol % lipid Z in SLV. As controls, we also used 20 mM putrescine as a competitive inhibitor, in which case the EP derivative is formed.

Following reaction, involucrin was fragmented with trypsin, and where applicable, lipid Z-attached peptides were extracted from the peptide pool by selective retardation on the C_4 HPLC column (Fig. 5*A*). Lipopeptides were quantified by amino acid analysis following acid hydrolysis. The remainder of the peptides was lyophilized and separated by C_{18} HPLC. Peaks harboring reactive Gln residues were collected based on their known chromatographic elution properties (44). Peaks of cross-linked peptides (Fig. 2*B*) 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 Gln^{107}, Gln^{118}, Gln^{122}, Gln^{133}, and Gln^{496} 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 CSO_4 in the absence of any other glutamyl-acceptor substrate, 51% of the Gln^{496} residue was modified, of which most was engaged in cross-link formation with Lys^{62} (Fig. 7*A*). Inclusion of increasing amounts of CSO_4 into SLV greatly decreased the percentage utilization of the Gln^{496} for cross-link formation, but steadily increased the amount of deamidation, so that in SLV with 10% CSO_4, 53% was deamidated and only 8% was used for cross-link formation. Similar deamidation rates were seen for the Gln^{133} residue which also participated in cross-link formation. For Gln^{107}, Gln^{118}, and Gln^{122} which did not engage in N^{ϵ}-(γ-glutamyl)lysine cross-link formation with Lys^{62}, most was likewise deamidated (Fig. 7*G*, other data not shown). Next, we used SLV containing 1 mol % of the synthetic ω-hydroxyceramide substrate lipid Z. In the case of Gln^{496}, 1 mol % lipid Z could not effectively compete out the isopeptide cross-link formation, but about 5% was used for ester formation in the absence of CSO_4 (27) (Fig. 7*B*). However, all was lost to deamidation by 6% membrane CSO_4. For Gln^{107}, Gln^{118}, Gln^{122}, and Gln^{133}, 20–30% was used for ester formation in the absence of CSO_4, and again, an increasing percentage of deamidation eradicated this reaction product by 8–10 mol % CSO_4 (Fig. 7*H* for Gln^{122}, other data not shown). Finally, addition of 20 mM putrescine to the above system led to near complete modification of Gln^{496} to EP and efficiently suppressed the cross-linking and deamidation by TGase 1 as expected (44). Even so, there was a small but significant increase in deamidation rate at the highest levels of CSO_4 tested (Fig. 7*C*). Essentially identical results were seen with the other four

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 (:γ-glutamylputrescine) ratios from sequencing yields. Numbers represent mean of three determinations rounded to whole percentage. NA, not available.

Substrates	CSO_4	Residue	Glutamine	Glutamate	γ-Glutamylputrescine	ε(γ-Glutamyl)lysine	(γ-Glutamyl)lipid Z
	mol			%		%	
Only involucrin (wild type)	0	496	49	5	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	1	79	0	NA
		133	34	0	66	0	NA
		122	31	1	68	0	NA
		118	39	0	61	0	NA
		107	72	0	38	0	NA
	4	496	21	2	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	NA	37	5
		133	73	3	NA	16	8
		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	46	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

TABLE III—continued

Substrates	CSO_4	Residue	Glutamine	Glutamate	γ-Glutamylputrescine	ε(γ-Glutamyl)lysine	(γ-Glutamyl)lipid Z
Only involucrin (K62N mutant)	0	496	19	81	NA	0	NA
		133	32	68	NA	0	NA
		122	32	68	NA	0	NA
		118	38	62	NA	0	NA
		107	32	68	NA	0	NA
	4	496	18	82	NA	0	NA
		133	30	70	NA	0	NA
		122	29	71	NA	0	NA
		118	37	63	NA	0	NA
		107	29	71	NA	0	NA
	6	496	16	84	NA	0	NA
		133	28	72	NA	0	NA
		122	26	74	NA	0	NA
		118	37	63	NA	0	NA
		107	27	73	NA	0	NA
	8	496	13	87	NA	0	NA
		133	26	74	NA	0	NA
		122	24	76	NA	0	NA
		118	35	65	NA	0	NA
		107	25	75	NA	0	NA
	10	496	11	89	NA	0	NA
		133	24	76	NA	0	NA
		122	23	77	NA	0	NA
		118	33	67	NA	0	NA
		107	22	78	NA	0	NA
Involucrin (K62N mutant) + 20 mM putrescine	0	496	13	0	87	0	NA
		133	28	1	71	0	NA
		122	29	0	71	0	NA
		118	36	1	63	0	NA
		107	30	1	69	0	NA
	4	496	14	1	85	0	NA
		133	27	1	72	0	NA
		122	28	2	70	0	NA
		118	35	3	62	0	NA
		107	30	2	68	0	NA
	6	496	12	4	84	0	NA
		133	27	4	69	0	NA
		122	27	5	68	0	NA
		118	34	6	60	0	NA
		107	29	7	64	0	NA
	8	496	11	11	88	0	NA
		133	26	9	65	0	NA
		122	26	9	65	0	NA
		118	34	8	58	0	NA
		107	27	11	62	0	NA
	10	496	9	16	75	0	NA
		133	25	15	60	0	NA
		122	26	14	60	0	NA
		118	33	15	52	0	NA
		107	26	15	59	0	NA
Involucrin (K62N mutant) + 1 mol % lipid Z	0	496	25	62	NA	0	13
		133	33	60	NA	0	7
		122	33	54	NA	0	13
		118	39	46	NA	0	15
		107	33	51	NA	0	16
	4	496	24	74	NA	0	2
		133	32	65	NA	0	3
		122	31	63	NA	0	6
		118	37	54	NA	0	9
		107	30	63	NA	0	7
	6	496	22	78	NA	0	0
		133	31	69	NA	0	0
		122	29	70	NA	0	1
		118	36	62	NA	0	2
		107	29	70	NA	0	1
	8	496	21	79	NA	0	0
		133	29	71	NA	0	0
		122	28	72	NA	0	0
		118	34	66	NA	0	0
		107	27	63	NA	0	0
	10	496	17	83	NA	0	0
		133	28	72	NA	0	0
		122	26	74	NA	0	0
		118	33	67	NA	0	0
		107	24	76	NA	0	0
		122	58	42	NA	0	0
		118	76	22	NA	0	2
		107	61	39	NA	0	0

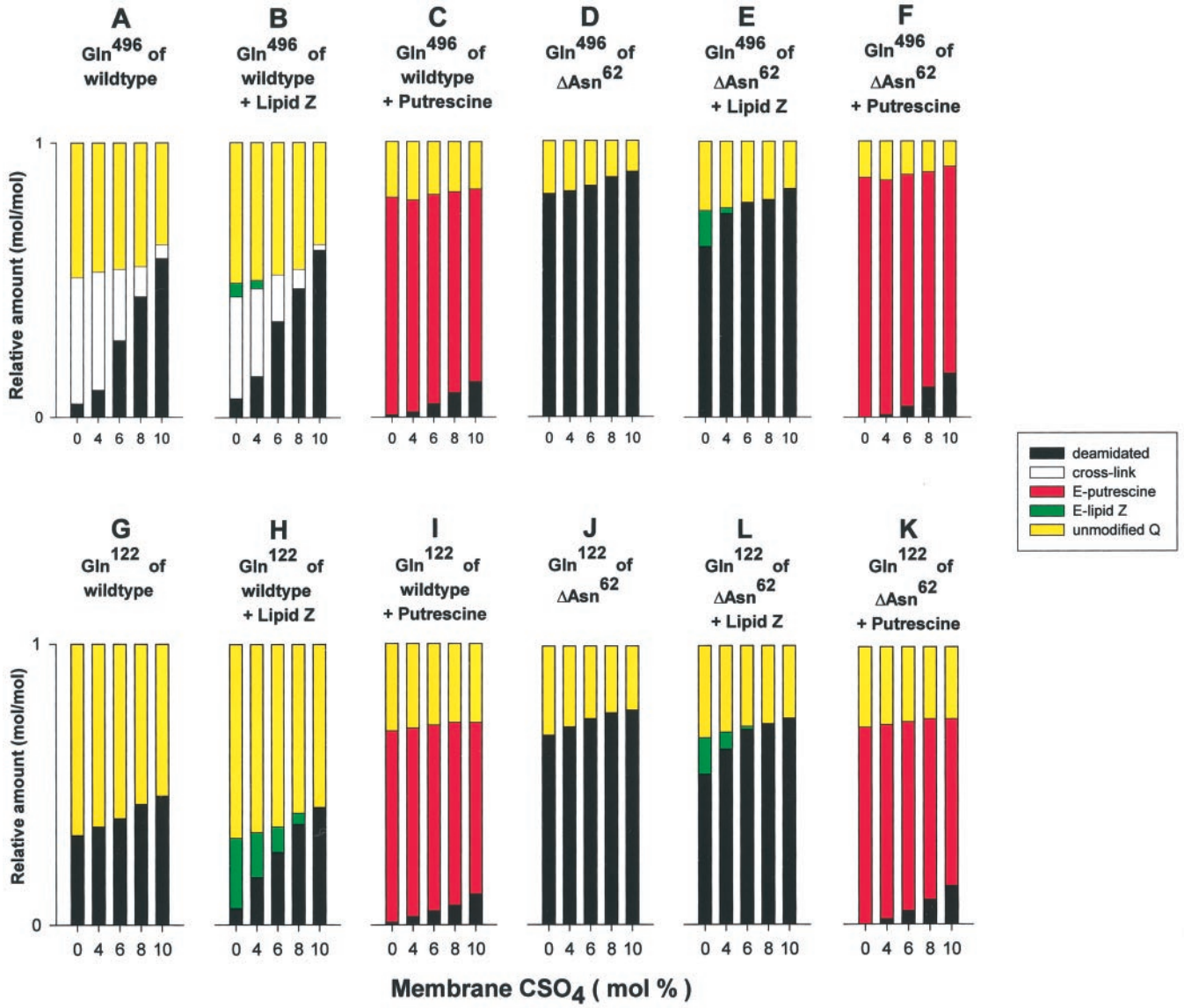

FIG. 7. **The fates of TGase 1 reactive residues Gln^{496} and Gln^{122} of involucrin with different substrates at increasing membrane CSO_4 levels.** The degrees of modifications of each Gln residue were quantitated as determined in Figs. 5*A* and 6. Shown here are the data for the most reactive Gln^{496} residue (*A-F*), which participates in both isopeptide and ester bond formation, and for Gln^{122} (*G-L*) which is involved only in ester formation under physiologically relevant conditions. The applied substrates and color codes are noted for each panel.

reactive Gln residues (Fig. 7*I*; 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 CSO_4 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 CSO_4, but this was lost to deamidation at ≥6 mol % CSO_4 (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 CSO_4, 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 Gln^{496}, Gln^{133}, and Lys^{62} can be used for N^{ϵ}-(γ-glutamyl)lysine 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 Lys^{62} to the acyl-enzyme complex when involucrin reacts with TGase 1 on its Gln^{107}, Gln^{118}, or Gln^{122}.

We also noted that cross-linking through Lys^{62} 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 Lys^{62} increased the yield of especially Gln^{107}, Gln^{118}, Gln^{122}, and Gln^{133} 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

(27). Thus it is possible that once an isopeptide bond is, practically irreversibly (52) formed through Lys^{62}, which is located near the reactive Gln residues in the head domain of involucrin, the bulk of the cross-linked involucrins "saves" ester bonds from further ester-hydrolase activity by TGase 1 enzyme.

How Does CSO_4 Facilitate Deamidation of Reactive Gln Residues?—The most striking observation in the foregoing data is that the presence of ≥6% CSO_4 in SLV membranes markedly diverts the TGase 1 reaction mechanism of Gln residues from isopeptide or ester formation to deamidation. One possible mechanism for this phenomenon is that excess membrane CSO_4 distorts the structure of the glutamyl acceptor substrate binding pocket so as to favor the access of water to the acyl-enzyme thioester intermediate. As water is in great molar excess over other glutamyl acceptor substrates, and the usage of water as a substrate is detectable under all assay conditions using TGases (Fig. 7) (23, 29), it is to be expected that even a slight conformational change should be sufficient to increase the opportunity of water to attack the acyl-enzyme thioester intermediate. The substrate binding pockets of TGases (based on crystallographic data of factor XIIIa (34, 53)) are imagined as grooves on the enzyme surface, permitting the binding of two protruding residues on bulky proteins and the consecutive release of the cross-linked dimer. The repelling of water from the active site is done by several juxtaposed hydrophobic side chain residues surrounding the active site, ideally making the concentration of water in the acceptor substrate-binding groove equal to the concentration of water in the saturated vapor of the reaction medium. In reality, the use of water may prevent TGases from being trapped to their glutaminyl substrates in the absence of other utilizable acceptors, so that this deamidation reaction route may have practical usefulness in regenerating the enzyme after interaction with decoy substrates (29). CSO_4 may thus enhance the opportunity for this natural phenomenon.

Apparently the highest level of membrane CSO_4 compatible with near-optimal cross-link and ceramide ester formation is ≤6 mol %, which based on the distribution of total stratum granulosum lipids, might be 4.5–5.5% by weight (54), although the actual local surface density of this metabolite might be lower owing to dilution by plasma membrane lipids or may be higher owing to the amphipathic character of CSO_4. Nevertheless, this estimate of 4.5–5.5% is right in the range found in the lowest layers of normal stratum corneum, and is severalfold less than that measured in XI epidermis (55).

Conclusions: Consequences for Pathogenesis of Ichthyosis Diseases—The precise mechanism by which excessive levels of CSO_4 cause defective skin barrier function and disease in XI has heretofore not been well understood. It was proposed that since CSO_4 has trypsin and chymotrypsin inhibitory properties *in vitro*, it might thereby affect breakdown of desmosomes, thus causing retention hyperkeratoses and abnormal scaling (18). The strong charge of its sulfate moiety conferring mild detergent properties to CSO_4 was shown to interfere with spontaneous sheet-formation of epidermal lipids *in vivo* and thus theorized to affect epidermal barrier function by deranging skin lipid layers, repressing cholesterol synthesis, and replacing cholesterol in the lipid sheets (16). More recently, it was demonstrated that CSO_4 can induce TGase 1 expression in cultured keratinocytes (19), but the connection between excess TGase expression and disease etiology remains unclear. Our present data offer an alternative biochemical explanation as to how accumulation of CSO_4 may cause XI disease. We show that CSO_4 inhibits the capacity of TGase 1 to perform isopeptide cross-linking as well as ester linkage of ceramides. Decreased isopeptide bonding is likely to disrupt the earliest stages of CE formation, by preventing cross-links between involucrin and desmoplakin or itself (41, 48) and thus derange the consecutive series of steps required for protein envelope formation. Furthermore, supraphysiological levels of CSO_4 inhibit lipid bound envelope formation and thus can interfere with the appropriate orientation and mechanical stability of the intercorneocyte lipid layers. In these ways, the net result is comparable to loss of TGase 1 function as observed in lamellar ichthyosis (34, 37, 38). Finally, it is possible that in addition to XI, other skin diseases having an ichthyosiform phenotype may also arise as a result of direct or indirect interference with effective TGase 1 function.

Acknowledgments—We are indebted to William W. Idler for expert assistance in expressing recombinant involucrins and are grateful to Peter M. Elias for stimulating discussions.

REFERENCES

1. Nemes, Z., and Steinert, P. M. (1999) *Exp. Mol. Med.* **3,** 5–19
2. Ishida-Yamamoto, A., and Iizuka, H. (1998) *Exp. Dermatol.* **7,** 1–10
3. Wertz, P. W., and Downing, D. T. (1991) in *Physiology, Biochemistry and Molecular Biology of the Skin* (Goldsmith, L. A., ed) Vol. 1, pp. 205–236, Oxford University Press, Oxford
4. Marekov, L. N., and Steinert, P. M. (1998) *J. Biol. Chem.* **273,** 17763–17770
5. Wertz, P. W. (1997) *Exper. Suppl.* (*Basel*) **78,** 227–237
6. Steinberg, D. S. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., and Frederickson, D. S., eds) 4th Ed., pp. 688–691, McGraw-Hill, New York
7. De Laurenzi, V., Rogers, G. R., Hamrock, D. J., Marekov, L. N., Steinert, P. M., Compton, J. G., Markova, N., and Rizzo, W. B. (1996) *Nat. Genet.* **12,** 52–57
8. Anton-Lamprecht, I. (1992) in *Diagnostic Ultrastructure of Non-neoplastic Diseases* (Papadimitrou, J. M., Henderson, D. W., Spagnolo, D. V., eds) pp. 459–551, Churchill Livingstone, New York
9. Shapiro, L. J., Weiss, R., Buxman, M. M., Vidgoff, J., Dimond, R. L., Roller, J. A., and Wells, R. S. (1978) *Lancet* **2,** 756–757
10. Epstein, E. H., Williams, M. L., and Elias, P. M. (1984) *J. Am. Acad. Dermatol.* **10,** 866–868
11. Ranasinghe, A. W., Wertz, P. W., Downing, D. T., and Mackenzie, I. C. (1986) *J. Invest. Dermatol.* **86,** 187–190
12. Long, S. A., Wertz, P. W., Strauss, J. S., and Downing, D. T. (1985) *Arch Dermatol. Res.* **277,** 284–287
13. Williams, M. L. (1992) *Semin. Dermatol.* **11,** 169–175
14. Rehfeld, S. J., Williams, M. L., and Elias, P. M. (1986) *Arch Dermatol. Res.* **278,** 259–263
15. Rehfeld, S. J., Plachy, W. Z., Williams, M. L., and Elias, P. M. (1988) *J. Invest. Dermatol.* **91,** 499–505
16. Zettersten, E., Man, M. Q., Sato, J., Denda, M., Farrell, A., Ghadially, R., Williams, M. L., Feingold, K. R., and Elias, P. M. (1998) *J. Invest. Dermatol.* **111,** 784–790
17. Lykkesfeldt, G., and Hoyer, H. (1983) *Lancet* **2,** 1337–1338
18. Sato, J., Denda, M., Nakanishi, J., Nomura, J., and Koyama, J. (1998) *J. Invest. Dermatol.* **111,** 189–193
19. Kawabe, S., Ikuta, T., Ohba, M., Chida, K., Ueda, E., Yamanishi, K., and Kuroki, T. (1998) *J. Invest. Dermatol.* **111,** 1098–1102
20. Chida, K., Murakami, A., Tagawa, T., Ikuta, T., and Kuroki, T. (1995) *Cancer Res.* **55,** 4865–4869
21. Ikuta, T., Chida, K., Tajima, O., Matsuura, Y., Iwamori, M., Ueda, Y., Mizuno, K., Ohno, S., and Kuroki, T. (1994) *Cell Growth Differ.* **5,** 943–947
22. Polakowska, R. R., Eickbush, T., Falciano, V., Razvi, F., and Goldsmith, L. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4476–4480
23. Folk, J. E., and Finlayson, J. S. (1977) *Adv. Protein Chem.* **31,** 1–133
24. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) *FASEB J.* **5,** 3071–3077
25. Gross, M., and Folk, J. E. (1974) *J. Biol. Chem.* **249,** 3021–3025
26. Parameswaran, K. N., and Lorand, L. (1981) *Biochemistry* **20,** 3703–3711
27. Nemes, Z., Marekov, L. N., Fésüs, L., and Steinert, P. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8402–8407
28. Folk, J. E., and Chung, S. I. (1985) *Methods Enzymol.* **113,** 358–375
29. Folk, J. E., and Chung, S. I. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol.* **38,** 109–191
30. Rice, R. H., Mehrpouyan, M., Qin, Q., and Phillips, M. A. (1994) in *The Keratinocyte Handbook* (Leigh, I. M., Lane, E. B., and Watt, F. M., eds) pp. 259–274, Cambridge University Press, Cambridge
31. Aeschlimann, D., Koeller, M. K., Allen-Hoffmann, B. L., and Mosher, D. F. (1998) *J. Biol. Chem.* **273,** 3452–3460
32. Candi, E., Melino, G., Mei, G., Tarcsa, E., Chung, S. I., Marekov, L. N., and Steinert, P. M. (1995) *J. Biol. Chem.* **270,** 26382–26390
33. Tarcsa, E., Candi, E., Kartasova, T., Idler, W. W., Marekov, L. N., and Steinert, P. M. (1998) *J. Biol. Chem.* **273,** 23297–23303
34. Candi, E., Melino, G., Lahm, A., Ceci, R., Rossi, A., Kim, I. G., Ciani, B., and Steinert, P. M. (1998) *J. Biol. Chem.* **273,** 13693–13702
35. Steinert, P. M., Kim, S. Y., Chung, S. I., and Marekov, L. N. (1996) *J. Biol. Chem.* **271,** 26242–26250
36. Rice, R. H., Rong, X. H., and Chakravarty, R. (1990) *Biochem. J.* **265,** 351–357
37. Russell, L. J., DiGiovanna, J. J., Rogers, G. R., Steinert, P. M., Hashem, N., Compton, J. G., and Bale, S. J. (1995) *Nat. Genet.* **9,** 279–283

38. Huber, M., Rettler, I., Bernasconi, K., Frenk, E., Lavrijsen, S. P., Ponec, M., Bon, A., Lautenschlager, S., Schorderet, D. F., and Hohl, D. (1995) *Science* **267,** 525–528
39. Matsuki, M., Yamashita, F., Ishida-Yamamoto, A., Yamada, K., Kinoshita, C., Fushiki, S., Ueda, E., Morishima, Y., Tabata, K., Yasuno, H., Hashida, M., Iizuka, H., Ikawa, M., Okabe, M., Kondoh, G., Kinoshita, T., Takeda, J., and Yamanishi, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1044–1049
40. Eckert, R. L., Yaffe, M. B., Crish, J. F., Murthy, S., Rorke, E. A., and Welter, J. F. (1993) *J. Invest. Dermatol.* **100,** 613–617
41. Steinert, P. M., and Marekov, L. N. (1997) *J. Biol. Chem.* **272,** 2021–2030
42. Green, H., and Djian, P. (1992) *Mol. Biol. Evol.* **9,** 977–1017
43. Jarnik, M., Simon, M. N., and Steven, A. C. (1998) *J. Cell Sci.* **111,** 1051–1060
44. Nemes, Z., Marekov, L. N., and Steinert, P. M. (1999) *J. Biol. Chem.* **274,** 11013–11021
45. Laemmli, U. K. (1970) *Nature* **227,** 680–685
46. Konigsberg, W. H., and Henderson, L. (1983) *Methods Enzymol.* **91,** 254–259
47. Tarcsa, E., and Fesus, L. (1990) *Anal. Biochem.* **186,** 135–140
48. Steinert, P. M., and Marekov, L. N. (1995) *J. Biol. Chem.* **270,** 17702–17711
49. LaCelle, P. T., Lambert, A., Ekambaram, M. C., Robinson, N. A., and Eckert, R. L. (1998) *Skin Pharmacol. Appl. Skin Physiol.* **11,** 214–226
50. Eckert, R. L., and Green, H. (1986) *Cell* **46,** 583–589
51. Steinert, P. M., and Marekov, L. N. (1999) *Mol. Cell. Biol.* **10,** 4247–4261
52. Parameswaran, K. N., Cheng, X. F., Chen, E. C., Velasco, P. T., Wilson, J. H., and Lorand, L. (1997) *J. Biol. Chem.* **272,** 10311–10317
53. Yee, V. C., Pedersen, L. C., Le Trong, I., Bishop, P. D., Stenkamp, R. E., and Teller, D. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7296–7300
54. Williams, M. L. (1991) *Adv. Lipid Res.* **24,** 211–262
55. Elias, P. M., Williams, M. L., Maloney, M. E., Bonifas, J. A., Brown, B. E., Grayson, S., and Epstein, E. H., Jr. (1984) *J. Clin. Invest.* **74,** 1414–1421

PROTEIN CHEMISTRY AND STRUCTURE:

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

Zoltán Nemes, Máté Demény, Lyuben N. Marekov, László Fésüs and Peter M. Steinert

J. Biol. Chem. 2000, 275:2636-2646.
doi: 10.1074/jbc.275.4.2636