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Effect of Oxazolidine E on Collagen Fibril Formation and Stabilization of the Collagen Matrix

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Oxazolidine E, an aldehydic cross-linking agent, is used to impart hydrothermal stability to collagen. The purpose of this study was to investigate the exact nature of oxazolidine E induced cross-links with collagen by using synthetic peptides having sequence homology with collagen type I. Tandem mass spectrometry revealed the formation of methylol and Schiff-base adducts upon reaction of oxazolidine E with the peptides. This was confirmed by allowing the reaction to proceed under reducing conditions using cyanoborohydride. Mass spectrometry (MS)−MS analysis clearly showed interaction of tryptophan and lysine residues with oxazolidine E and demonstrated that arginine could be cross-linked with glycine in the presence of oxazolidine E through the formation of a methylene bridge. Collagen fibrils regenerated from monomers in the presence and absence of oxazolidine E were studied using atomic force microscopy to investigate morphological alterations. Regenerated fibrils showing the typical 65 nm D-banding pattern were obtained from those formed both in the presence and absence of oxazolidine E, and there was no evidence of a change in the D-periodicity of these fibrils. This indicated that oxazolidine E does not hinder collagen molecules from correctly aligning to form the quarter-stagger structure.

KEYWORDS: Collagen; AFM; MS/MS; reconstituted collagen fibrils

INTRODUCTION

The extracellular matrix, a complex structural entity found within mammalian tissues, is essentially made up of collagen, water, proteoglycans, and elastic fibers. Collagen is the main structural component of the extracellular matrix and as such is the most abundant structural protein in animal connective tissues. Collagen molecules require the correct alignment of three polypeptides, consisting of Gly-Xaa-Yaa repeats, to form the characteristic triple helical structure of the protein (*1*). Collagens exist in various morphological forms ranging from complex interwoven structures to long fibrillar forms (*2*).

Collagen monomers, which are rod-shaped semiflexible molecules ∼1 nm in diameter and ∼280 nm in length, are known to self-assemble under favorable conditions (*3*). Type I collagen, the main constituent of skin, exhibits a fibrillar organization that is characterized by a banding pattern with a periodicity of 65 nm, and its fibrillogenesis has been studied extensively using a wide variety of techniques (*4*). Fibrils formed in vitro exhibit the characteristic 65 nm banding pattern of native fibrils and hence have been used to investigate the effect of various external factors such as temperature, ionic strength, and cross-linking agents on their formation (*5*, *6*).

It is possible to stabilize collagen by chemically cross-linking the fibrils with, for example, formaldehyde or chromium III (*7*, *8*). Chemical cross-linking agents in the biomedical, pharmaceutical, and biochemical fields have been extensively studied because of the many and varied applications of this chemistry. They have been used, for example, to inactivate toxins for vaccine production (*9*−*11*) and to produce materials for bioprostheses such as cardiovascular devices (*12*). The physical properties of formaldehyde induced intramolecular cross-linking in collagens have been investigated by Veis and Drake (*13*). More recently, Gayatri et al. (*6*) studied the effect of chromium III on the structural assembly of collagen.

In nature, collagens can be cross-linked through proteoglycans (which also play a mechanical role in tissue) by strong ionic interactions (*14*−*16*). Although a large number of studies have been carried out to characterize the effect of chemically induced covalent cross-linking on a macroscopic level (*17*, *18*), fewer reports have focused on characterizing the intermolecular interactions.

In the present study, the effect of the putative cross-linking agent oxazolidine E or II (1-aza-3,7-dioxabicyclo-5ethyl (3,3,0)

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octane) on collagen was investigated. Oxazolidines are heterocyclic derivatives obtained by the reaction of amino-hydroxy compounds with aldehydes. Oxazolidine E, which is predicted to cross-link protein molecules through the formation of a carbocationic intermediate formed as a result of ring opening (*19*), has been previously shown to impart hydrothermal and mechanical stability to collagen (*19*−*21*), as manifested by the higher shrinkage temperature of the treated hide (*19*, *20*).

To fully elucidate the interactions between the amino acids of collagen and the cross-linking agent, synthetic peptides having identical sequences to those found in collagen were subjected to mass spectrometric analysis both before and after reaction with oxazolidine E. Although many studies have been carried out to identify the amino acids involved in aldehyde-assisted cross-links (*22*, *23*) and the reactions that cause them (*20*, *21*, *24*), there is little evidence in terms of mass spectrometry or Edman sequencing to show that they actually occur.

Atomic force microscopy (AFM) was also used to study the effect of oxazolidine E on collagen. AFM is useful for analyzing biological systems at the ultrastructural level mainly because minimal sample preparation is required and little perturbation of the sample occurs during analysis (*25*). Because AFM has very good height resolution, particularly in deflection images, it is possible to differentiate minute ultrastructural and hence morphological differences between samples that are not as easily determined using electron microscopy (*26*). The in-vitro assembly of collagen fibrils in the presence of chromium has been previously studied using AFM (*6*). This technique was therefore deemed suitable to provide an insight into the oxazolidine E assisted in-vitro assembly of collagen fibrils including the possible structural changes in self-assembled collagen.

Association of collagen molecules into a fibrillar structure is an entropy driven self-assembly process that is characterized by a lag phase, a propagation phase, and an equilibrium phase between fibril formation and dissolution into monomers (*27*). Lag and growth phases measured during in-vitro collagen polymerization studies have shown that structures formed during the lag phase serve as nuclei helping subsequent polymerization of collagen (*28*). These studies have also shown that the self-assembly of collagen I monomers into fibrils is dependent on the interactions between different regions of the monomers and involves specific binding sites. Since self-assembly of collagen monomers is entropy driven, hydrophobic sequences in the triple helix are likely to contain specific binding sites as shown by Prockop and Fertala (*29*). The process of fibrillar rearrangement of collagen monomers can be studied either by enzymatic cleavage of collagen propeptides generated de novo or by incubating a neutral solution of extracted collagen from tissues at an elevated temperature (*29*−*31*). The process of fibril formation is similar in both cases although the shape and size of the fibrils formed are characteristic of the procedure used (*32*). Fibril formation involves both apolar and ionic interactions (*33*). The importance of hydrophobic interactions and hydrogen bonding in fibril formation is shown by the fact that hydrophobic alkyl ureas (*34*) and dihydroxy alcohols inhibit fibril formation (*35*). Aldol cross-links formed between two lysines (or hydroxylysine side chains) are known to stabilize side-by-side interaction of collagen helices leading to fibril formation (*36*).

In this study, it is shown that collagen monomers self-assemble into fibrils despite the presence of oxazolidine E, indicating that oxazolidine E does not result in adverse structural changes to collagen during cross-linking and does not disable correct alignment of the monomers and hence the formation of fibrils.

MATERIALS AND METHODS

Materials. Zolidine ZE (80% Oxazolidine E or II, 1-aza-3,7-dioxabicyclo-5-ethyl (3,3,0) octane) was from Angus Chemicals (Dow Chemicals), Chicago, IL. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium cyanoborohydride, acetic acid, formic acid, paraformaldehyde, picric acid, osmium tetroxide, ethylene-diamine-tetra-acetic acid (EDTA), and Tris(hydroxymethyl)aminomethane were purchased from Sigma (St. Louis, MO). Acetonitrile was from SDS (France). Phenylmethylsulfonyl fluoride (PMSF) and *n*-(2-hydroxyethyl) piperazine-*n*′-(2-ethanesulfonic acid) (HEPES) were from BDH Chemicals Ltd (Poole, United Kingdom). All other chemicals were of analytical grade. Synthetic peptides were obtained from Auspep Pty. Ltd. (Parkville, Australia).

Methods. *Reaction of Peptides with Oxazolidine E.* The reaction of a synthetic peptide with oxazolidine E was carried out according to the procedure described by Metz et al. (*37*). Ten microliters of a 10 mM peptide solution in water was added to 10 μL of 1 M potassium phosphate buffer at pH 7.5. To this, 5 μL of either $NaCNBH_3$ or water was added, and the volume of the reaction mix was made up to 95 μL with water. The reaction was initiated by adding 5 μL of an aqueous solution of 1.0 M oxazolidine E and was allowed to proceed at 35 °C for 48 h. Samples were stored at −20 °C before analysis.

Cross-Linking Glycine with Peptides Using Oxazolidines. The ability of the peptides to form cross-links with glycine was investigated by reacting all the peptides individually for 48 h in the presence of 50-fold excess of glycine and oxazolidine E. After the reaction, the samples were desalted and analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Removal of Excess Oxazolidine and Salts from Peptide Reaction Mix. Excess oxazolidine and salts were removed from the reaction mix by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Phenomenex Jupiter C4 300 angstrom (250 × 4.6 mm) column. Samples were diluted with water to a peptide concentration of 200 μM, and 40 μL of this diluted solution was injected onto the column. The column was washed in solvent A (0.1 M acetic acid in water) for 7 min before a linear gradient from 0 to 30% solvent B (0.1 M acetic acid in acetonitrile) was applied over 20 min to elute the peptide. The elution was monitored at 214 nm, and peaks were collected, were dried under vacuum (Savant Speed-vac, SC-100), and then were redissolved in 200 μL of water and were stored at −20 °C.

Electrospray Ionization (ESI)-MS Studies. ESI-MS studies were carried out using a Micromass ZMD mass detector in postive ion mode (m/z 400−2000). The capillary voltage was set at 3.5 kV and the cone voltage at 25 V.

ESI-MS/MS Studies. Positive ion ESI MS/MS was performed with a QStar XL (Applied Biosystems, United States). The samples were infused into the spectrometer through a fused silica capillary at a rate of 3 μL/min using a syringe pump (Harvard Apparatus, South Natick, MA, Model 2400-001). MS/MS parameters for the fragmentation experiments were optimized by using a declustering potential of 60 V, a focusing potential of 265 V, an ion spray voltage of 5500 V, and a collision energy of 40 V.

MALDI-TOF MS of Collagen Peptides. The rapid evaporation method was used for the sample preparation for MALDI-TOF analysis (*38*). One microliter of peptide matrix/nitrocellulose solution (10 mg nitrocellulose and 40 mg α-cyano-4-hydroxy-trans-cinnamic acid in 1 mL acetone and 1 mL isopropanol, prepared fresh) was applied to the sample target and was allowed to evaporate to dryness. Two microliters of the peptide solution was then concentrated and desalted using ZipTips (Millipore, USA, Cat. # ZTC18S024) according to the manufacturer's instructions. One microliter of the peptide solution eluted from the ZipTip was directly pipetted onto the matrix and was evaporated to dryness at ambient temperature.

The sample target was inserted into the MALDI-TOF mass spectrometer (M@LDI Micromass) and was analyzed in positive ion reflectron mode.

Preparation of Soluble Collagen. Skin samples were cut into small pieces and were homogenized using a blender (Watson Victor Ltd, Model # 32BL88) in 10 volumes of cold isotonic medium consisting of 0.25 M sucrose, 10 mM HEPES, pH 7, 2 mM EDTA, and 1 mM

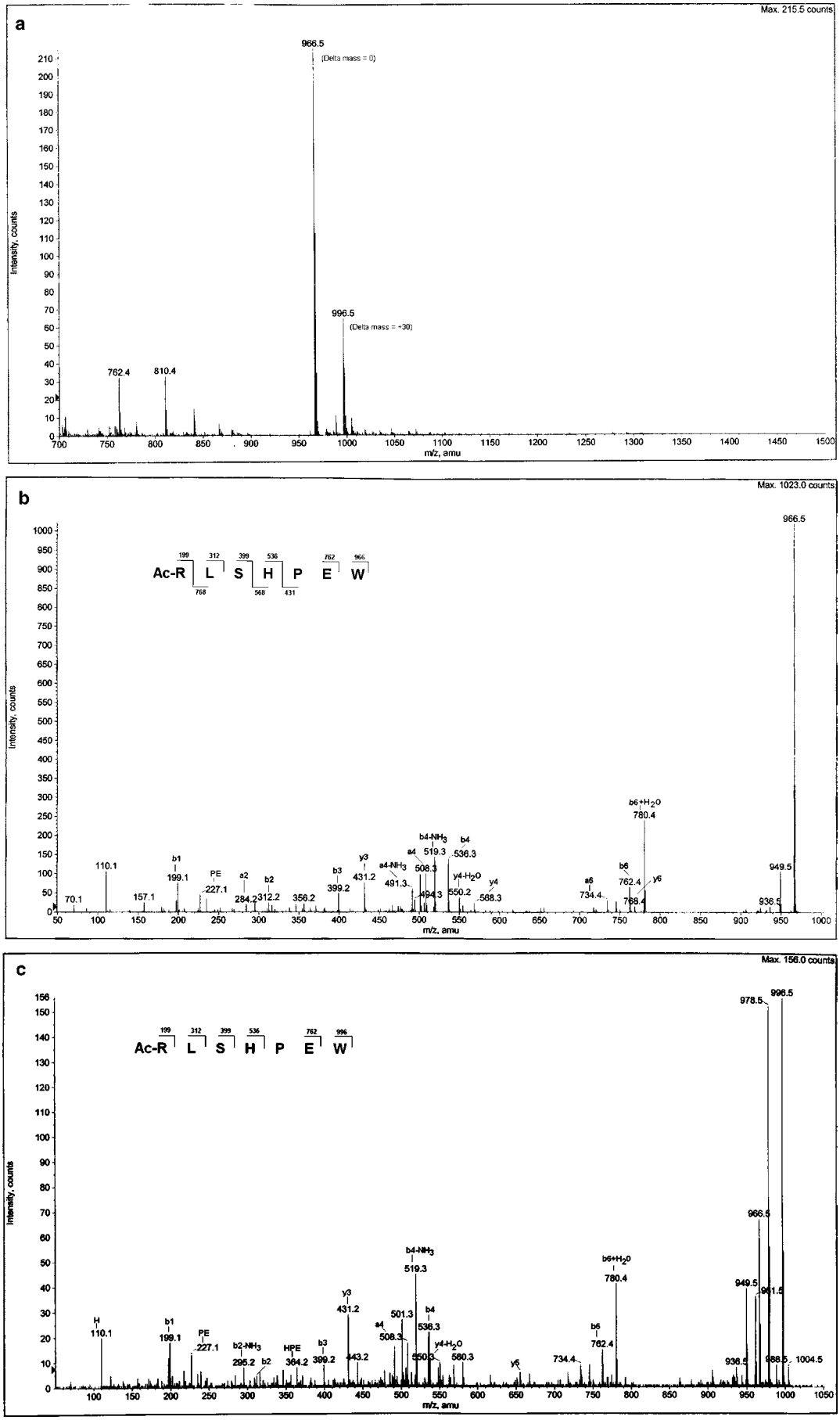

Figure 1. (**a**) MS spectra of peptide 2 (*m*/*z* 966.5) showing the formation of a product with a mass increase of 30 Da (*m*/*z* 996.5) upon reaction with oxazolidine E in the presence of $NaCNBH_3$. (**b**) MS/MS spectra of the peak at *m*/*z* 966.5. (**c**) MS/MS spectra of the peak at 996.5 formed after oxazolidine E reaction in the presence of $NaCNBH_3$, showing the modification at the tryptophan residue.

PMSF for 2 min at 4−5 °C. The homogenate was then centrifuged for 30 min at 7500*g*, and the residue was suspended in 10 volumes of extraction buffer containing 0.275 M NaCl, 50 mM Tris-HCl, pH 8, and 1 mM PMSF with stirring at 4−5 °C for 4 h. Insoluble material was removed by centrifugation at 20000*g* for 30 min, and the supernatant was collected and stored at −20 °C until further use (*39*).

Soluble collagen was prepared according to the method of Chandrakasan et al. (*40*). The extracted collagen was dialyzed overnight in 0.5 N acetic acid, and aggregates were removed by centrifugation at 7500*g*. The supernatant was then dialyzed against several changes of 0.02 M Na_2HPO_4. The precipitate formed was collected and dissolved in 0.5 N acetic acid and was dialyzed extensively against 0.1 N acetic acid.

Preparation of Reconstituted Collagen Fibrils for Microscopy. Collagen fibrils were reconstituted from collagen solution according to the method of Suzuki et al. (*41*). Collagen in 5 mM acetic acid was neutralized in a disposable polystyrene cuvette by addition of phosphate-buffered saline (3× concentrated) with gentle shaking on ice, and its final concentration was adjusted to 0.25 mg/mL. The neutralized collagen solution (pH 7.4) was incubated at 37 °C and was monitored at 530 nm to track fibril development.

Preparation of Collagen Fibrils Reconstituted with Oxazolidine E for Microscopy. Collagen fibrils were also produced in the presence of oxazolidine E using the same method as above, except that the solution was made up in 2% oxazolidine E. The pH was maintained at 8, and the solution was kept at 37 °C for at least 48 h to allow fibril formation.

Treatment of Reconstituted Collagen Fibrils with Cross-Linking Agents. Reconstituted collagen fibrils, prepared as described above (in the absence of oxazolidine E), were suspended in 1 mL of 2% oxazolidine E in an Eppendorf tube, and the pH was adjusted to 7.5 with formic acid. The cross-linking reaction was allowed to proceed for 24 h at 37 °C after which time the fibrils were rinsed with distilled water, were mounted on glass slides, and were air-dried at room temperature for AFM imaging.

Preparation of Samples for Scanning Electron Microscopy (SEM) Studies. To investigate the effect of oxazolidine on collagen fibrils in skin, thin sections of pickled skins (skins treated with sodium sulfide and lime, bated with enzymes, and then preserved in NaCl and sulfuric acid) and pickled skins treated with 2% oxazolidine E at pH 7.5 were cut using a mictrotome (NR 362306, Reichert, Austria). These sections were then fixed for 30 min with modified Zamboni's solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The samples were further fixed for 30 min with 1% osmium tetroxide in Zamboni's solution (*3*), then were prepared as circular samples (10 mm), were dehydrated, and were dried in a critical point dryer (Balzars SD050, Lichtenstein). The samples were then analyzed under a scanning electron microscope (Cambridge 250 mk3, Cambridge Instruments, Cambridge, United Kingdom).

AFM Procedure. An atomic force microscope (MFP-3D Asylum Research, Santa Barbara, CA) was used to image the samples described above. Silicon cantilevers Ultrasharp CSG-11 (NT-MDT, Zelenograd, Moscow, Russia) were used to study the samples with contact mode in air. The tip, nominally 10 nm in radius, was located on the free end of a cantilever 350 μm long, 35 μm wide, and 0.7−1.3 μm thick with a nominal force constant of 0.01−0.08 N/m. A tip speed of 40 μm/s was used to scan the samples.

RESULTS AND DISCUSSION

Reaction of Oxazolidine E with Synthetic Peptides. Three model peptides were chosen to systematically map the oxazolidine E induced modifications and to determine whether the collagen cross-linking does occur as a result of the action of this compound. The three peptides used to investigate possible cross-linking mechanisms were Ac-PGVKGEP-OH (peptide 1), Ac-RLSHPEW-OH (peptide 2), and Ac-GDFYRAD-OH (peptide 3). The amino acid sequences of all the three peptides appear commonly in collagen type I from mammalian sources. For example, peptide 1 corresponds to residues 193−199, peptide 2 to residues 1165−1171, and peptide 3 to residues 1112−1118, all from bovine collagen I (α2).

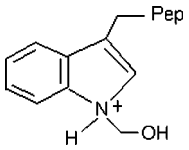

Figure 2. Schematic diagram showing the formation of a methylol adduct of tryptophan upon reaction with oxazolidine E.

Peptide 1 was chosen because it has an internal lysine which is one of the amino acids that supposedly reacts with cross-linking agents. The other amino acids in the sequence appear frequently in collagen and are not known to react with cross-linking agents. Peptide 2 was chosen because it contains histidine and tryptophan, residues that had both previously been shown to react with cross-linking agents (*37*, *42*), as well as arginine, which had been previously been shown to be modified by methylol groups in the presence of cross-linking agents (*37*). Peptide 3 was chosen because it contained tyrosine which is known to cross-link with labile Schiff's bases and arginine.

When oxazolidine E was reacted with peptides 2 and 3, the only change observed was the modification of tryptophan by the addition of a methylol group. It was somewhat surprising that arginine, histidine, and tyrosine did not appear to be affected by reaction with this reagent. The evidence for this finding is as follows.

First, it was observed that the product of the reaction between oxazolidine E and peptide 2 had a mass 30 Da greater than that of the peptide itself (**Figure 1a**). Comparison of the total ion count (TIC) indicated that there was a 35% conversion to this higher molecular weight product, which matched the addition of a methylol group to the peptide. At this stage, it was not known which residue had been modified, but fragmentation analysis showed that the increase in mass was due to modification of the tryptophan residue in peptide 2 by the addition of a methylol group (**Figures 1b**, **1c**, and **2**). The b- and their associated a-ions (b-ion, − CO) produced by collision-induced dissociation (CID) were easily distinguishable in the MS−MS spectra, while the y-ion series was less obvious. However, upon reaction with oxazolidine, some y-ions that had lost ammonia were detected. Although formaldehyde has been reported to produce imine derivatives of tryptophan (*42*), the dehydration of the methylol-tryptophan adduct to produce an imine was not observed in this study.

No evidence was obtained from our MS studies for adduct formation with arginine and histidine residues which is surprising, since Metz et al. (*37*) observed that methylol adducts could be formed with both arginine and histidine residues as well as with tryptophan. Similar results were seen with peptide 3, which contained both arginine and tyrosine but showed no changes after reaction with oxazolidine E. It is possible that the lack of modification of histidine and arginine may be a result of their position in the peptide sequences. Although this seems unlikely, it has previously been observed that specific peptide sequences seem to affect the equilibrium of the reaction of peptides with cross-linking agents such as formaldehyde, influencing the concentrations of methylol and imine adducts formed (*37*).

When peptide 1 was reacted with the oxazolidine E, a product with a mass increase of 12 Da was detected, which can be attributed to the formation of a Schiff base. To confirm this, the reaction was carried out in the presence of $NaCNBH_3$, which resulted in the formation of two products, with mass increases of 14 and 28 Da corresponding to methylation and dimethylation

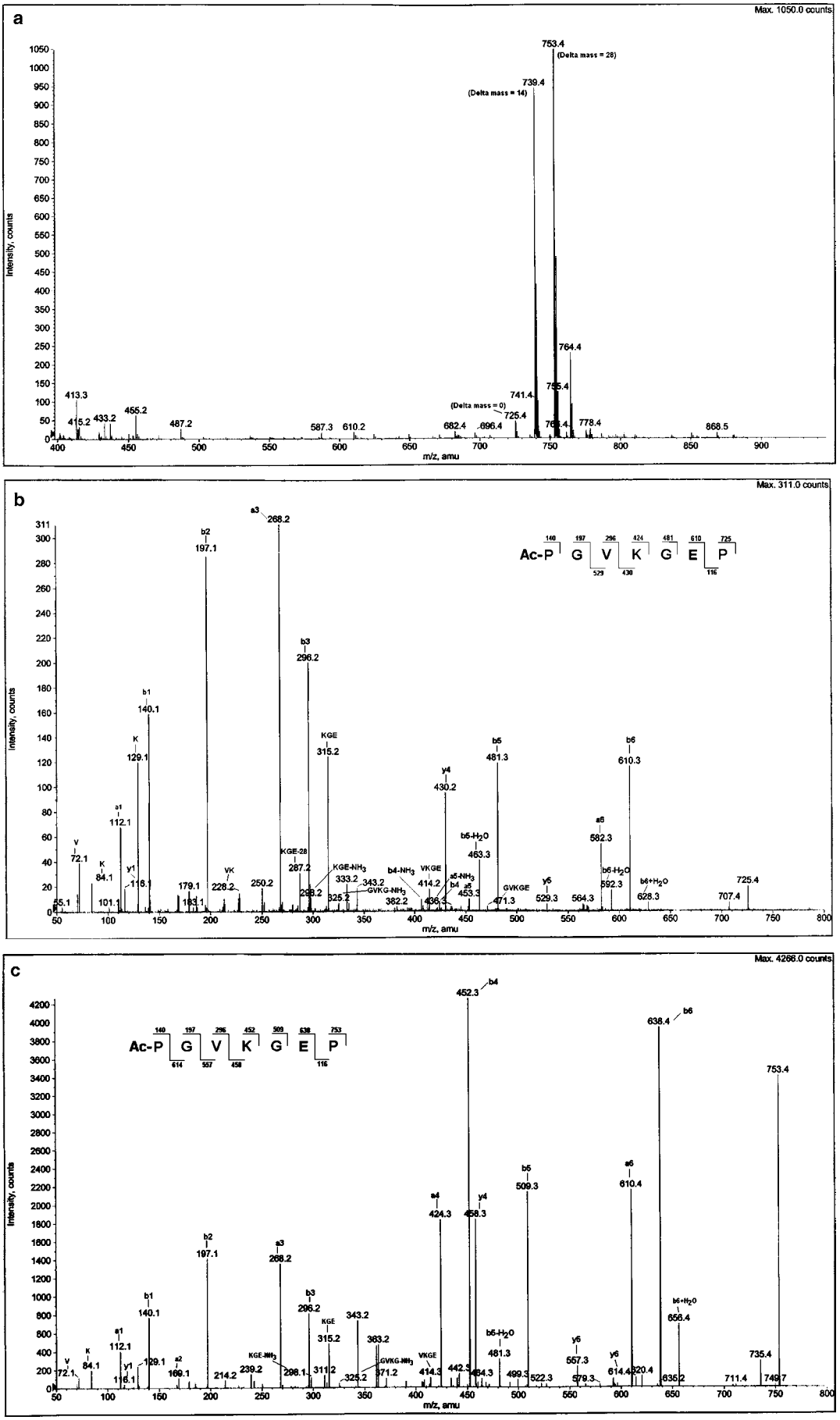

Figure 3. (**a**) MS spectra of peptide 1 (*m*/*z* 725.4) showing the formation of three products with mass increases of 14 Da (*m*/*z* 739.4), 28 Da (753.4), and 30 Da (755.4) upon reaction with oxazolidine E in the presence of $NaCNBH_3$. (**b**) MS/MS spectra of the peptide 1 (*m*/*z* 725.4). (**c**) MS/MS spectra of the peak *m*/*z* 753.4 formed after reaction of peptide 1 with oxazolidine E in the presence of $NaCNBH_3$, showing the modification of the lysine residue.

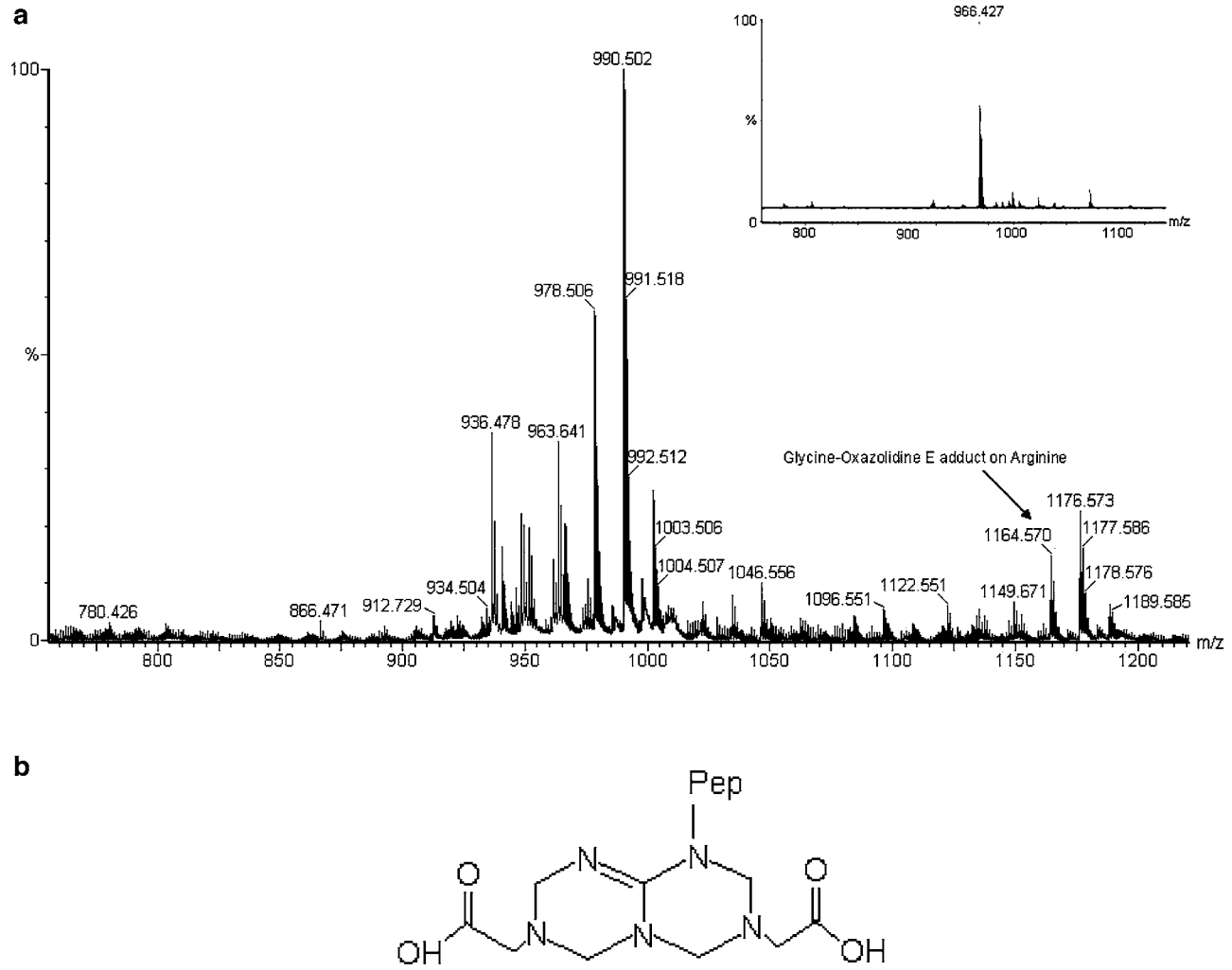

Figure 4. (**a**) MALDI-TOF MS showing the modification of the arginine residue in peptide 2 because of the attachment of two glycine-oxazolidine E adducts resulting in a mass increase of 198 Da. The major peak at *m*/*z* 990.5 represents the breakdown of the product by the loss of two molecules of $C_3H_5NO_2$ (*37*), probably induced by the ionization process. The inset shows the MALDI-TOF MS of the peptide before the reaction. (**b**) The proposed structure of the di-glycine-oxazolidine E adduct with arginine.

of the peptide, respectively (**Figure 3a**). MS/MS analysis of the peak with showing the mass increase of 28 Da (*m*/*z* 753.4) revealed that the modification resided on the lysine (**Figure 3b**, **3c**). A mass increase of 30 Da at *m*/*z* 755.4 was also detected, indicating the formation of a methylol adduct of the lysine residue.

Up to this point, there had been no real evidence of cross-linking, merely the suggestion that it was possible through the modification of lysine and tryptophan residues by oxazolidine E. The ability of oxazolidine E to cross-link a single amino acid to peptides 1−3 was therefore investigated. If the four potential cross-linking amino acids (arginine, histidine, tyrosine, and tryptophan) form methylol adducts in the presence of oxazolidine E, and if these are subsequently dehydrated to form Schiff's bases, then they should, in turn, react with a primary amino group. Glycine was chosen because it had been shown to form cross-links through a Schiff's base in a previous study (*37*).

No glycine/oxazolidine E adduct was found for peptide 1, which contained the internal lysine. This could be attributed to the instability of the methylene bridges in the acidic environment used during MS analysis and is in agreement with the results obtained by Metz et al. who reported the absence of any modifications of lysine residues of a peptide reacted with glycine in the presence of formaldehyde (*37*). However, a mass increase of 198 Da was obtained for peptides 2 and 3 (both of which contain arginine) indicating that a residue present in both peptides formed an adduct with glycine. The MALDI-TOF MS of the reaction with peptide 2 is shown in **Figure 4a**, and a schematic diagram for the proposed glycine-peptide cross-link is shown in **Figure 4b**. The difference in mass increase can be attributed to the coupling of two glycine residues to a single arginine residue via methylene bridges. As there was no modification of the arginine in peptides 2 and 3, it is reasonable to assume that the cross-link occurred because of modification of the α-amino group of the glycines by oxazoladine to form reactive imine adducts which were then able to interact with the arginine in peptides 2 and 3.

As noted above, peptide 3 containing tyrosine did not produce any detectable reaction product with oxazolidine E although previous work had shown that tyrosine residues in skin peptides were modified upon reaction with oxazolidines (*43*). This modification was not the result of a bridging interaction between oxazolidine tyrosine and another amino acid, but occurred because an imine, formed as a result of the interaction of oxazolidine with the primary amino side chain of lysine, was able to make a covalent bond with the side chain of tyrosine. Similar interactions have been reported between lysine adducts and arginine and to a lesser extent glutamine, asparagine, tryptophan, and histidine (*37*).

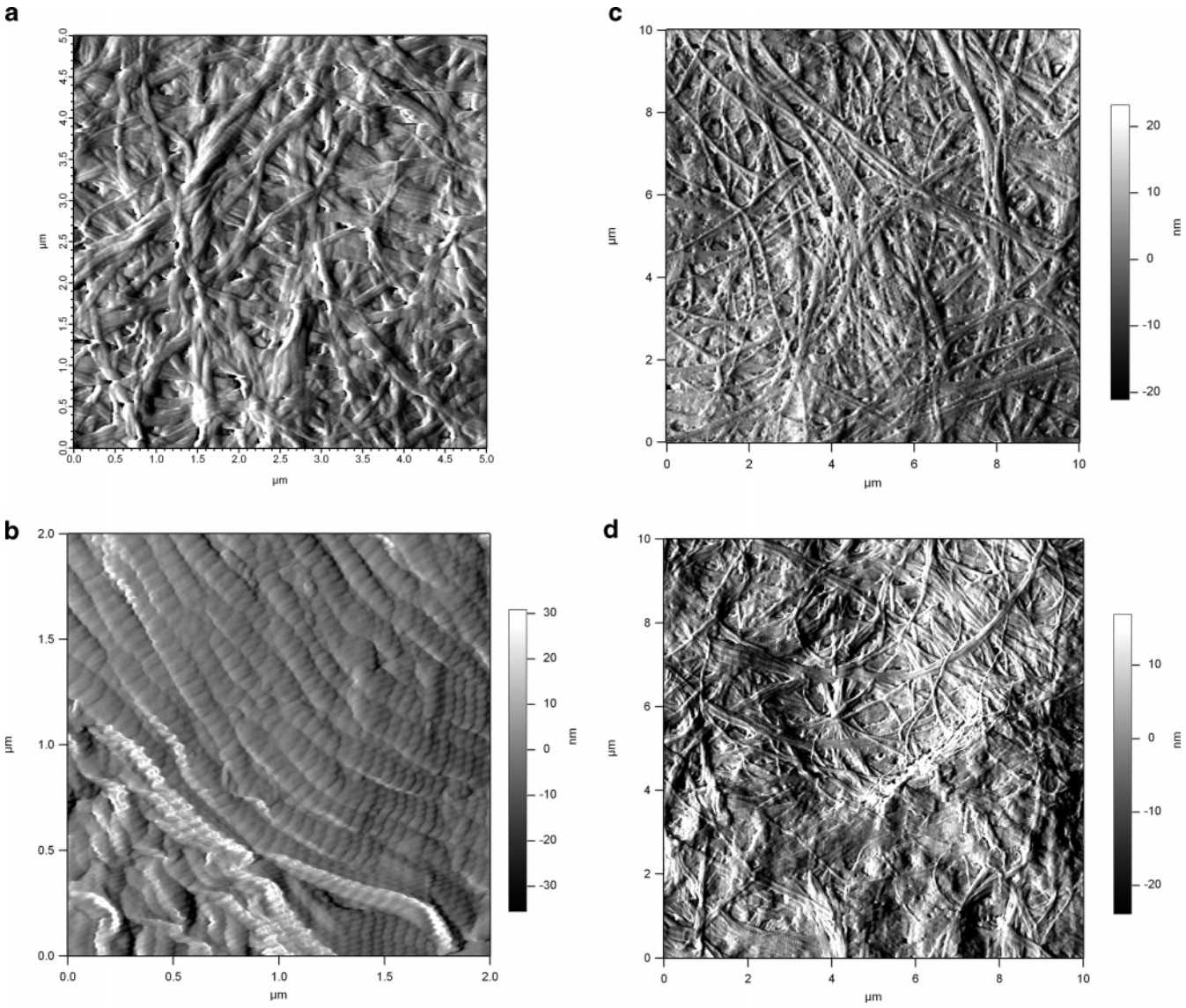

Figure 5. (**a**) AFM image of collagen fibrils self-assembled from the monomers in the presence of oxazolidine E. (**b**) AFM image of a thin section of skin showing collagen fibrils. (**c**) AFM image of regenerated fibrils from collagen monomers in the absence of oxazolindine E. (**d**) AFM image of regenerated collagen fibrils subsequently cross-linked with oxazolidine E showing a well-oriented structure.

Although the results obtained from this study do not provide evidence for oxazolidine bridges forming cross-links between collagen molecules, or indeed within the same molecule, they have shown that oxazolidine is able to form adducts that can then interact with amino acid side chains from the same or other polypeptide chains to form either inter- or intramolecular links. The inability to detect such cross-links between the peptides used in this study could have been due to the experimental conditions used or to the lower reactivity of the amino acids in the particular peptides used. As previously reported, these reactions can be influenced by various factors such as the local environment of each reactive amino acid in the protein, the rate of the cross-linking reaction, the pH, and the reactant concentrations (*37*).

AFM Studies of Self-Assembly of Collagens. Self-assembly of collagen monomers in the presence of oxazolidine has been attempted for the first time in this present study. Cross-linking of collagen fibrils by oxazolidine is thought to be the reason for the increased stability of the treated collagen fibrils that is manifested by a higher shrinkage temperature (*19*, *20*). Collagen monomers clearly formed fibrils in the presence of phosphate ions at pH 8 and in the presence of oxazolidine E as shown by the AFM images where the typical collagen banding pattern of grooves and ridges can be seen (**Figure 5a**).

The width of the D-periodicity of collagen fibrils from skin and regenerated collagen fibrils did not appear to be significantly different. Also, there was not a difference when the fibrils were formed in the presence or in the absence of oxazolidine E. Measurements of collagen fibrils in skin (**Figure 5b**) showed them to have a mean D-periodicity of 62 nm with a standard deviation of 9 ($n = 54$); measurements of fibrils formed in the absence of oxazolidine E (**Figure 5c**) had a mean D-periodicity of 67 (*9*) nm ($n = 13$); those formed in the presence of oxazolidine E (**Figure 5a**) had a mean D-periodicity of 61 (*15*) nm ($n = 20$); and those formed in the absence of oxazolidine E and then treated with oxazolidine E (**Figure 5d**) had a mean D-periodicity of 66 (*8*) nm ($n = 42$). These distances are not significantly different and are characteristic of the axial periodicity of collagen fibrils. The quarter-staggered fibrils were formed from the collagen monomers only under alkaline conditions (pH > 7.5), as a gel was obtained at lower pH without any evidence of fibrillar arrangement.

While the D-periodicity did not vary, neither was there a statistically significant difference between the widths of the

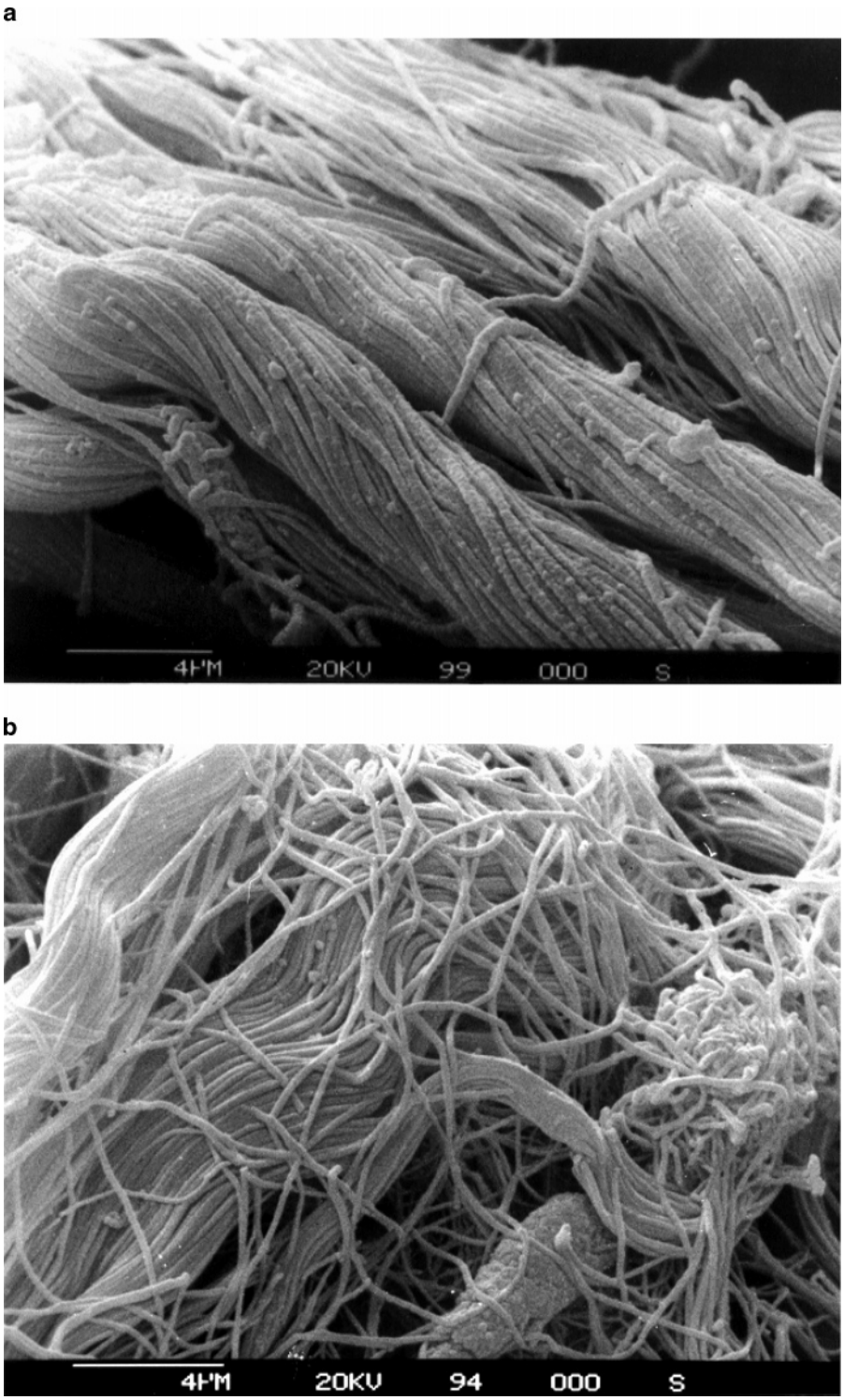

Figure 6. (**a**) SEM image of collagen fibrils in skin cross-linked with oxazolidine E showing well-oriented collagen fibril bundles. (**b**) SEM image of collagen fibrils in skin.

fibrils. Measuring widths of fibrils is more difficult than measuring the D-periodicity and is also more subjective as the fibrils form overlapping networks where the sides of a fibril may not always be clearly determined. The fibril widths measured can therefore only be considered as approximate. Measurements of collagen fibrils in skin (**Figure 5b**) showed that they had a mean width of 123 nm with a standard deviation of 20 ($n = 16$); fibrils formed in the absence of oxazolidine E (**Figure 5c**) had a mean width of 82 (*23*) nm ($n = 7$); fibrils formed in the presence of oxazolidine E (**Figure 5a**) had a mean width of 72 (*12*) nm ($n = 16$); fibrils formed in absence of oxazolidine E and then treated with oxazolidine E (**Figure 5d**) had a mean width 92 (*17*) nm ($n = 17$). These values lie within the range from 70 to 140 nm as has been previously reported (*44*, *45*). A means *t*-test comparing the values for the width of the collagen fibrils in skin with any of the regenerated fibrils calculated that in each case there is around a 50% probability that the means are different and, considering the subjective nature of the measurements, are insufficient to claim any real difference.

The ability of the collagen monomers to align in a quarter-stagger arrangement in the presence of oxazolidine E (**Figure**

5a) shows that the size of these molecules does not hinder the formation of collagen fibrils. The effects of cross-linking reagents on collagen triple-helix formation have been studied by previous workers (*46*) who reported that the helical content of regenerated collagen fibrils in the presence of cross-linkers was greater than that of regenerated fibrils in the absence of cross-linkers. They attributed this increase to the ability of the cross-linking agents to facilitate the assembly of collagen into the triple helices by bringing the polypeptide chains closer to each other. Although it has been shown that oxazolidine E results in the formation of reactive adducts of some amino acid residues found in collagen, which in turn may result in the formation of inter- or intramolecular cross-links between collagen molecules, it does not appear to affect the structure or the arrangement of the collagen fibrils.

A similar study performed with Cr(III) complexes showed that though Cr(III) dimers helped form the quarter-stagger structure, Cr(III) trimers induced a fibrous network of aggregated collagen molecules that lacked the D-periodicity, possibly because of size constraints (*6*). Gelman et al. (*47*) reported that the presence of aldehydes during lateral assembly of collagen filaments decreased the rate of lateral disassembly thus helping a faster net rate of stable fibril formation. In our study with oxazolidine E, we do not observe an increase in collagen molecules lacking D-periodicity or a fibrous aggregated network. On the contrary, AFM analysis of regenerated collagen fibrils cross-linked with oxazolidine E revealed a well-ordered structure with the fibrils properly oriented and well aligned compared to collagen fibrils in the absence of oxazolidine E (**Figure 5c**, **5d**).

SEM Analysis of Collagen Fibrils. AFM images of collagen fibrils cross-linked with oxazolidine E showed a well-ordered structure, but the effect the cross-linker was more pronounced when thin pieces of skin in the presence or in the absence of oxazolidine E were magnified through scanning electron microscopy. In the presence of oxazolidine E, the collagen fibrils of skin appeared compact and well oriented (**Figure 6a**) compared to the untreated sample (**Figure 6b**). This effect of oxazolidine on the ordering of collagen fibrils was also noted by D'Aquino et al. while studying the effect of oxazolidine on shrinkage temperature of collagen fibrils of skin (*19*).

In this study, we have demonstrated the interaction of the amino acids present in collagen with oxazolidine E, using MS/MS analysis. Synthetic peptides having sequence homology with collagen were used to simplify the study of reaction of the amino acids with the cross-linker. MS/MS analysis clearly showed the formation of methylol adducts with tryptophan and lysine residues but not with arginine, histidine, or tyrosine. The cross-linking reactions with lysine were carried out in a reducing atmosphere in the presence of $NaCNBH_3$ and resulted in methylation of the Schiff's base formed by lysine residues. Intermolecular cross-links between peptides were not observed in our study, but this could have been due to factors such as the position and local environment of each reactive amino acid in the peptide, the pH, the reactant concentration, and the rate of the reaction. When oxazolidine E was added to a solution containing a model peptide and glycine, two glycines were cross-linked to the peptide through methylene bridges indicating that it is possible for this molecule to produce cross-links between primary amine groups such as those found on lysine and the side chains of other amino acids.

Regeneration of collagen fibrils from monomers in the presence of oxazolidine E was attempted for the first time in this study. The regenerated fibrils, studied using AFM, showed the formation of the quarter-stagger structure, with a 65 nm banding pattern that was preserved in fibrils regenerated both in the presence and in the absence of oxazolidine E. Alignment of the collagen monomers into a quarter stagger in the presence of oxazolidine E shows that the aldehyde does not impose a size constraint in the self-assembly process. SEM analysis indicated that the oxazolidine E may in fact help in ordering the collagen fibrils.

Therefore, in conclusion, we have provided a better understanding of the reaction of oxazolidine E with the amino acids present in collagen. Our results have shown the involvement of lysine and tryptophan in oxazolidine E induced cross-linking. We have also provided evidence of the role played by the cross-linker in the self-assembly and proper alignment of the collagen molecules.

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