Elastin is heterogeneously cross-linked

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Edited by Gerald W. Hart

Elastin is an essential vertebrate protein responsible for the elasticity of force-bearing tissues such as those of the lungs, blood vessels, and skin. One of the key features required for the exceptional properties of this durable biopolymer is the extensive covalent cross-linking between domains of its monomer molecule tropoelastin. To date, elastin's exact molecular assembly and mechanical properties are poorly understood. Here, using bovine elastin, we investigated the different types of cross-links in mature elastin to gain insight into its structure. We purified and proteolytically cleaved elastin from a single tissue sample into soluble cross-linked and noncross-linked peptides that we studied by high-resolution MS. This analysis enabled the elucidation of cross-links and other elastin modifications. We found that the lysine residues within the tropoelastin sequence were simultaneously unmodified and involved in various types of cross-links with different other domains. The Lys–Pro domains were almost exclusively linked via lysinonorleucine, whereas Lys–Ala domains were found to be cross-linked via lysinonorleucine, allysine aldol, and desmosine. Unexpectedly, we identified a high number of intramolecular cross-links between lysine residues in close proximity. In summary, we show on the molecular level that elastin formation involves random cross-linking of tropoelastin monomers resulting in an unordered network, an unexpected finding compared with previous assumptions of an overall beaded structure.

Elasticity and resilience are fundamentally important characteristics of dynamic tissues. Within vertebrates, with the exception of cyclostomata (1), elastic fibers enable organs such as lungs, arteries or skin to stretch and bend with recoil and thus to maintain their physiological function. The fibers consist of an outer mantle of fibrillin-rich microfibrils and a dense elastin core that composes over 90% of the volume. Elastin is an extremely long-lived protein with remarkable properties, including a very low elastic modulus. It is hydrophobic and insoluble, but its hydration is a requirement for the elastic properties (2). The structural basis for understanding its reversible elasticity has been elusive. However, a fundamental feature is the generation of covalent cross-links between the tropoelastin (TE)[4] monomers. The resultant network distributes the stress and strain forces throughout the biopolymer during deformation. TE consists of alternating hydrophobic and more hydrophilic domains. In the hydrophobic domains, the small nonpolar amino acids Gly, Leu, Val, and Pro dominate, whereas the hydrophilic domains contain Lys–Ala (KA) and Lys–Pro (KP) motifs, which are involved in cross-linking. After secretion, TE undergoes self-association by interactions between hydrophobic domains of the monomers (3, 4), leading to the formation of distinct globular aggregates on the cell surface (5). Lysyl oxidase (LOX) and LOX-like enzymes then catalyze the oxidative deamination of the ϵ-amino group of Lys residues to the highly reactive α-aminoadipic acid$-\delta$-semialdehyde, also termed allysine (further denoted as Lya) (6). In KA domains, Lys residues occur as pairs or triplets separated by two or three Ala residues (KAAK and K*X*AAK), and in KP domains the Lys residues are separated by at least one Pro residue (K*X*PK, KP*X*K, and KPK) (7, 8). After oxidation, covalent cross-links are formed spontaneously by nonenzymatic condensation of either two Lya residues via aldol condensation forming allysine aldol (AA) or by the reaction of a Lya residue with the ϵ-amino group of another Lys residue via Schiff base reaction resulting in the formation of dehydrolysinonorleucine (ΔLNL) (9, 10). Such reducible cross-links then further condense with each other, partly with participation of unmodified Lys residues or with other intermediates, to form the trifunctional cross-links dehydromerodesmosine and cyclopentenosine (11–13) as well as the tetrafunctional cross-link desmosine (DES) and its isomer isodesmosine (IDES)

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[4] The abbreviations used are: TE, tropoelastin; AA, allysine aldol; DES, desmosine; FA, formic acid; IAA, iodoacetamide; IDES, isodesmosine; IF, isoform; ΔLNL, dehydrolysinonorleucine; LNL, lysinonorleucine; Lya, allysine; MD, molecular docking; PE, porcine pancreatic elastase; ACN, acetonitrile; LOX, lysyl oxidase; PTM, post-translational modification; AGC, automatic gain control.

Native cross-links in elastin
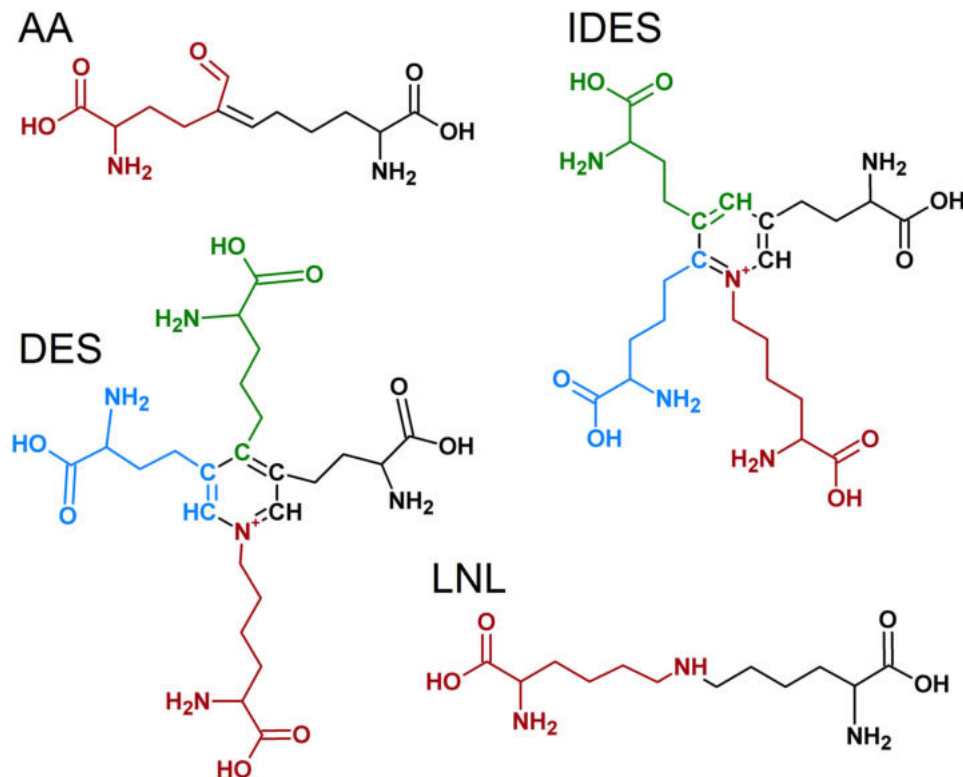
Figure 1. Chemical structures of cross-linking amino acids present in elastin: allysine aldol, isodesmosine, desmosine, and lysinonorleucine. The original lysine residues composing these amino acids are indicated in *black, red, green,* and *blue,* respectively.

(14–16). The majority of TE's Lys residues are modified in the course of maturation (17). The different types of cross-links coexist in mature elastin, and over time, dehydrolysinonorleu-cine and dehydromerodesmosine further get reduced to lysi-nonorleucine (LNL) and merodesmosine, respectively (18). The most abundant cross-linking amino acids in elastin are the bifunctional and tetrafunctional amino acids (19) shown in Fig. 1. LNL and AA are also found in collagens, but DES and IDES are unique to elastin (20). Although the types of cross-links present in elastin are well studied, very little is known about the exact pattern of cross-linking and hence the overall molecular organization of elastin. A better comprehension of its overall structure is required to understand the mechanical properties of elastic fibers as well as their mechanisms of formation and breakdown, especially as the latter contributes to pathologies such as pulmonary emphysema or cardiovascular diseases. However, the analysis of elastin's cross-links is very challenging due to their diversity, the tremendous number of possible combinations, and the repetitive nature of the precursor's primary structure. Complicated to deal with are further elastin's insol-ubility and splice variants, the resistance to specific proteases, and the presence of other post-translational modifications (PTMs).

The only exact cross-linking sites were determined more than 2 decades ago by Mecham and co-workers (21). In that study, Brown-Augsburger *et al.* (21) created an incompletely cross-linked elastin by impeding the activity of LOX/LOX-like enzymes in pigs. This trick made it possible to specifically digest elastin and to investigate some cross-link–containing peptides by amino acid analysis and Edman sequencing. Thus, the

authors demonstrated the involvement of domains 10, 19, and 25 in three cross-links. These results are also the basis of the proposed head-to-tail model (22). However, these cross-links represent only a small part of the matter as elastin features a total of 10 KA and 6 KP domains, which can be covalently linked with each other by a multitude of different cross-links. To sup-plement the current knowledge and to attain a better under-standing of the elastogenesis, we examined in this study the cross-links in unaltered and mature elastin. The application of state of the art high-resolution MS as well as in-house–developed software (16, 23, 24) enabled us to gain new struc-tural insight into the transition from TE to elastin.

Results

Both KP and KA domains are incompletely cross-linked in mature elastin

The identity of linear (noncross-linked) peptides can indi-rectly provide important information about putative cross-linking sites. After cleaving mature elastin by pancreatic elas-tase (PE), we identified 310 linear elastin peptides covering 78% of TE's sequence. The exact coverage and cleavage sites of PE are shown in Fig. 2. The majority of linear peptides were released from hydrophobic domains, which are not involved in cross-linking. We identified partial hydroxylation of 16 Pro res-idues located in G*X*PG motifs, where *X* was Val, Ile, Leu, or Phe, and in one case within the motif LPA. We found no hydroxy-prolines within any of the cross-linking motifs.

We observed that linear peptides containing motifs capable of being involved in cross-linking occurred at lower frequency
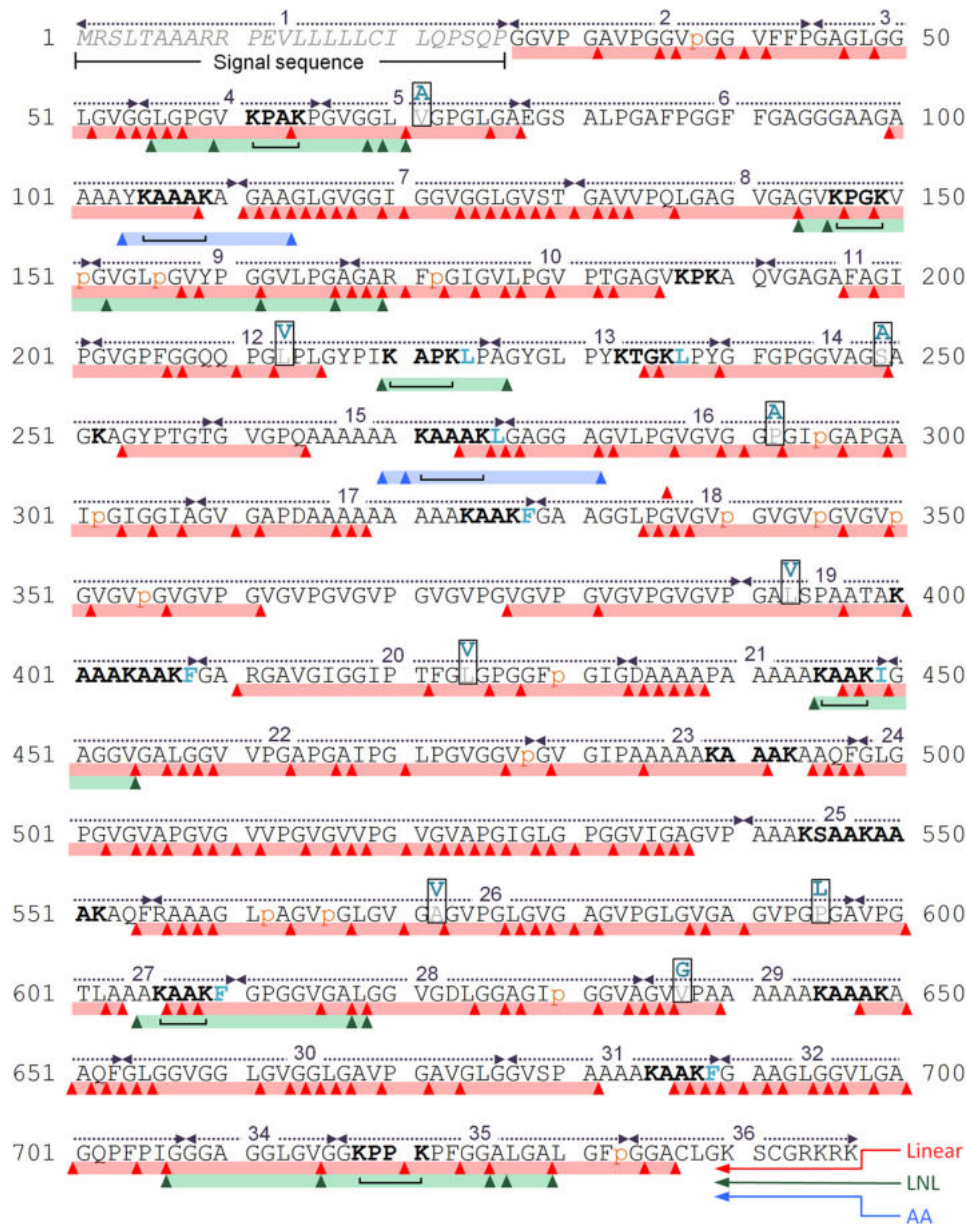
Figure 2. Bovine elastin's primary structure (IF1, UniProt no. P04985-1). Cleavage sites induced by PE are indicated by *triangles*, and all regions covered by peptides are marked with *solid lines below* the sequence. Noncross-linked peptides are shown in *red*, whereas peptides that are intramolecularly cross-linked via LNL and AA are shown in *green* and *blue*, respectively. Identified hydroxylated proline residues are shown as *lowercase letter p* in *orange* and cross-linking motifs are highlighted in *bold*. Identified amino acid substitutions are indicated with *turquoise capital letters* above the respective residue.

than those released from hydrophobic domains. Nevertheless, we found that Lys residues belonging to KP domains 4, 8, 13, and 35 as well as to KA domains 6, 15, 19, 21, 23, 27, 29, and 31 were partly unmodified. This demonstrates that most KA and KP domains were not completely involved in cross-linking. However, the abundance of those peptides was rather low indicating that the degree of cross-linking was generally high. In most cases, the C-terminal Lys of the pairwise-occurring Lys residues was found in linear peptides, and consequently, it was neither deaminated nor cross-linked. The other, N-terminal Lys residue, which typically belongs to the AAKAA motif, was partly unmodified only in KA domains 6, 19, and 23. Because elastin was isolated from adult animals, we conclude that some Lys residues are not oxidatively deaminated in the course of the elastic fiber maturation and remain unmodified throughout

life. We found no peptides containing free Lya residues, even though we considered this modification in the sequencing approach. Furthermore, we found no linear peptides covering cross-linking motifs of KP domains 10 and 12 and KA domains 17 and 25, and no peptides originated from the C-terminal RKRK motif, which is highly conserved among species.

It is worth mentioning that the MS analysis of linear peptides revealed nine amino acid substitutions with respect to the canonical amino acid sequence of bovine TE (IF1, UniProt no. P04985), displayed in Table 1. Although six of the residue replacements were already described earlier in the UniProt database (25, 26), we report here three new substitutions in bovine elastin. All nine mutations were present in hydrophobic regions of elastin and hence did not affect any sites of cross-linking.

Table 1
Amino acid substitutions discovered in bovine elastin

Mutation	Domain	Described in UniProt
V71A	5	No
L213V	12	Yes
S249A	14	No
P292A	16	Yes
L393V	19	Yes
L424V	20	Yes
A572V	26	Yes
P595L	26	No
V637G	29	Yes

Majority of Lys residues are modified

Amino acid analysis of isolated elastin revealed a high correlation with the theoretical composition of bovine TE with the exception of Pro and Lys residues (see Fig. S1). Pro was reduced by 10% and coincided with the detection of the same amount of hydroxyproline. Lys, however, was decreased by 92%, indicating a high cross-linking degree.

Bifunctional cross-links are formed inter- and intramolecularly in KA and KP domains

The identification of cross-linked peptides can provide insights into the spatial proximity of certain amino acids and thus into the overall protein structure. Among the different types of cross-links in elastin are the bifunctional cross-links LNL and AA. We identified 41 peptides, each containing one of the two cross-links. We detected no peptides with ΔLNL, indicating the intermediate was fully reduced to LNL. The sequences revealed the remarkable fact that cross-links were not only formed intermolecularly but also intramolecularly. The latter was proven for the Lys pairs in KP domains 4, 8, 12, and 35 (K*XX*K motif) as well as in KA domains 21 and 27 (AKAAK*X* motif; *X* is a large hydrophobic residue here), which were all linked by an intramolecular LNL. A representative product ion spectrum of one of the intramolecularly cross-linked peptides is shown in Fig. 3*A*. Interestingly, for nearly all KP and some KA motifs, we identified peptides that show that both adjacent Lys residues can condense to LNL without participation of other domains (Fig. 2). The condensation to ΔLNL requires one Lys to be oxidatively deaminated, whereas the other must have remained unmodified. As shown in Fig. 1, LNL is a symmetric molecule, and hence there is no way to determine which of two original Lys residues was previously oxidized by LOX. Besides LNL, we found AA to cross-link the adjacent Lys residues in the KA domains 6 and 15. The aldol condensation requires two Lya residues, which means that Lys-105, Lys-109, Lys-271, and Lys-275 undergo enzymatic deamination by LOX. Another interesting finding is that while two residues separate the Lys residues in the identified LNL-containing domains, three residues separate the two Lys residues in the AA-containing domains.

Strikingly, we identified numerous interpeptidal cross-links, which connect KP and KA motifs. Table 2 summarizes all identified intermolecularly and bifunctionally cross-linked peptides. Such peptides consist of two continuous peptide chains that are covalently connected only by the cross-link. For all interpeptidal cross-links, it was impossible to determine whether the two chains were linking two domains of the same

or two different monomers *in vivo*. An example is the cross-linked peptide AGKAGYPT_AKLGAGGA, the fragment spectrum is shown in Fig. 3*B*. It represents two AA cross-linked strands of domains 14 and 15, respectively. Even though the peptide consists of two noncontinuous chains, it is also possible that they, due to their spatial proximity, originate from the same monomer and were simply cleaved by PE. Other peptides prove the formation of cross-links between domains 4 and 12 (both KP), 6 and 14 (both KA), as well as 12 (KP) and 27 (KA). Furthermore, we found many peptides that comprised one chain with a C-terminal Lys residue of a cross-linking domain and another very short peptide chain. The latter were only two to five amino acid residues in length, often providing insufficient information for an unambiguous assignment. For instance, Lys-61 of domain 4 (KP) was found to be intramolecularly linked via LNL to Lys-64 of the same domain (indicated in Fig. 2), intra- or intermolecularly linked via LNL to Lys residues located in the chains AKAA and KF (both KA domains) and intra- or intermolecularly linked via AA to AKAA (KA domain). However, the motifs AKAA and KF occur in different KA domains of bovine TE 12 and 4 times, respectively.

KA domains are involved in the formation of desmosine or isodesmosine

The discovery and exact sequence determination of DES/IDES-containing peptides are considerably more difficult than the identification of AA- and LNL-containing peptides. Both DES and IDES can theoretically link up to four peptide chains, even though it was proposed that DES/IDES may only link two peptide chains together *in vivo* (27, 28). The sequence determination is further complicated on the bioinformatics level by the complex fragmentation behavior of such peptides in tandem MS and the unpredictable proteolytic cleavage of elastin. In recent years, we studied numerous elastases with regard to their cleavage characteristics, but none of them showed any conducive site specificity (29–33). Thus, in this study we used pancreatic elastase, which rapidly cleaves mature elastin.

For the discovery of DES/IDES-peptides, we employed an LC-MS/MS–based workflow, which we recently introduced. This approach is based on the detection of emerging marker fragments when pyridinium-containing molecules such as DES/IDES are fragmented (24). We enriched the detected peptides by LC and fraction collection and then thoroughly investigated them by tandem MS. We interpreted the complex spectra manually and/or in combination with the in-house software PolyLinX, which we developed earlier for the identification of polyfunctionally cross-linked peptides (16).

This approach enabled the exact sequence determination of three DES/IDES– cross-linked elastin peptides. The isomers DES and IDES could not be distinguished, because they yield the same product ions in collision-induced dissociation (34), which is the fragmentation method of choice for peptide identification. An annotated product ion spectrum of a DES/IDES-containing peptide along with its reporter ions released at a higher collision energy is shown in Fig. 4. This peptide as well as the two others (Fig. S2, *A* and *B*) were found to be composed of three chains. The amino acid sequences of these chains are reported in Table 3. The identified DES/IDES-peptides have in
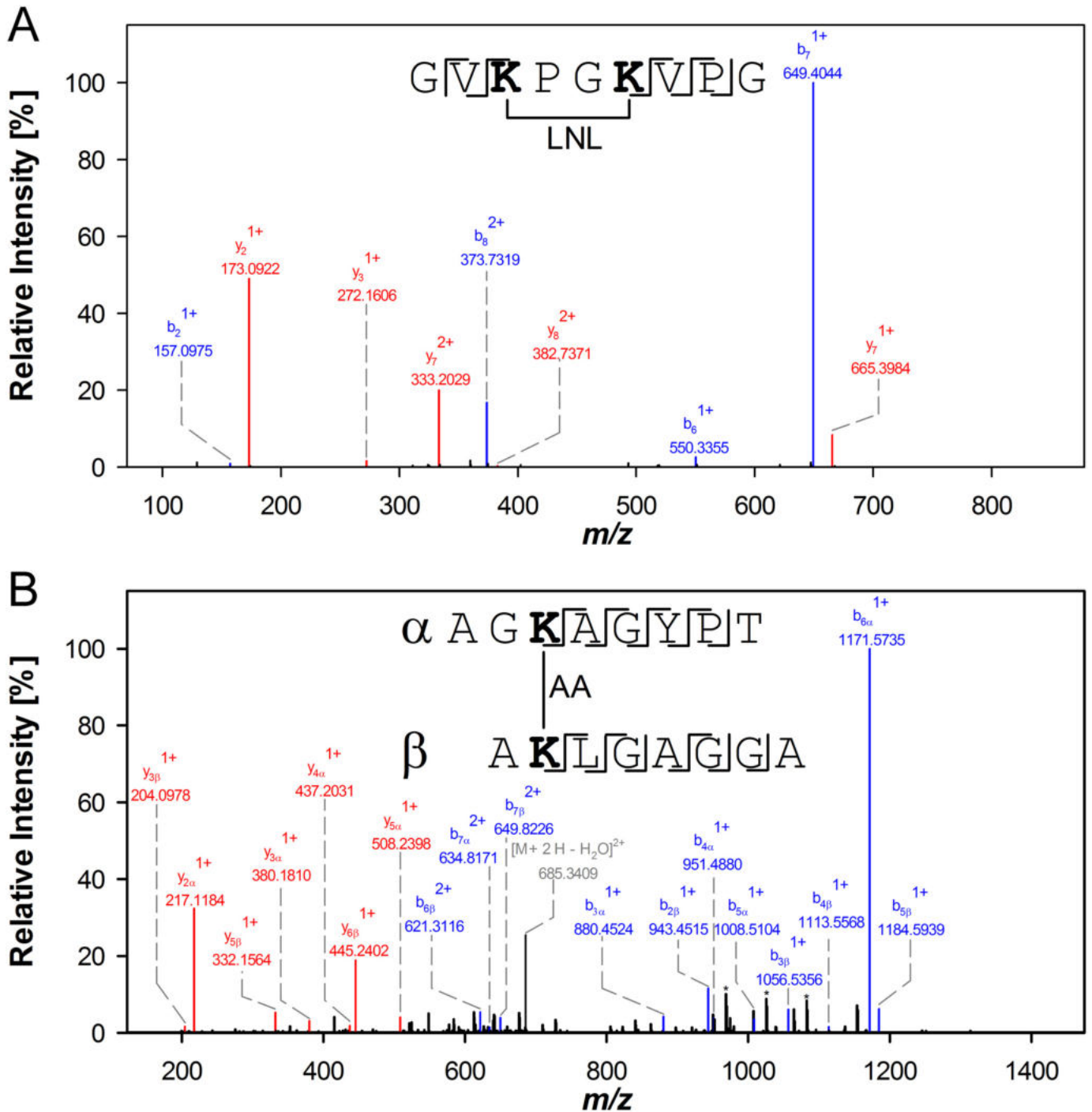
Figure 3. Representative product ion mass spectra of bifunctionally cross-linked elastin peptides. Peptide sequences are displayed within the figure, and cross-links are shown as *solid lines*. *A* shows the fragment spectrum of an intramolecularly cross-linked peptide released of domain 8 at *m/z* 411.2475 (2+) with an LNL, and *B* shows the fragment spectrum of an intermolecularly cross-linked peptide at *m/z* 694.3467 (2+) with an AA. For the sake of simplicity, fragments derived from two or more dissociation events are labeled with an *asterisk*. Corresponding y ions are highlighted in *red*, and b ions and a ions are highlighted in *blue*. The nomenclature used is according to Schilling *et al.* (64).

common that they contain one peptide sequence with a large hydrophobic residue C-terminal to one Lys residue followed by some other small residues such as Ala, Gly, or Pro. They cover KA domains 15, 27, and 17 or 31. Hence, these domains are involved in the formation of DES/IDES. We could not elucidate the other domains involved, as the respective sequences were again too short for an assignment. Nevertheless, the short sequences rich in Ala residues reveal that they originate from

KA domains. We found no DES/IDES-peptides with chains assignable to any of the KP domains. A schematic overview based on a domain map summarizing all identified cross-links is depicted in Fig. 5.

Pancreatic elastase cleaves in regions of cross-linking

The finding of DES/IDES-containing peptides, connecting three discontinuous peptide chains, raises the question of

Table 2
Bifunctionally cross-linked peptides derived from mature aortic elastin
Lysine residues involved in cross-link formation are highlighted in bold. KP and KA domain numbers are shown in blue and red, respectively.

[M+H]+ observed	[M+H]+ calculated	Δm/ ppm	Peptide 1	Domain 1	Type	Domain 2	Peptide 2
1080.6404	1080.6412	-0.7	LGPGVKPA	4	LNL	15,17,19,21,23,25,27,29,31	AKAA
1014.5972	1014.5982	-1.0	LGPGVKPA	4	LNL	17,19,27,31	KF
1077.5940	1077.5939	0.2	LGPGVKPA	4	AA	15,17,19,21,23,25,27,29,31	AKAA
969.5716	969.5728	-1.2	AKPGVGGL	4	LNL	6,15,17,19,21,23,25,27,29,31	AAK
969.5720	969.5728	-0.7	AKPGVGGL	4	LNL	6,15,17,19,21,23,25,27,29,31	KAA
869.5452	869.5455	-0.3	KPGVGGL	4	LNL	12	IK
733.4237	733.4243	-0.8	GVKPG	8	LNL	17,19,27,31	KF
867.4567	867.4571	-0.4	GVKPG	8	AA	15,17,19,21,23,25,27,29,31	AAKAA
777.4500	777.4505	-0.6	GKLPY	13	LNL	6,10,12,14,15,17,19,21,23,25,27,29,31	KA
791.4654	791.4662	-0.9	KLPY	13	LNL	6,15,17,19,21,23,25,27,29,31	KAA
1089.5213	1089.5211	0.2	AGKAGYPT	14	AA	6	AKAG
1174.5744	1174.5739	0.4	AGKAGYPT	14	AA	15,17,19,21,23,25,27,29,31	AAKAA
1387.6859	1387.6852	0.5	AGKAGYPT	14	AA	15	AKLGAGGA
1032.4998	1032.4997	0.2	KAGYPT	14	AA	6	AKAGA
1054.5531	1054.5527	0.4	AAAKLGAGGA	15	AA	6,15,17,19,21,23,25,27,29,31	AKA
920.5196	920.5200	-0.4	AKLGAGGA	15	LNL	17,19,27,31	KF
886.5352	886.5356	-0.5	AKLGAGGA	15	LNL	12,13,15,21	KI / KL
903.4567	903.4571	-0.4	AAKFGAA	17,31	AA	6,15,17,19,21,23,25,27,29,31	KAA
769.4236	769.4243	-0.5	AKFGA	17,19,31	LNL	17,19,27,31	KF
735.4395	735.4400	-0.5	KFGAA	17,31	LNL	12,13,15,21	KI / KL
872.5197	872.5200	0.2	AKIGAGGV	21	LNL	6,10,12,14,15,17,19,21,23,25,27,29,31	KA
877.5138	877.5142	-0.4	KIGAGGV	21	LNL	17,19,27,31	KF
1130.5944	1130.5953	-0.8	AKAQFRAA	25	AA	6,15,17,19,21,23,25,27,29,31	KAA
1003.5563	1003.5571	-0.8	AKFGPGGV	27	LNL	6,15,17,19,21,23,25,27,29,31	AAK
903.5294	903.5298	-0.5	KFGPGGV	27	LNL	12	IK
863.4982	863.4985	-0.4	KFGAAGL	31	LNL	6,15,17,19,21,23,25,27,29,31	AK

whether this indicates the formation of the cross-link between three domains. Alternatively, proteolytic cleavage must have occurred between the two adjacent Lys of one cross-link motif. To prove this possibility, we conducted molecular docking and molecular dynamics (MD) simulations (see "Experimental procedures" for details). As shown in Fig. 6, we found that docked DES/IDES-peptides, joining two peptide chains, are able to come into close proximity of the catalytic domain of PE. This would allow for enzymatic cleavage of the Ala–Ala bonds and explain the presence of the observed peptides.

Discussion

Elastogenesis is the complex and still poorly understood sequence of events that eventually leads to the deposition of elastic fibers in our connective tissues. One of the many steps is the enzyme-induced cross-linking, which is fundamental for the conversion of TE to the functional polymer elastin.

Although recent studies on TE and *in vitro* cross-linked TE provided valuable information on structural properties of elastin's precursor in solution (35–39), very little is known with respect to the organization of mature elastin in the elastic fiber core. Earlier reports that dealt with the identification of cross-linked elastin peptides focused on the identification of a single or very few peptides because of technical limitations (40, 41). Such peptides were enriched in labor-intensive approaches and subsequently analyzed by Edman degradation (27). The most recent of these studies was carried out in 1995 by Brown-Augsburger *et al.* (21) on porcine elastin. The authors deduced three cross-links connecting domains 10 (KP) and 19 and 25 (both KA). However, it remained unknown to what extent the respective Lys residues are incorporated in these very cross-links, whether the lowering of the LOX activity influenced the cross-linking qualitatively and which role the other 13 cross-linking domains play in the maturation. On the basis of this single cross-link in pig elastin along with low-resolution structural data of recombinant human TE without PTMs, a head-to-tail model for elastin's assembly was proposed by Baldock *et al.* (22), suggesting an ordered, beaded assembly. Yet, there is no experimental evidence for the interaction of the monomers in the suggested manner.

In our study, we addressed the above-mentioned questions using an untargeted sequencing approach via LC-MS/MS. We report the identification of numerous cross-linked peptides derived from almost all KA and KP domains of naturally
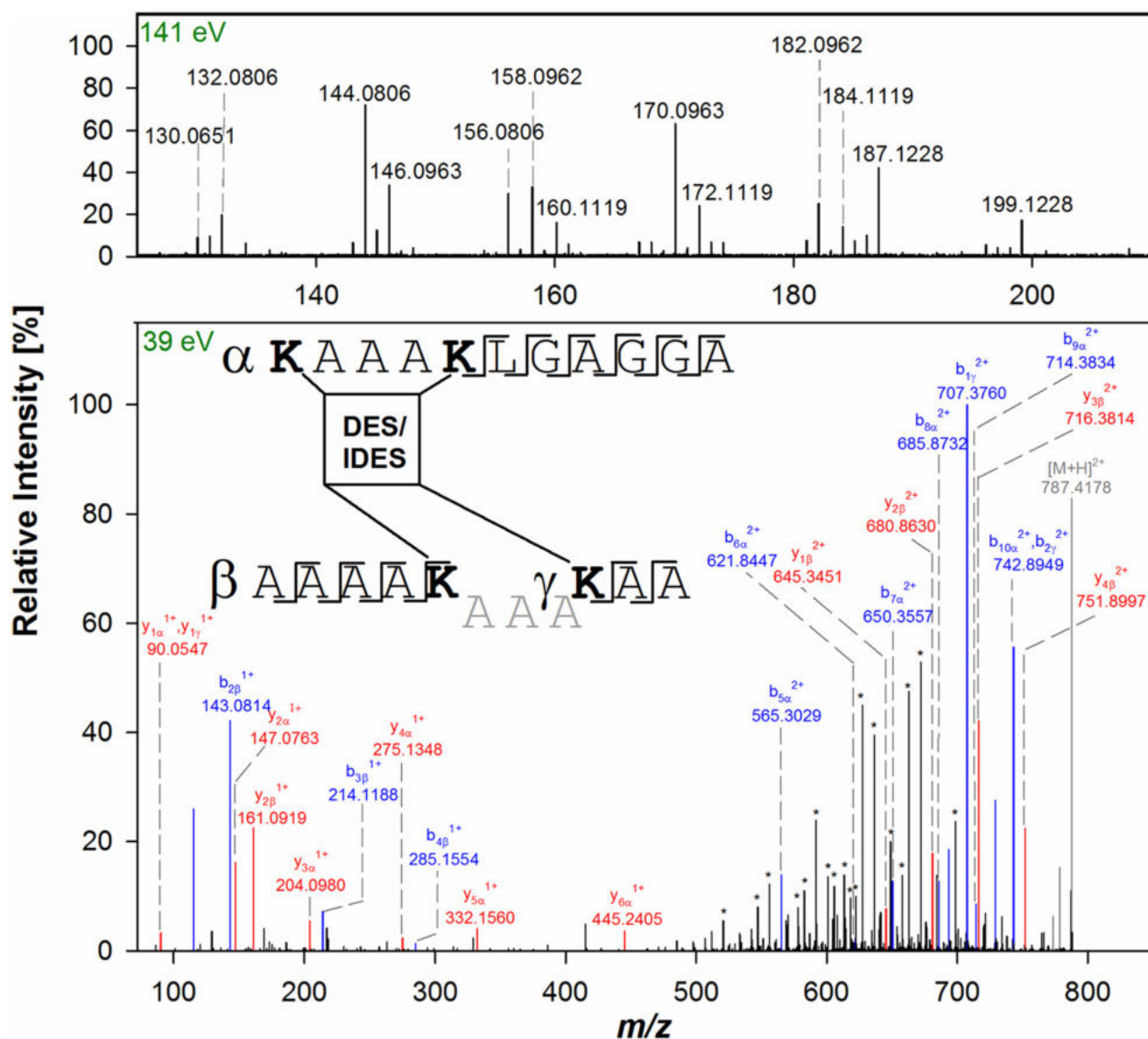
Figure 4. Representative high-resolution tandem mass spectrum of a tetrafunctionally cross-linked elastin peptide with *m/z* 787. 4178 (2+) dissociated at different collision energies. The *upper panel* shows low-mass DES/IDES-specific reporter ions released at elevated energies (24). The *lower figure* shows the release of sequence-specific product ions, released at lower energies. Respective energies are specified in the figures. Amino acid sequences of the DES/IDES– cross-linked peptide consisting of three peptide chains are displayed within the figure. For the sake of simplicity, product ions derived from two or more dissociation events are labeled with an *asterisk*. Corresponding y ions are shown in *red*, and b ions and a ions are shown in *blue*.

Table 3

**Tetrafunctional cross-links in mature aortic elastin**

Lysine residues involved in cross-link formation are highlighted in bold. KA domain numbers are shown in red.

| M+ observed	M+ calculated	Δm [ppm]	Peptide 1	Domain 1	Cross-linking type	Peptide 2	Peptide 3
1573.8321	1573.8333	-0.8	**K**AAA**K**LGAGGA	15	DES/IDES	AAAA**K**	**K**AA
1280.6609	1280.6633	-1.9	**K**AA**K**FGAA	17, 31	DES/IDES	A**K**A	AA**K**
1732.9016	1732.9016	0.0	A**K**AA**K**FGPGGV	27	DES/IDES	AAA**K**A	A**K**AA

cross-linked elastin. The peptides revealed yet unknown intra- and intermolecular cross-links present in both KA and KP domains and shed new light on elastin's molecular assembly and structure.

Gerber and Anwar (27) suggested that the condensation of two bifunctional cross-links leads to the formation of DES and IDES and that these cross-links are not necessarily the ultimate result. The authors (27) rather stated that about half of the
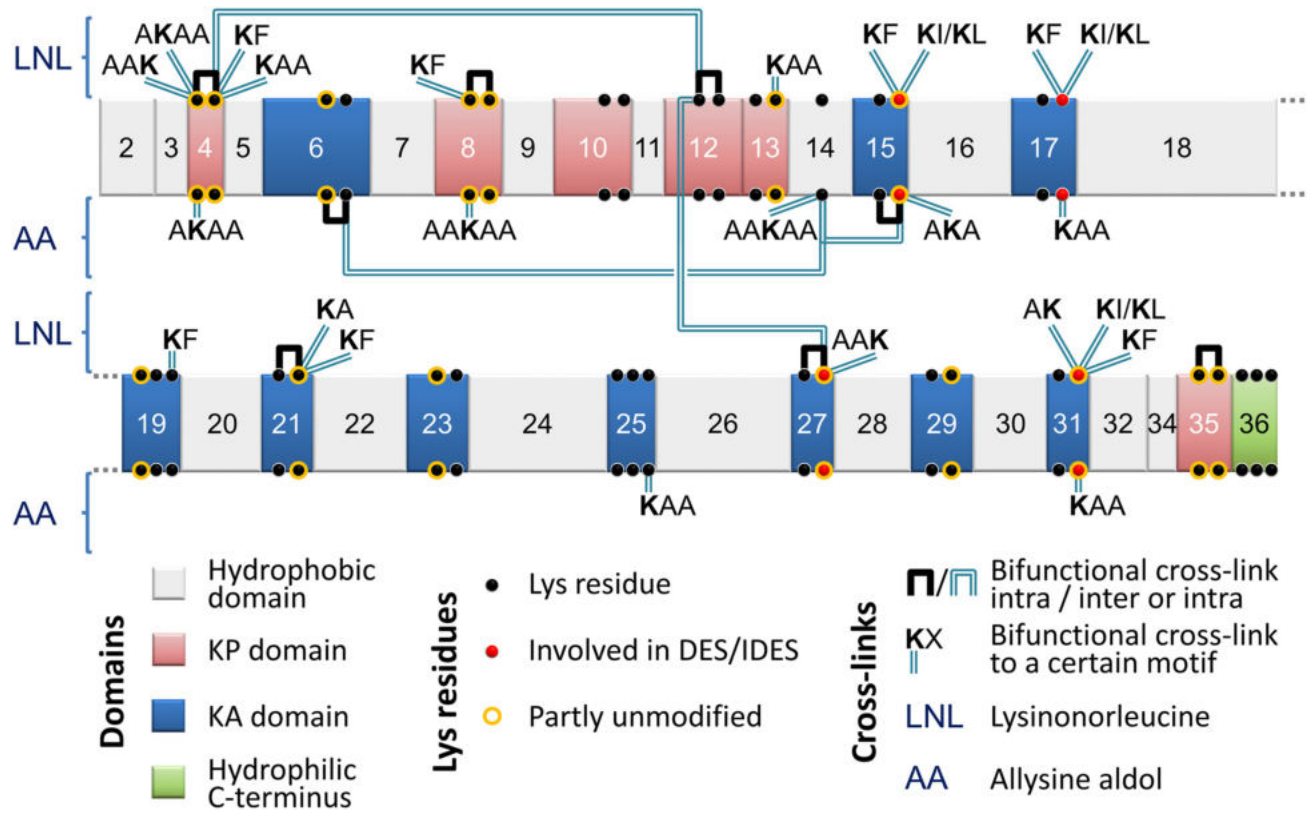
Figure 5. Domain structure of bovine tropoelastin (IF1) showing all identified cross-linking sites. Hydrophobic domains are shown as *gray boxes*, and hydrophilic domains with KP and KA motifs are displayed as *red* and *blue boxes*, respectively. The *width* of the boxes corresponds to the relative length of the domains, and their numbering is based on exon assignment. Lys residues are shown as *black filled circles,* and those found to be involved in DES/IDES formation are shown in *red.* Lys residues found to be partly unmodified are *yellow-rimmed.* Intramolecularly and intermolecularly bifunctionally cross-linked Lys residues are connected by *black solid* and *blue double lines*, respectively. For clarity, all links are shown as intramolecular cross-links. Identified peptide chains of cross-linked peptides belonging to two or more possible domains are shown in the amino acid one-letter code.
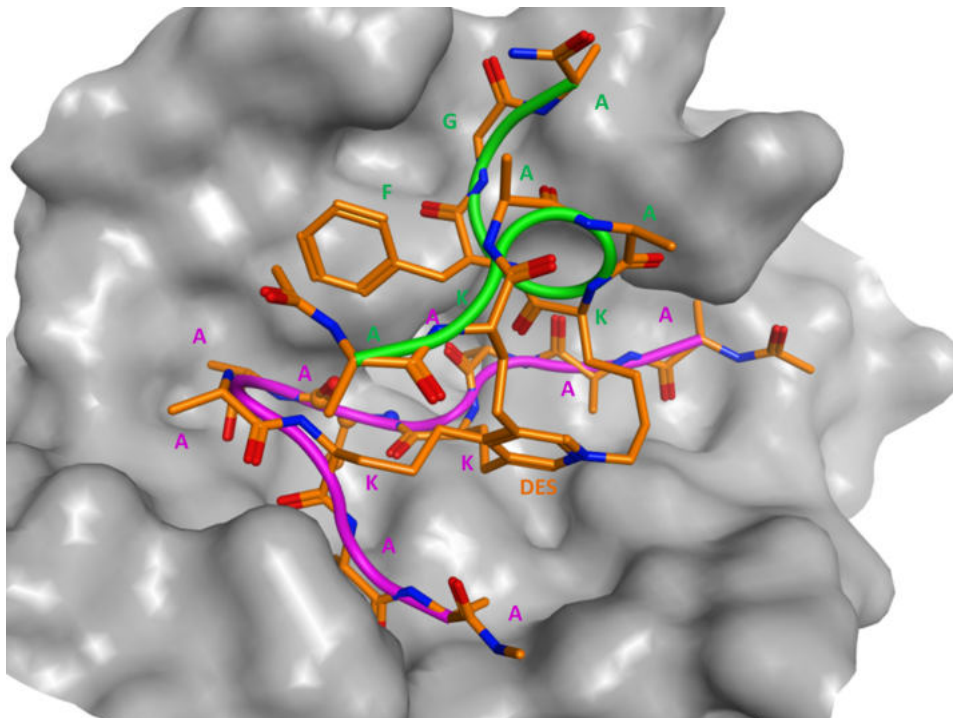
Figure 6. Docking pose of DES linking two peptide chains in complex with the catalytic site of PE. Carbon atoms of the cross-linked peptide are represented as *orange sticks.* The two peptide backbone chains are colored *magenta* and *green* and corresponding amino acids are labeled. PE's van-der-Waals surface is shown in *gray.*

previously formed bifunctional cross-links do not further condense. Our experimental data support these suppositions by revealing the existence of a high number of LNLs and AAs in elastin of full-grown animals. With the detection of intramolecular bifunctional cross-links, we obtained clear evidence that these amino acids are formed as intermediates in the course of the maturation. We conclude that some of them do not meet due to steric hindrance and in turn remain in the elastic fiber. However, damage of the fibers caused by excessive mechanical stress or proteolytic cleavage during aging or disease may cause subsequent condensations.

The observation of intramolecular LNL in KP domains raises the question whether these domains are involved in DES/IDES formation. It was proposed earlier that their contribution is unlikely because of steric constraints caused by the Pro residues (21). Several studies have shown that Pro impedes the formation of α-helical conformations, which are thought to be required to bring the Lys side chains in close proximity (42, 43). However, Baig *et al.* (44) identified a peptide, belonging either to domain 4 or domain 8 (both KP) in bovine elastin, that was involved in the formation of DES/IDES. Furthermore, quantitative analyses of the DES/IDES content in amphibian elastin, whose cross-linking sites are found almost exclusively in KP motifs (8, 45), showed that its level is comparable with those of teleosts (46), in which KA motifs prevail. These facts together with our results suggest that KP domains may directly contribute to DES/IDES formation, even though the extent could be less when compared with KA domains.

Another intriguing question concerns the homogeneity of the cross-linking, *i.e.* whether the same domains are always cross-linked with each other. The opposite is the case; the peptide sequences reveal a high diversity in the formation of cross-links in elastin of the same tissue for several unambiguously assignable Lys residues. This behavior appears to be independent of the involved domain type. We discovered for instance that Lys-61 of domain 4 (KP) was cross-linked to the sequence chain AKAA by both AA and LNL, respectively. This means that for the formation of AA, both Lys residues underwent deamidation by LOX, whereas in the case of LNL only one Lys residue was modified to Lya. It was previously shown that neighbor residues play a critical role for the susceptibility of Lys residues by LOX (47). Although adjacent Ala residues favor the oxidation of Lys residues, the susceptibility decreases with the neighboring residues in the following order: Ala > Val > Leu > Lys > Phe > Tyr. This suggests for the observed peptides that the Lys residue of the first chain (Lys-61) was the one being partially oxidized. We further verified this by the identification of linear peptides containing Lys-61. A close proximity of the respective residue with the aldehyde group of A*K*AA then resulted in the formation of both LNL and AA.

Based on these findings for the specificity of LOX, it was previously proposed that Lys residues, which are followed by a bulky amino acid such as Leu, Ile, Phe, or Tyr, do not undergo oxidation in elastin (48). This assumption is used for explaining the formation of DES and IDES *in vivo* by the condensation of one Lys and one Lya residue on one and two Lya residues on another TE molecule (21). Our peptide data are in line with this assumption. For example, we found intramolecularly cross-

linked Lys residues, localized within the same domain, to be cross-linked either by AA or by LNL. We observed a correlation between the type of cross-linking amino acid formed and the sequence motif involved. We found that Lys residues of the motif K*XX*K*Y*, where *Y* was often a bulky amino acid, were exclusively cross-linked via LNL. In contrast, AA was only formed between the two Lys residues of K*XXX*K motifs, when the Lys residues were not C-terminally flanked by aromatic residues. Our results further show that KP domains also undergo intrachain formation of LNL. We also observed that Lys residues with adjacent Gly or Ala residues were linked to other Lys residues also followed by Gly or Ala via AA, showing that both Lys residues were oxidized prior to the condensation. In contrast, we found that Lys residues followed by bulky residues were connected almost exclusively via LNL as also described above for intramolecular cross-links. However, we identified one peptide consisting of the chains AKFGA and KF linked via an LNL. Thus, Lys residues adjacent to Phe can undergo oxidation induced by LOX. This exception to the rule demonstrates how spontaneous and unregulated the cross-linking takes place. Another prime example for the high complexity of the cross-linking represents Lys-275 of domain 15 (KA). We found this Lys residue to be (*a*) unmodified; (*b*) cross-linked via LNL to a chain KF; (*c*) cross-linked via LNL to another chain KI/KL; (*d*) cross-linked via AA with Lys-252 in domain 14; (*e*) cross-linked via AA with a chain AKA; (*f*) intramolecularly cross-linked with the other Lys (Lys-271) of the same domain; and (*g*) involved in a DES/IDES cross-link. All these findings are insofar remarkable as they show that a single Lys residue can be involved in many different cross-links or even remains unmodified. This in turn proves that not only a large variety of combinations exists but also that *in vivo* they actually coexist in the same tissue, which is surprising as it is in contrast with prior assumptions of a rather ordered cross-linking in mature elastin.

Lys-252 in domain 14 of TE is an exception insofar as it is located within the sequence motif AGKAG, which is unlike typical KA and KP domains. Furthermore, Lys-252 is not accompanied by another Lys residue. A previous study by Baig *et al.* (44) revealed the involvement of Lys-252 in the formation of DES/IDES suggesting that even Lys residues, which do not occur pairwise, can form tetrafunctional cross-links. Although we did not identify DES/IDES-peptides proving that, we confirm the involvement of Lys-252 in various AA cross-links together with Lys residues of KA domains 6 and 15. The proximity of domains 14 and 15 suggest this cross-link could be formed also intramolecularly and that PE cleaved between the domains. This is in line with a previous work, which showed that domains 14 and 15 of *in vitro* cross-linked human TE were intramolecularly cross-linked to a high degree (35).

The study of mature elastin by MS requires its prior hydrolysis. We used PE as a tool to generate soluble peptides with lengths suitable for LC-MS–based investigations. PE preferentially cleaves on the carboxyl side of Ala, Val, and Ile but also other residues (49). Hence, we observed multiple cleavage sites near or within KA motifs, suggesting that PE predominantly cleaves the oligo-Ala sequences in elastin. The released cross-linked peptides in turn contain only a few Ala residues, and thus

the respective chains are often not clearly assignable. We found this for many intermolecularly cross-linked peptides in this study.

The three DES/IDES– cross-linked peptides were found to be derived exclusively from KA domains. The repetitive nature of the domains and the above-mentioned unfavorably located cleavage sites did not allow for a distinct domain annotation for all joining peptide chains. The finding that three peptide chains joined the cross-links was unexpected. Although we have recently shown that the formation of DES from three Lya-containing peptides is actually possible (16), it is commonly accepted that DES and IDES only link two chains in mature elastin. The reason for the peptide composition most likely lies in the protease treatment. Several studies have shown the potential of different elastases to cleave within DES/IDES-peptides (40) or even to release free DES/IDES from intact elastin (50). Our docking simulations between a DES– cross-linked peptide and PE revealed the ability of the protease to cleave between residues within the cross-link. This can lead to the formation of these three cross-linked peptides. However, our results do not allow us to conclude whether the oligo-Ala peptides attached to DES/IDES arise from a single domain or not.

The second abundant PTM in elastin is the hydroxylation of Pro residues (51, 52) and represents another source of variability. We observed partial modification for ~20% of all Pro residues and an overall hydroxylation degree of 10%. The enzyme responsible for the intracellular modification is prolyl 4-hydroxylase. It modifies Pro residues in G*X*PA or G*X*PG motifs and is an important element in collagenesis. We have recently shown that prolyl hydroxylation is a feature present in elastin for various vertebrates, but it varies with species and tissue (52). Although the exact role of the PTM in elastin is unknown, several studies on elastin-mimetic peptides suggest that it may lead to structural changes and thus influence the assembly of elastic fibers and their stability (53, 54).

Applying the SPIDER algorithm to our data further allowed us to gain insight into amino acid substitutions within the primary TE sequence, which may also influence the physicochemical properties of elastin. We assume that all observed novel amino acid substitutions in elastin, arising from point mutations, are of minor importance for the overall properties of elastin and reflect the genetic pool of the investigated bovine subpopulation.

The observed lack of the C-terminal domain with its polybasic RKRK motif is consistent with other studies dealing with mass spectrometric investigation of peptides released from elastin by proteolytic degradation (29, 37). This motif is supposed to mediate cell adhesion of TE via binding to integrin $\alpha_v\beta_3$ (55, 56). It has been proposed that it is either cleaved afterward or undergoes post-translational modifications (57).

In summary, we demonstrate with this study the high heterogeneity of the cross-linking pattern in unaltered, mature elastin. Members of the LOX family merely catalyze the oxidative deamination of Lys residues. This process is followed by unregulated condensation reactions not involving any catalysts, which mainly depend on the spatial proximity of the Lys and Lya residues. We provide extensive data on the contribution of eight KA, five KP, and one further domain to intra- and inter-

molecular cross-linking, showing the occurrence of multiple condensation reactions at the same residues of different monomers. Hence, we conclude that the assembly of elastin takes place in a more unpredictable manner than previously assumed. It may be possible that a first-ordered alignment and cross-linking of TE molecules as proposed by the head-to-tail model defines the overall longitudinal structure of elastic fibers and is followed by an unordered and spontaneous formation of further cross-links, which determine the lateral structure of the fibers. With regard to the functional significance of an unordered network of covalently bound tropoelastin molecules, it has been shown that the extension of disordered, cross-linked peptide chains during mechanical stress leads to a global decrease of the conformational energy in a "rubber-like" polymer (58). Furthermore, a recent study using MD simulations of hydrophobic sequence motifs of elastin supports our model of a disordered aggregate (59).

Overall, our results reveal surprising new facets of the cross-linking in elastin that confute previous assumptions on a regularly ordered cross-linking pattern in elastin.

Experimental procedures

Materials

Porcine PE was obtained from Elastin Products Co. (Owensville, MO). Ammonium bicarbonate, cyanogen bromide, 2-mercaptoethanol, urea, dithiothreitol (DTT), sodium azide, sodium chloride, and trypsin from porcine pancreas were obtained from Sigma (Steinheim, Germany). Iodoacetamide (IAA) was purchased from AppliChem GmbH (Darmstadt, Germany); hydrochloric acid was purchased from Grüssing GmbH (Filsum, Germany), and analytical grade Tris, trifluoroacetic acid (TFA), and formic acid (FA) were obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile (VWR Prolabo, Leuven, Belgium) and doubly distilled water were used. All other chemicals were of analytical grade or better.

Isolation of bovine aortic elastin

Bovine aorta was obtained from a single, healthy full-grown (3-year-old) Fleckvieh cow at a local slaughterhouse. Prior to isolation, the aortic sample was cut into small pieces with lateral lengths between 5 and 10 mm. Isolation of intact aortic elastin was carried out as described previously (60). Purified elastin was dried at room temperature and stored at −26 °C until analysis.

In-solution digestion of elastin and enrichment of cross-linked peptides

Elastin was dispersed in 50 mM Tris-HCl, pH 8.5, at a concentration of 5 mg ml^{-1} followed by reduction with 10 mM DTT at 56 °C and alkylation with 30 mM IAA for 1 h at room temperature. PE was added to a final enzyme-to-substrate ratio of 1:50 (w/w), and the samples were subsequently incubated at 37 °C for 24 h. Proteolysis was stopped by adding TFA to a final concentration of 0.5% (v/v). Samples were stored at −26 °C prior to further analysis. Fractionation of elastin digests was performed using an Agilent 1100 system (Waldbronn, Germany) coupled to a Fraction Collector II (Waters, Manchester, UK). For chro-

matographic separation, the sample was loaded onto a Repro-Sil-Pur 120 column (C18, 3 μm, 2 mm inner diameter $\times$ 150 mm; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and eluted using a solvent system of solvent A (0.1% FA in H$_2$O) and solvent B (0.1% FA in ACN/H$_2$O 80:20 (v/v)) by applying the gradients 10–40% solvent B in 40 min to 90% solvent B in the next 5 min and by maintenance at 90% solvent B for 10 min. The flow rate was 200 μl min^{-1}, and fractions were collected in 30-s intervals.

Liquid chromatography coupled to MS

Collected fractions and purified digests were separated on an Ultimate 3000 RSLCnano system (ThermoFisher Scientific). Peptide mixtures were loaded on the trap column (Acclaim PepMap RP-C18, 300 μm $\times$ 5 mm, 5 μm, 100 Å) and washed with water containing 0.1% TFA for 15 min (30 μl min^{-1}), before the peptides were separated on the separation column (Acclaim PepMap RP-C18, 75 μm $\times$ 250 mm, 2 μm, 100 Å) using gradients from 1 to 35% solvent B (90 min), 35 to 85% B (5 min) followed by 85% B (5 min), and with solvent A: 0.1% FA in H$_2$O, and solvent B: 0.08% FA in ACN. The nano-HPLC system was coupled to an Orbitrap Fusion Tribrid mass spectrometer (ThermoFisher Scientific) equipped with a Nanospray Flex Ion Source. Data were acquired using data-dependent MS/MS mode. Full scans were acquired in the Orbitrap with a resolution of 120,000 over *m/z* 300 to 1500, an automatic gain control (AGC) target value of 4 $\times$ 10^5, and a maximum ion injection time of 50 ms. Precursors were selected based on signal intensity and subjected to collision-induced dissociation with 35% normalized collision energy. Product ions were analyzed in the Orbitrap with a resolution of 15,000 using the following settings: AGC target value 5 $\times$ 10^4; maximum injection time 200 ms; and quadrupole isolation window of 2 *m/z*. Dynamic exclusion for 60 s (mass window $\pm$ 2 ppm) was enabled to allow analysis of a less abundant species. Data acquisition was controlled with Xcalibur 3.0.63.

Manual tandem MS experiments

DES/IDES-containing peptides were found to be more hydrophilic than other peptides based on the identification of specific reporter ions. This enabled the selective enrichment of those tetrafunctionally cross-linked peptides by collecting early eluting fractions using C18 chromatography. Selected fractions containing putative tetrafunctionally cross-linked peptides were further investigated by manual MS/MS experiments using static nanoelectrospray ionization. Optimization of the higher-energy collisional dissociation conditions and extended acquisition times allowed improving the spectral quality. Experiments were carried out on an Orbitrap Velos Pro (ThermoFisher Scientific) mass spectrometer. The capillary and spray voltages were set to 50 V and 3.5 kV, respectively. Tandem mass spectra starting at *m/z* 50 were acquired with a resolution of 100,000. A method recently introduced by our group was used to detect DES/IDES-containing peptides based on specific product ions generated when elevated collision energies up to 161 eV are applied (24). Classical tandem MS experiments for identification purposes were carried out by incre-

mentally raising the collision energy up to 50 eV until optimum product ion spectra were obtained.

Peptide sequencing

Noncross-linked and intramolecularly cross-linked peptides were identified by automated *de novo* sequencing followed by matching to the Swiss-Prot database using the software PEAKS (version 7; Bioinformatics Solutions, Waterloo, Canada). The enzyme was set to "none," and the search was taxonomically restricted to *Bos taurus*. Hydroxylation of Pro (mass shift of +15.9949 Da) and oxidative deamination of Lys (mass shift of −1.0316 Da) were chosen as variable modifications. Mass error tolerances for precursor and fragment ions were set to 8 ppm and 0.015 Da, respectively. The peptide score threshold was decreased until a false discovery rate of ≤1% on the peptide level was reached. Amino acid substitutions were identified using the SPIDER algorithm (61). A minimum of two identified peptides having a mutation was used as constraint for unbiased identification. Intramolecular cross-links were detected in PEAKS by using a modified sequence of bovine elastin IF1. More precisely, cross-linking motifs were reduced to a single Lys residue, and the mass difference from this residue to the corresponding intramolecularly cross-linked motif was implemented as a variable PTM. Processed spectra that did not result in satisfying sequence information or only in sequence tags were exported as MGF files and further analyzed using the programs StavroX (23) and PolyLinX to identify intermolecular cross-links (16). LNL and AA were both included as cross-links with mass decreases of 17.0265 and 20.0738 Da, respectively. Lys residues were considered as potential sites of cross-linking, and the amino acid sequence of bovine TE isoform 1 (UniProt no. P04985-1) was used as basis for the peptide identification in StavroX and PolyLinX.

Amino acid analysis

An insoluble elastin pellet was hydrolyzed in 6 N HCl at 110 °C for 24 h. Dried hydrolysates were redissolved in sodium citrate loading buffer, pH 2.2, and amino acid analysis was performed by ion-exchange chromatography with postcolumn derivatization with ninhydrin (Biochrom 30; Biochrom, UK).

Molecular docking and dynamics

Conformations of the peptide (AKAAKFGAA_AAKAAA-KAAA) were sampled by using the Monte Carlo search method and the AMBER EHT12 force field implemented in MOE (MOE 2012.10; Molecular Operating Environment). In total, 156 conformations of the DES peptide using an energy window of 20 kcal mol^{-1} were generated and used for further docking studies. GOLD 5.2 (62) was used to dock the peptide into the binding pocket of porcine pancreatic elastase (Protein Data Bank code 1H9L). The active site Ser-214 and all residues within a 15-Å radius were used to define the binding pocket. Goldscore was used as scoring function. 10 poses for each of the 156 conformations were calculated. All docking poses were subjected to a cluster analysis using the peptide heavy atoms for calculating root mean square deviation values. Three favorable clusters were retrieved and subjected to MD simulations using the AMBER14 package (63). Parameters for nonstandard

amino acids, Lya and Lys residues connecting to form DES ring, were first prepared. The modified amino acids were built and energy-minimized using the HF/6-31G* method. Then, parameter libraries of these residues were generated. Before performing the MD simulations, force field and charges for the DES-containing peptides were assigned (AMBER03 force field for all standard amino acid residues and the extra-generated parameters for the nonstandard amino acid residues). Water (TIP3P model) and counter ions were then added and the protein–peptide complexes were energy-minimized (63). MD simulations were performed at 310 K, and thus the temperature of the system was gradually increased from 0 to 310 K during the first few picoseconds. Then the temperature was kept at 310 K by applying Langevin dynamics with a collision frequency of 1 ps^{-1}. In the final step, free MD simulation was carried out from 100 ps to 20 ns using the NPT ensemble, which means that a constant pressure was set at 1 bar, and the temperature was kept constant at 310 K. A time step of 2 fs with SHAKE algorithm was also applied. The Particle-Mesh-Ewald method was used for computing electrostatic interactions, whereas nonbonded interaction was calculated using a cutoff radius at 10 Å.

Author contributions—C. U. S. data curation; C. U. S., P. M., B. K. M., J. B., and W. S. formal analysis; C. U. S., B. K. M., J. B., and C. E. H. S. investigation; C. U. S., B. K. M., W. S., and C. E. H. S. visualization; C. U. S. and C. E. H. S. methodology; C. U. S. and C. E. H. S. writing-original draft; A. H. and C. E. H. S. supervision; A. H. and C. E. H. S. funding acquisition; A. H. and C. E. H. S. writing-review and editing; B. K. M. software; C. E. H. S. conceptualization; C. E. H. S. project administration.

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