Vol. 186, No. 1, 1992
July 15, 1992

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 549-555

The Cysteine Residues in the Carboxy Terminal Domain of Tropoelastin Form an Intrachain Disulfide Bond that Stabilizes a Loop Structure and Positively Charged Pocket

Patricia L. Brown, Lisa Mecham, Clarina Tisdale, and Robert P. Mecham

Departments of Cell Biology and Medicine, Respiratory and Critical Care Division,
Jewish Hospital at Washington University Medical Center,
216 South Kingshighway, St. Louis, MO 63110

Received June 2, 1992

Summary Analysis of purified bovine tropoelastin with Ellman's reagent and [^{14}C]iodoacetamide demonstrated that the only two cysteine residues in the molecule form an intrachain disulfide bond. Molecular modeling suggests that the cysteine residues are juxtaposed as the result of a tight turn that produces an antiparallel beta structure. Protruding from the C-terminal end of the turn is the sequence Arg-Lys-Arg-Lys which forms the floor of a positively charged pocket created by the extension of the arginine and lysine side chains on opposite sides of the peptide chain perpendicular to the plane of the turn. The side chain of a conserved lysine residue in the disulfide-bonded loop forms the top of the pocket. This positively charged pocket may define a binding site for acidic microfibrillar proteins that mediate elastic fiber assembly.

Elastin contains alternating lysine-rich domains that form crosslinks between molecules and hydrophobic domains that impart to the protein properties of elastic recoil (1). These domains are each encoded by individual exons in the gene. It was not until the elastin gene was cloned, however, that a sequence at the carboxy terminus (GFPGGA-CLGKSCGRKRK), atypical of other regions of elastin, was identified (2). This domain contains the molecule's only two cysteine residues and ends in four basic amino acids. Like other functional domains in elastin, a single, highly conserved exon encodes the C-terminal sequence (3).

The presence of cysteine residues and the conserved nature of the C-terminal domain has led to speculation that this region of the molecule may play a critical role in fiber assembly. Several investigators have proposed that the cysteine residues in elastin form disulfide bonds with cysteine-rich proteins in microfibrils, serving to align tropoelastin monomers prior to crosslinking (2,4). In this report, we show that the cysteine residues in tropoelastin are disulfide bonded within the molecule, precluding their direct

Abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; DTNB (Ellman's reagent), 5,5'-dithiobis-2-nitrobenzoic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TBS, Tris-buffered saline; HPLC, high performance liquid chromatography.

0006-291X/92 $4.00

participation in inter-molecular associations. Molecular modeling of the region around the disulfide bonds suggests the formation of a positively charged pocket that may define the binding site for acidic microfibrils.

Experimental Procedures

Materials

[^{14}C]Iodoacetamide (sp. act. 16 mCi/mmol) and Enhance were from Dupont NEN, (Boston, MA). Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) was from Pierce Chemical (Rockford, IL). All other reagents were from Sigma (St. Louis, MO).

Tropoelastin isolation from fetal bovine ligamentum nuchae

The ligamentum nuchae from a near term fetal calf was minced and incubated overnight at 37 °C in DMEM containing 3% fetal calf serum and 100 μg/ml β-aminopropionitrile (5). The tissue mince was then pelleted by centrifugation, washed thoroughly with cold water to remove serum components, and homogenized in 0.05 N acetic acid using a Brinkmann Polytron. After extraction overnight at 4 °C, the sample was centrifuged at 16,000 xg for 90 min, and the pellet reextracted with acetic acid containing 50 mM DTT. Tropoelastin concentrations in the acetic acid extracts were determined by direct-binding ELISA (6), and tropoelastin was isolated using propanol-butanol as described (6).

Analysis of disulfide bonds with Ellman's reagent

Tropoelastin (700 μg, 10 nmoles) was dissolved in 1 ml 0.1 M sodium phosphate buffer, pH 7.3, containing 6.4 M guanidinium chloride and 5 mg/ml EDTA (the buffer was sparged with nitrogen for 30 min before use). The absorbance at 412 nm was recorded and 30 μl of DTNB solution (40 mg DTNB in 10 ml of 0.1 M sodium phosphate buffer, pH 7.3) was added. The color was developed for 15 min and read at 412 nm (7). Buffer without protein incubated with DTNB for 15 min served as a blank. An equal molar concentration of bovine β-lactoglobulin served as the positive control. A molar extinction coefficient of 13,700 M^{-1} cm^{-1} (8) was used to determine free sulfhydryl concentration.

Alkylation of tropoelastin

Lyophilized tropoelastin isolated from the ligamentum nuchae was dissolved in a denaturing buffer containing 8 M urea, 0.1 M Tris, pH 8.1 and separated into two equal aliquots. One aliquot was reduced by the addition of 1 mM (final concentration) DTT for 45 min at 37 °C under nitrogen. The second sample was treated similarly but without DTT. Both samples were then precipitated with 3 volumes of ice cold acetone, and the protein collected by microcentifugation. The pellets were washed once with 3 volumes of ice cold 75% acetone and then resuspended in 0.1 ml of urea/Tris buffer. Alkylation was performed by adding 10 μl (6.25 μmoles) of [^{14}C]iodoacetamide per 100 ml reaction volume and incubating the samples for 30 min under nitrogen at room temperature in the dark.

HPLC separation of tropoelastin

HPLC analysis of radiolabeled tropoelastin was performed on a 0.41 x 15 cm Hamilton PRP-3 column at 0.5 ml/min flow rate. The column was developed using a linear 45 min gradient from 100% water-0.05% TFA to 80% n-propanol-0.05% TFA. Column fractions were counted for radioactivity in a Beckman LS8000 scintillation counter to localize the position of the ^{14}C peak. The elution position of tropoelastin was verified by assaying each column fraction by direct-binding ELISA.

Electrophoresis, Autoradiography, and Immunoblotting

Samples in SDS sample buffer were analyzed by SDS-PAGE using 0.75 mm thick 7.5-12% gradient gels. Gels with radioactive samples were fixed with 5% methanol-7% acetic acid for 30 min, impregnated with Enhance, dried, and exposed to Kodak XAR-2 film at -70 °C. Non-radioactive samples were transferred from the SDS-PAGE gel to nitrocellulose at 200 mA for 90 min at 4 °C. The nitrocellulose with transferred proteins was incubated for 2 h at room temperature with 5% (w/v) nonfat dry milk in TBS to block unbound sites and then washed twice with TTM [TBS containing 0.1% (v/v) Tween-20 and 0.5% (w/v) nonfat dry milk] for 15 min at room temperature. Tropoelastin antiserum

at a dilution of 1:300 in TTM was added and the blot incubated for 1 h. After washing twice with TTM, a 1:500 dilution of horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin in TTM was added and the blot incubated for 1 h. Color development reagent (4-chloro-naphthol) was then added after washing the blot twice with TBS for 15 min. The color reaction was stopped by rinsing the membrane with distilled water, and the blot was air dried for photography.

Results & Discussion

With the inhibition of lysyl oxidase-derived crosslinking, tropoelastin can be isolated from elastin-rich tissues with either neutral salt buffers (9,10) or dilute acetic acid (5). Interestingly, neither of these extraction protocols include reducing agents, suggesting that disulfide bond reduction is not a requirement for tropoelastin extraction. When tropoelastin was extracted from fetal ligamentum nuchae incubated with BAPN, virtually all of the extractable protein (1.43 mg) was obtained in a nonreducing acetic acid extract. Essentially no new tropoelastin (0.003 mg) was obtained when the acid extracted pellet was reextracted with buffers containing the reducing agent DTT. SDS-PAGE of tropoelastin isolated from the ligament (Figure 1) demonstrated the characteristic multiple isoforms described in earlier publications (5). These isoforms contain the carboxy-terminal peptide (5,11) and have an intact amino terminus. Under nondenaturing conditions, tropoelastin remained monomeric and did not form high molecular weight multimers (Figure 1).

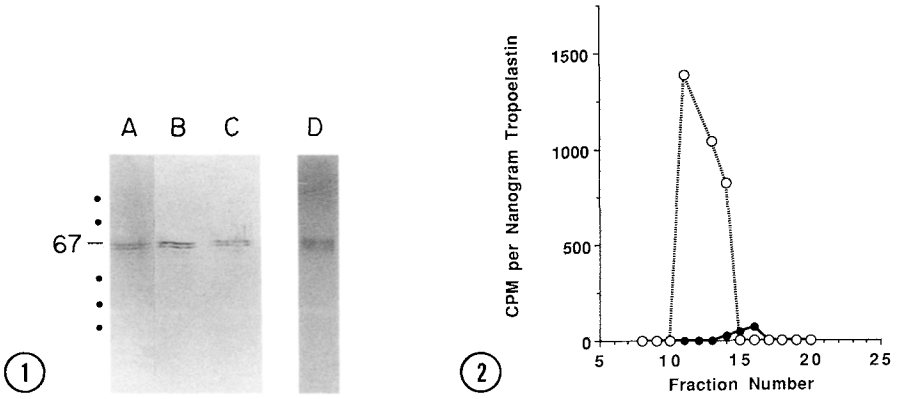

Figure 1. SDS-PAGE of purified tropoelastin.
SDS-PAGE of tropoelastin purified from bovine ligamentum nuchae. Lane A is a silver-stained gel showing the typical tropoelastin isoforms. Lanes B and C are western blots of purified tropoelastin run under reducing and nonreducing conditions, respectively. Lane D is an autofluorograph of reduced tropoelastin alkylated with [^{14}C]iodoacetamide and analyzed by SDS-PAGE. Molecular weight markers, indicated by dots, are, from top to bottom, 106, 80, 32.5, 27.5, and 18.5 kDa.

Figure 2. HPLC elution of alkylated tropoelastin.
Reduced and native tropoelastin were incubated with [^{14}C]iodoacetamide in the presence of urea and analyzed by HPLC using a PRP-3 reverse phase column. The column was developed with a linear gradient starting with water/0.05% TFA progressing to 80% propanol/0.05% TFA over 45 min. Scintillation counting of the eluted fractions demonstrated [^{14}C]iodo-acetamide incorporation into reduced but not nonreduced tropoelastin. Coelution of radioactivity with tropoelastin was confirmed by immunoblot analysis of each column fraction.

To determine the redox state of the cysteine residues, we incubated purified tropoelastin with Ellman's reagent (DTNB) which reacts with sulfhydryl groups liberating 1 mole of 4-nitrothiophenolate anion per mole of sulfhydryl. No increase in absorption over background was found when tropoelastin was incubated with DTNB, although an equivalent molar concentration of β-lactoglobulin gave the appropriate value for two free sulfhydryl groups. The above results suggest that the cysteine side-chains of tropoelastin are not free sulfhydryls and, hence, may be joined as a disulfide bond. This was confirmed by demonstrating that tropoelastin cannot be carboxyamidated with [^{14}C]iodoacetamide unless first reduced with DTT. Figure 2 shows ^{14}C radioactivity associated with reduced and alkylated tropoelastin eluting from a PRP-3 reverse phase HPLC column. Few counts were found associated with nonreduced tropoelastin incubated with [^{14}C]iodoacetamide whereas prior reduction of tropoelastin resulted in efficient incorporation of the ^{14}C label. Interestingly, the carboxyamidated protein eluted from the reverse phase column slightly earlier that the unmodified protein.

Figure 3 compares the C-terminal region of tropoelastin in the five species for which sequence data are available. The first cysteine in human, bovine, and sheep tropoelastin is preceded by the sequence Phe-Pro-Gly-Gly-Ala. In all species, an aromatic residue, usually phenylalanine, is conserved at position 1. The glycine residue at position

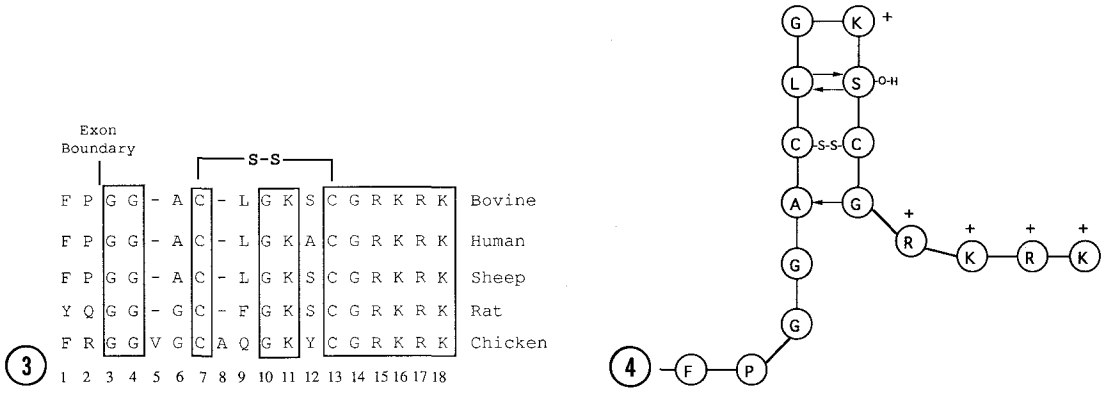

Figure 3. Comparison of C-terminal sequences in elastin.
Homology at the carboxy terminus of human (14,15), bovine (16), sheep (4), chick (17) and rat (18) tropoelastin is indicated by boxed sequences. Also shown is the boundary between exons 35 and 36 in bovine, rat, sheep, and chick tropoelastin and exon 33 and 36 in human (exons 34 and 35 are missing from human elastin).

Figure 4. Proposed structure for the C-terminal domain of tropoelastin. Molecular modeling of the region around the disulfide bond in tropoelastin suggests a hairpin loop resulting in an antiparallel beta structure. The strands are stabilized by hydrogen bonds (arrows) formed between the carboxyl group of alanine (position 6--refer to Figure 3 for numbering) and amino group of glycine (position 14) and between both the amino and carboxyl hydrogens on leucine (position 9) and serine (position 12). The side chain of leucine at position 9, lysine at position 11, and serine at position 12 are on the same side of this relatively planar turn structure with the disulfide crosslink underneath. If ϕ=90° and ψ=0° for the glycine residue at position 14, the succeeding Arg-Lys-Arg-Lys sequence protrudes at a right angle away from the incoming chain with the basic side chains extending from opposite sides of the strand forming the floor of a positively charged pocket. The side chain of the conserved lysine at position 11 extends into the top of the pocket.

3 marks the beginning of exon 36, and, although human tropoelastin lacks exon 35, the last two amino acids of human exon 34 (Phe-Pro) that join with exon 36 are identical with the last two amino acids of bovine exon 35. The proline residue at position 2 induces a 60° bend placing the cysteine-containing loop at an angle from the incoming peptide chain. A proline residue at this position does not appear essential, however, since glutamine and arginine substitute for proline in rat and chicken tropoelastin, respectively.

Although disulfides have an important role in stabilizing native protein conformation, other aspects of secondary structure must bring the reduced cysteine side chains into proximity for disulfide bond formation to occur; cysteine residues alone do not determine which parts of the molecule will end up as neighbors (12). Molecular modeling of bovine tropoelastin suggests that the cysteine residues are juxtaposed as the result of a tight turn formed by the intervening amino acids in positions 9-12 (Figure 4). The resulting antiparallel beta structure is stabilized by hydrogen bonds formed between the carboxyl group of alanine (position 6) and amino group of glycine (position 14) and between both the amino and carboxyl hydrogens on leucine (position 9) and serine (position 12). The side chains of leucine at position 9, lysine at position 11, and serine at position 12 all lie on the same side of this relatively planar turn structure with the disulfide crosslink underneath.

The six amino acids following the second cysteine are strictly conserved in all species. Although it is difficult, without experimental data, to deduce the conformation of the sequence after the loop, several predictions can be made based on known properties of the included amino acids. One possible structure is shown in Figure 4. The sequence begins with a glycine residue (position 14), the amino group of which is in the correct position to hydrogen bond with the carbonyl group of the alanine residue that precedes the first cysteine (position 6). In many proteins, glycines are conserved where the R-chain of the alpha carbon is directed inwards where only a hydrogen can be accommodated. With this assumption for the glycine at position 14, and setting ϕ=90° and ψ=0°, models show that the Arg-Lys-Arg-Lys sequence protrudes at a right angle away from the incoming chain (Figure 4). The arginine and lysine side chains extend from opposite sides of the peptide chain, perpendicular to the disulfide bonded loop, forming the floor of a positively charged pocket. At the top of the pocket is the side chain of the conserved lysine at position 11. The serine side chain at position 12 is also in a position to hydrogen bond with molecules that might fit into the pocket. Although the positively charged side chains of the Arg-Lys-Arg-Lys residues most likely remain in an extended position because of charge repulsion, freedom of rotation around the ψ bond of glycine at position 14 allows a great deal of flexibility for this positively charged sequence. Whatever the conformation of the Arg-Lys-Arg-Lys sequence, a consequence of forming the disulfide-stabilized loop is the positioning of the lysine at position 11 and the side chain at position 12 in proximity to the arginines and lysines to form the three dimensional pocket.

A function for the C-terminal domain is suggested by the requirements of elastic fiber assembly and interactions of tropoelastin with microfibrils. It is generally assumed

that microfibrils direct the morphogenesis of elastic fibers by acting as a 'scaffold' on which elastin is assembled. It is intriguing to speculate that the Arg-Lys pocket on tropoelastin provides a noncovalent binding site for interactions with acidic microfibrillar proteins and thus, is responsible for the critical step in fiber formation. This speculation is supported by several in vitro studies showing that a large proportion of tropoelastin not incorporated into elastic fibers and found in culture medium lacks the carboxy-terminal domain (5,11). Similarly, immunohistochemical studies have demonstrated the presence of the carboxy terminal domain in insoluble elastin fibers (5,13). Serving a critical role in fiber assembly would explain the high degree of conservation evident in the carboxy-terminal sequence; changes that alter the ability of the C-terminal domain to form the disulfide-bonded bend or that alter the orientation of the Lys-Arg side chains would have detrimental effects on fiber assembly.

Acknowledgments

This work was supported by NIH grants HL26499 and HL41926. Technical help was provided by Noreen Person. The excellent secretarial assistance of Terese Hall is gratefully acknowledged. We thank William C. Parks for helpful discussion.

References

1. Mecham, R.P. and Heuser, J.E. (1991). In: Cell Biology of Extracellular Matrix, 2nd Edition. Edited by Hay, E.D. Plenum Press, Inc., New York.
2. Cicila, G., May, M., Ornstein-Goldstein, N., Indik, Z., Morrow, S., Yeh, H.S., Rosenbloom, J., Boyd, C., Rosenbloom, J. and Yoon, K. (1985). Biochemistry. 24, 3075-3080
3. Indik, Z., Yeh, H., Ornstein-Goldstein, N. and Rosenbloom, J. (1990). In: Genes for extracellular matrix proteins. Edited by Sandell, L. and Boyd, C. 221-250. Academic Press, New York.
4. Yoon, K., Davidson, J.M., Boyd, C., May, M., LuValle, P., Ornstein-Goldstein, N., Smith, J., Indik, Z., Ross, A., Golub, E. and Rosenbloom, J. (1985). Arch. Biochem. Biophys. 241, 684-691
5. Wrenn, D.S., Parks, W.C., Whitehouse, L.A., Crouch, E.C., Kucich, U., Rosenbloom, J. and Mecham, R.P. (1987). J. Biol. Chem. 262, 2244-2249
6. Prosser, I.W., Whitehouse, L.A., Parks, W.C., Stahle-Bäckdahl, M., Hinek, A., Park, P.W. and Mecham, R.P. (1991). Connect. Tiss. Res. 25, 265-279
7. Habeeb, A.F.S.A. (1972). Methods Enzymol. 25, 457-464
8. Riddles, P.W., Blakeley, R.L. and Zerner, B. (1983). Methods Enzymol. 91, 49-60
9. Sandberg, L.B., Weissman, N. and Smith, D.W. (1969). Biochemistry. 8, 2940-2945
10. Whiting, A.H., Sykes, B.C. and Partridge, S.M. (1974). Biochem. J. 179, 35-45
11. Franzblau, C., Pratt, C.A., Faris, B., Colannino, N.M., Offner, G.D., Mogayzel, P.J.J. and Troxler, R.F. (1989). J. Biol. Chem. 264, 15115-15119
12. Richardson, J.S. and Richardson, D.C. (1989). In: Prediction of Protein Structure and the Principles of Protein Conformation. Edited by Fasman, G.D. 1-98. Plenum Press, New York.
13. Rosenbloom, J., G., W., Abrams, W., Ornsten, G.N., Indik, Z. and Kucich, U. (1986). Collagen Relat. Res. 6, 423-33
14. Indik, Z., Yoon, K., Morrow, S.D., Cicila, B., Rosenbloom, J.C., Rosenbloom, J. and Ornstein-Goldstein, N. (1987). Connect. Tiss. Res. 16, 197-211

15. Indik, Z., Yeh, H., Ornstein-Goldstein, N., Sheppard, P., Anderson, N., Rosenbloom, J.C., Peltonen, L. and Rosenbloom, J. (1987). Proc. Natl. Acad. Sci. USA. 84, 5680-5684
16. Yeh, H., Ornstein-Goldstein, N., Indik, Z., Sheppard, P., Anderson, N., Rosenbloom, J.C., Cicila, G., Yoon, K. and Rosenbloom, J. (1987). Collagen Rel. Res. 7, 235-247
17. Bressan, G.M., Argos, P. and Stanley, K.K. (1987). Biochemistry. 26, 1497-1503
18. Pierce, R.A., Deak, S.B., Stolle, C.A. and Boyd, C.D. (1990). Biochemistry. 29, 9677-9683