Volume 212, number 2, 229–232 FEB 04480 February 1987

Identification of disulfide bonds in carboxy-terminal propeptides of human type I procollagen

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Received 18 November 1986

Intra-chain and inter-chain disulfide bonds within the carboxy-terminal propeptides of human type I procollagen were studied using cyanogen bromide cleavage of the propeptides and electrophoresis on SDS-polyacrylamide/glycerol gels. The results could provide a better understanding of the assembly of proα-chains into procollagen.

Type I procollagen; Disulfide bond; Carboxy-terminal propeptide

1. INTRODUCTION

Type I procollagen consists of two proα1(I)-chains and one proα2(I)-chain. The amino-terminal end contains a globular domain in which intra-chain disulfide bonds exist, whereas no disulfides are found in the long triple helical domain. The carboxy-terminal domain features both intra-chain and inter-chain disulfide bonds [1–4]. About half of the total of 23 cysteine residues found in the carboxy-terminal propeptides form inter-chain disulfides linking the three chains together [5].

The assembly of proα-chains into procollagen is thought to begin in the carboxy-terminal propeptides by disulfide bond formation [1,3]. The rate limiting step seems to be the *cis/trans*-isomerization of the peptide bonds [6,7]. The carboxy-terminal disulfides may enhance triple helix formation by serving as a nucleation site in that the three proα-chains are in order alignment for correct triple helix folding [8].

The primary structure of the human proα1(I)-chain and proα2(I)-chain have recently been deduced by cDNA sequencing [9,10]. Human proα1(I)-chain contains a carboxy-terminal propeptide (C1) consisting of 244 amino acids, eight of which are cysteine residues at positions 40, 46, 63, 72, 80, 151, 195 and 242 [9]. The carboxy-terminal propeptide of the human proα2(I)-chain (C2) consists of 247 amino acids, the seven cysteine residues being at positions 44, 67, 76, 84, 153, 198 and 245 [10]. There is a high degree of homology between propeptides C1 and C2 in the locations of the cysteine residues, except for the fact that cysteine residue 46 in C1 is missing in C2.

It is not exactly known how the disulfide bonds are arranged within the carboxy-terminal propeptides. Olsen [11] concludes by cyanogen bromide (CNBr) peptide mapping experiments that two intra-chain disulfides lie between the residues 80–242 and 151–195 in the C1-propeptide, while Kuhn [8] proposes on theoretical grounds that inter-chain disulfides may lie between residues 40–46 and 44–46, linking the two C1-propeptides and the C2-propeptide together.

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Abbreviations: DTT, dithiothreitol; CNBr, cyanogen bromide

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

In this study CNBr fragments of isolated human type I carboxy-terminal propeptides were produced from native propeptides, propeptides containing only intra-chain disulfides and totally reduced propeptides. Peptide maps of these CNBr fragments were then used to resolve the disulfide bonds of the propeptide chains.

2. EXPERIMENTAL

Iodinated carboxy-terminal propeptides of human type I procollagen were kindly donated by Dr Juha Risteli, Department of Medical Biochemistry, University of Oulu, Finland [12]. The radiolabeled material was reduced totally by 10 mM DTT or partially by 0.5 mM DTT, which is known to reduce only inter-chain disulfides [5]. Free cysteine residues were blocked by adding iodoacetic acid to a final concentration of 0.1 M. The DTT and iodoacetic acid were removed by centrifugation in Sephadex G-25 gels [13].

The unreduced, partially reduced and totally reduced propeptides were digested with CNBr at room temperature for 24 h in 70% formic acid, after which they were lyophilized and submitted to electrophoresis on SDS-polyacrylamide/glycerol gels as described in [14]. After drying, the gels were exposed to Kodak X-Omat films to identify the radiolabeled propeptides and/or fragments of these. The films were scanned by densitometry.

All theoretically possible CNBr-derived fragments of unreduced, partially reduced or totally reduced carboxy-terminal propeptides were deduced from the primary sequence of human proα1(I) and proα2(I) carboxy-propeptides and then from different possibilities for inter-chain and intra-chain pairing of cysteine residues within these. The experimental results were compared to theoretical possibilities to resolve how the disulfide bonds lie within the carboxy-terminal propeptide of human type I procollagen.

3. RESULTS AND DISCUSSION

The SDS-polyacrylamide/glycerol slab gels used [14] showed an overall resolution of peptides with molecular masses between 2 and 100 kDa. The R_f value versus logarithm of molecular mass was linear within this range (fig.1). No confusing broadening of bands of low molecular mass was seen, and peptides differing by only about 500 Da could be separated by this method.

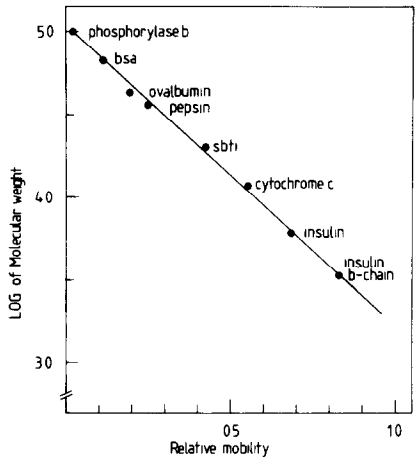

Fig.1. Standards used for determination of the molecular masses of propeptide and its CNBr fragments on SDS-polyacrylamide/glycerol gels. Standards (M_r) were phosphorylase *b* (92500), bovine serum albumin (bsa, 66200), ovalbumin (45000), pepsin (35000), soya bean trypsin inhibitor (sbti, 21500), cytochrome *c* (11700), insulin (5800) and the b-chain of insulin (3400).

The unreduced radiolabeled carboxy-terminal propeptide of human type I procollagen seemed to be of 100 kDa, which fits in well with the value expected, in view of the fact that propeptides prepared in the manner followed here contain some amino acids belonging to the telopeptide region [12]. This unreduced material also appeared to contain dimeric and monomeric propeptides with an apparent molecular mass of 64 and 34 kDa, respectively (fig.3D).

Theoretically, eleven different peptide fragments are obtained by CNBr digestion of totally reduced propeptides (fig.2). Peptides I–VI are gained from the C1 propeptide and peptides VII–XI from C2. When totally reduced radiolabeled propeptides were digested with CNBr only three bands were seen on the slab gels (fig.3A), with apparent molecular masses of 4.3, 3.5 and 2.8 kDa. The theoretical calculations suggest that the 4.3 kDa fragment could be peptide III, the 3.5 kDa fragment peptide II and the 2.8 kDa fragment peptide V (see fig.2). The molecular mass of peptide V is as calculated from the amino acid composition 2.1 kDa, but this contains a car-

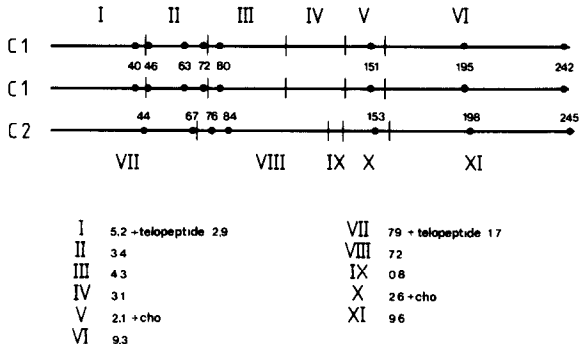

Fig.2. Schematic representation of the carboxy-terminal propeptides of the human type I proα1(I)-chain (C1) and proα2(I)-chain (C2). The vertical bars indicate the positions of methionine residues and the dots the positions of cysteine residues. CNBr fragments of the propeptides are marked by Roman numerals I–XI and their theoretical molecular masses given in kDa. Cho = carbohydrate moiety.

bohydrate moiety in the asparagine residue at position 146, which would give it a higher molecular mass [9]. The other peptides were not labeled because the radioiodination was not complete, although all the peptides except peptide IX contain tyrosine residues capable of being iodinated.

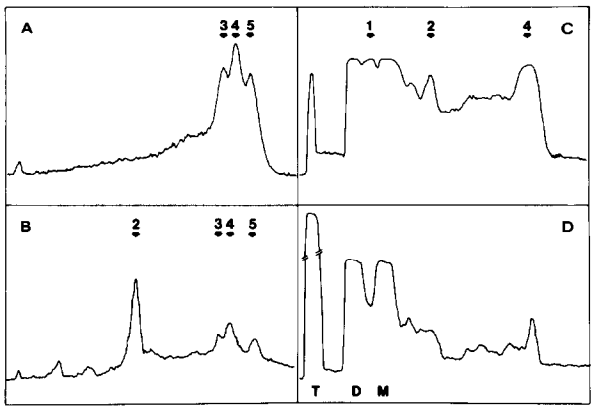

Fig.3. Scanning curves for (A) totally reduced propeptide after CNBr digestion, (B) partly reduced propeptide after CNBr digestion, and (C) unreduced propeptide after CNBr digestion, (D) unreduced, undigested propeptide. The numbers indicate the following molecular mass (in kDa): 1, 44; 2, 16.6; 3, 4.3; 4, 3.5; 5, 2.8. T, trimeric propeptide; D, dimeric propeptide; M, monomers of the propeptide.

After partial reduction with 0.5 mM DTT, which is capable of reducing only inter-chain disulfide bonds, an additional band with a molecular mass of 16.6 kDa occurred (fig.3B) and the proportions of the 4.3 and 2.8 kDa bands diminished. This new fragment must thus contain peptides III + V + VI, indicating that there are intra-chain disulfides between residues 80–242 and 151–195 in the C1 propeptide (fig.4).

In order to resolve the inter-chain disulfides between the propeptides, unreduced propeptides were cleaved by CNBr. Slab gels now showed two additional bands with apparent molecular masses of 44 and 16.6 kDa (fig.3C) as compared with the unreduced, undigested material (fig.3D). The 16.6 kDa fragment was theoretically assumed to be composed of CNBr peptides III + V + VI and the 44 kDa fragment could have been composed only of peptides I + II + II + VII + VIII + X + XI, indicating that the inter-chain disulfide bonds lie between residues 40–46, 44–46, 63–72, 67–72 and 63–76 (fig.4).

Since the unreduced material also contained monomeric and dimeric propeptides (fig.3D), the cyanogen bromide peptides derived from these should be noted. In fact, the 16.6 and 3.5 kDa peptides seen in fig.3C are probably obtained from the C1 propeptide, while from dimers consisting of the C1 + C2, peptides II + VII + VIII + X + XI and III + V + VI corresponding to molecular masses of 33.2 and 16.6 kDa should be seen. The former have the same molecular mass as the monomers

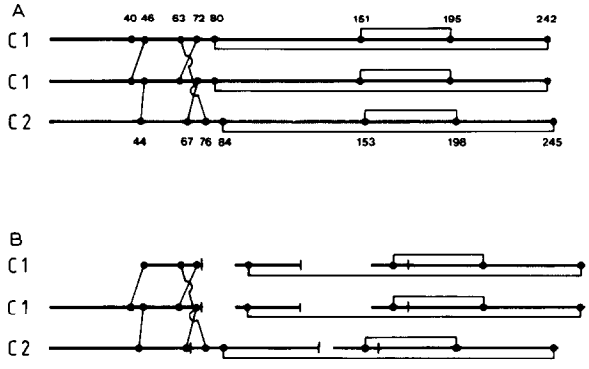

Fig.4. Schematic representation of disulfides in propeptides (A) and fragments after digestion of unreduced propeptides by CNBr (B), showing the 44 and 16.6 kDa fragments.

and are seen in the region of the monomer-sized protein. Dimers consisting of two C1 propeptides give fragments $2\times(I+II)$ and $III+V+VI$ and yield an additional band of 23.2 kDa (the faint band between the monomers and the 16.6 kDa fragment in fig.3C).

ACKNOWLEDGEMENTS

The author wish to thank Juha Risteli for preparing the propeptides, and Raili Myllylä for critical reading of the manuscript. The technical assistance of Miss Liisa Äijälä is also highly appreciated.

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