Oligomeric forms of the 148 kDa cartilage matrix protein

Reema ZEINELDIN, Suzanne EKBORG and John BAKER¹

Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, 1808 7th Avenue South, Birmingham, AL 35294, U.S.A.

The 148 kDa cartilage matrix protein (CMP), composed of three disulphide-bonded subunits, is a cartilage-specific glycoprotein found in association with fibrils of type II collagen and possibly with aggrecan. It is probable that CMP serves a structural role. As cartilage ages, an increasing proportion of the CMP becomes insoluble and resistant to extraction. In the present study, the isolation of CMP has been improved by inclusion of a hydrophobic chromatography step, thereby removing the remaining traces of collagen and proteoglycan. Evidence of self-association of CMP is presented. Higher-molecular-mass forms of CMP,

ranging in apparent molecular mass from 270 to 510 kDa and separated by SDS/PAGE, were located using a specific anti-CMP monoclonal antibody. Both CMP and its oligomeric forms are reducible to 52 kDa subunits, and only trace amounts of other proteins. The formation of oligomers, which may constitute 23% of the total cartilage matrix protein, could occur as a byproduct of the normal biosynthetic trimerization of subunits. Alternatively, the oligomers may represent a step toward the age-related cross-linking and insolubilization of CMP.

INTRODUCTION

The 148 kDa cartilage matrix protein (CMP) was first identified [1] and isolated from bovine tracheal cartilage [2]. It is a trimeric glycoprotein with subunits of approx. 52 kDa [2]. From the cloning and sequencing of the gene for chick CMP, it is evident that the subunit possesses two repeated domains that are homologous to domains in von Willebrand factor A. They are separated by an epidermal growth factor-like domain in CMP, and, additionally, there is a 43-residue C-terminal segment [3]. Of the 12 cysteine residues of the CMP subunit, two are found in this C-terminal segment and have been shown to be necessary and sufficient for the formation of inter-subunit disulphide bonds [4].

Some findings on the form and interactions of CMP have provided clues to its physiological role(s). Interactions with aggrecan in the extracellular matrix of cartilage may occur, as CMP and proteoglycan co-fractionate [1] and interaction *in vitro* has been reported [5]. CMP has been immunolocalized on collagen fibrils within the extracellular matrix of cultured chondrocytes [6,7]. Recently, CMP has been reported to form two types of filament, one of which associates with type II collagen [8]. Such interactions of CMP with collagen and aggrecan may be important for cartilage matrix organization. Nevertheless, it must be remembered that articular cartilage contains no CMP [9], but is properly organized.

There is considerable interest in identifying specific markers for diseases of cartilage degeneration [10]. CMP is a cartilage-specific product and can be immunoassayed in serum or synovial fluid. Some evidence suggests that elevated levels of CMP antigen in sera may be a feature of generalized degenerative disorders of cartilage metabolism [11].

CMP undergoes quantitative, as well as qualitative, changes with age. For example, there is an increase in the insolubility and total amount of CMP in tracheal cartilage of cattle during the first 10 years of life [12]. For our own studies of changes in CMP with age, it was necessary to isolate and purify CMP from bovine tracheal cartilage. Using the published method of isolation, which involves two gel-filtration steps, traces of higher-molecular-mass forms of CMP were noted. When size-based fractionation steps were avoided, the apparent higher-molecular-

mass forms of CMP were more evident. Some features of these oligomers have been examined and their possible significance discussed.

EXPERIMENTAL

Materials

Guanidine hydrochloride, dithiothreitol (DTT), CHAPS and 4-chloro-1-naphthol were from Sigma (St. Louis, MO, U.S.A.). Sephadex G-200, Sepharose CL-6B, octyl-Sepharose CL-4B gels and PD-10 columns were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). Pansorbin, a preparation of killed cells of *Staphylococcus aureus*, was from Calbiochem-Novabiochem (San Diego, CA, U.S.A.), and rabbit anti-mouse Ig (catalogue number AXL232) was obtained from Accurate Chemical and Scientific (Westbury, NY, U.S.A.). Horseradish peroxidase-conjugated goat anti-mouse Ig was from Biorad Laboratories (Hercules, CA, U.S.A.). Iodo-gen was purchased from Pierce (Rockford, IL, U.S.A.). Na¹²⁵I (17 Ci/mg) was from Du Pont NEN Research Products (Boston, MA, U.S.A.). Protran nitrocellulose membranes (BA85) were from Schleicher and Schuell (Keene, NH, U.S.A.).

Monoclonal antibodies (mAbs) were raised in mice to CMP, which had been isolated and purified from bovine trachea as previously described [2]. Four different mAbs to CMP, including mAb 55.9, were identified and characterized using methodologies previously described [13]. The mAb that was employed in the present study, mAb 55.9, was shown to have specificity for both CMP and species (bovine).

Extraction of CMP from bovine tracheal cartilage and chromatography on octyl-Sepharose CL-4B

In initial studies (see Figure 1), CMP was isolated from bovine tracheal cartilage by a published method [2]. An additional and final step, hydrophobic chromatography on octyl-Sepharose CL-4B, under previously described conditions [14] (and see below), was employed to free the product of remaining collagen and proteoglycan.

Recovery of higher oligomers of CMP was enhanced by utilizing the following procedure. Bovine tracheal cartilage (2 g),

Abbreviations used: CMP, the 148 kDa cartilage matrix protein; DTT, dithiothreitol; mAb, monoclonal antibody.

¹ To whom correspondence should be addressed.

which had been milled under liquid N₂ to pass a 100 mesh sieve and stored at -80 °C, was extracted in 4 M guanidine hydrochloride/0.1 M 6-aminocaproic acid/10 mM EDTA/5 mM sodium phosphate, pH 6.5 (20 ml) for 17 h at 4 °C. The extract was centrifuged at 25000 *g* for 2 h at 4 °C to remove tissue debris. The supernatant was diluted with an equal volume of 4 M guanidine hydrochloride containing protease inhibitors, and pumped onto an octyl-Sepharose CL-4B column (0.9 cm × 8.0 cm) at a flow rate of 6 ml/h. The column was then washed with 4 M guanidine hydrochloride/5 mM sodium phosphate, pH 6.5 until proteoglycan was no longer detected in the column effluent. Elution was with a linear gradient (100 ml total volume) of CHAPS (0–2%) in 4 M guanidine hydrochloride/5 mM sodium phosphate, pH 6.5 at a flow rate of 6 ml/h, and 2 ml fractions were collected.

The protocol was repeated, but on a larger scale, for experiments subsequent to that of Figure 2. Thus the extract from 50 g of cartilage was applied to an octyl-Sepharose CL-4B column (2.5 cm × 23 cm) and eluted with a 1 litre gradient at a flow rate of 60 ml/h (9 ml fractions).

Separation of CMP and higher oligomeric forms of CMP by chromatography on Sepharose CL-6B

CMP-containing fractions from the octyl-Sepharose CL-4B column (see Figure 3, fractions 19–35) were pooled and precipitated with ethanol (4 vol., 18 h at 4 °C). The pellet was recovered and dissolved in 4 M guanidine hydrochloride (10 ml) and applied to a Sepharose CL-6B column (2.5 cm × 200 cm), which was developed with 4 M guanidine hydrochloride/5 mM sodium phosphate, pH 7.6 at a flow rate of 15 ml/h. After a 200 min delay, fractions of 7.5 ml were collected.

Immunoprecipitation of CMP and the higher oligomeric forms of CMP

Proteins in an aliquot (40 µl; approx. 50 µg of protein) of the CMP oligomer pool (see the legend to Figure 4) were precipitated with ethanol (4 vol.), recovered following centrifugation and dissolved in 100 µl of 0.5% SDS in Tris-buffered saline (20 mM Tris and 500 mM NaCl, pH 7.4) before ¹²⁵I labelling using Iodogen [15]. A fraction of this ¹²⁵I-labelled oligomeric CMP pool (5 × 10⁵ c.p.m.) was immunoprecipitated with the anti-bovine CMP mAb 55.9 in the presence of rabbit anti-mouse Ig and Pansorbin as described previously [16]. The immunoprecipitate, bound to Pansorbin, was washed extensively with Tris-buffered saline containing 0.25% (w/v) sodium deoxycholate and 0.1% (w/v) Nonidet P40. Then, the immunoprecipitate was dissociated and dissolved in Laemmli sample buffer (160 µl) at 100 °C for 5 min.

SDS/PAGE and Western blotting

SDS/PAGE [17], electroblotting [18] and immunolocation (Bio-Rad instruction pamphlet) were carried out according to published procedures. Except where stated otherwise, gels (1.5 mm thick) were 7.5% (w/v) acrylamide plus bis-acrylamide.

RESULTS

CMP isolated from bovine tracheal cartilage in the manner previously reported [2] has a purity of less than 95%. The major impurities are collagen and proteoglycan. For purposes of characterizing mAbs to CMP and for studies of CMP's interactions, CMP was purified further. The additional step, hydrophobic chromatography on octyl-Sepharose CL-4B, was effective in reducing the hydroxyproline and galactosamine contents of

Figure 1 SDS/PAGE of CMP preparations

CMP was isolated by the published procedure [2] (lanes 1 and 3) or further purified by chromatography on octyl-Sepharose CL-4B (lanes 2 and 4). Samples were run in the absence (A) or presence (B) of DTT. Protein bands were localized by staining with Coomassie Blue.

the CMP preparation from 35 nmol and 28 nmol/mg, respectively, to undetectably low levels. Furthermore, a band with the mobility of collagen type II β chain (Figure 1, lane 3) was no longer evident after the additional purification step, despite overloading the gel (Figure 1, lane 4). Therefore, the octyl-Sepharose CL-4B step is useful for the removal of these final contaminants from isolated CMP. Although at this stage the CMP had been through a five-step purification process, traces of bands of molecular mass higher than CMP could still be discerned by SDS/PAGE (Figure 1, lanes 1 and 2). These higher-molecular-mass bands were not seen when the samples were reduced (Figure 1, lanes 3 and 4).

Further work was aimed at determining whether the higher-molecular-mass bands are possibly of higher oligomeric forms of CMP. It was supposed that some of the higher-molecular-mass forms could have been lost during the size-fractionation steps of the CMP-purification protocol. Therefore a simpler isolation procedure, which depends upon binding of CMP to octyl-Sepharose CL-4B and does not include fractionation according to size, was employed (see the Experimental section). The procedures were carried out at pH 6.5 and as rapidly as possible in order to minimize the risk of higher-molecular-mass CMP forming by disulphide exchange. Aliquots of eluant fractions from the octyl-Sepharose CL-4B column were separated by SDS/PAGE and electroblotted to nitrocellulose. CMP was immunolocated using a mouse anti-bovine CMP mAb, 55.9 (Figure 2). Eluting early in the gradient (fractions 8–21) were the doublet bands of CMP at > 212 kDa. Additionally, there were at least four bands of higher molecular mass, which also specifically bound to this antibody (Figure 2a). Densitometric scanning of

Figure 2 Fractionation of CMP by chromatography on octyl-Sepharose CL-4B

Aliquots of fractions from the gradient elution of CMP from a column (0.9 cm $\times$ 8.0 cm) of octyl-Sepharose CL-4B (see the Experimental section) were examined by SDS/PAGE in the absence (a) or presence (b) of DTT. Protein bands from the gels were electroblotted to nitrocellulose membranes, and CMP was immunolocalized using the mouse mAb 55.9. As commonly found, both CMP and CMP subunit resolve into two bands. (On more heavily loaded gels, e.g. Figure 1, this resolution is not seen.) #, number.

the CMP and the higher-molecular-mass bands indicated that the latter constituted 23 % of the extracted CMP. The antibody has also located two lower-molecular-mass fragments of CMP. After reduction with DTT before electrophoresis, the only bands that were immunolocalized were the two bands with the mobility of CMP subunits (Figure 2b). These findings support the suggestion that the higher-molecular-mass bands are of CMP oligomers, and further indicate that the oligomerization occurs through disulphide bonding.

It was shown that this one-step procedure was effective in separating CMP from the bulk of proteoglycan and collagen in the extract. Aggrecan and collagen type II did not bind to the column (see the legend to Figure 3), whereas CMP did bind and was separated from other major cartilage proteins: link proteins, albumin and a 36 kDa matrix protein (Figure 3).

CMP is not separated from its higher-molecular-mass oligomers by chromatography on octyl-Sepharose CL-4B, although the oligomers appeared on average to be retarded relative to CMP (Figure 2). Fractionation by gel filtration on a column of Sepharose CL-6B also only gave, as determined by SDS/PAGE of fractions, a partial separation (Figure 4). There are at least four bands of oligomeric forms of CMP. The apparent molecular masses of these four oligomeric forms of CMP were determined in an SDS/PAGE separation on a 5 % gel (results not shown) to be 510, 430, 390 and 270 kDa. These bands are labelled 1, 2, 3 and 4 respectively (Figure 4).

Figure 3 Separation of CMP from other major proteins of the cartilage matrix by chromatography on octyl-Sepharose CL-4B

A 4 M guanidine hydrochloride extract of cartilage was loaded on a column (2.5 cm $\times$ 23 cm) of octyl-Sepharose CL-4B, the column was washed with 4 M guanidine hydrochloride/5 mM sodium phosphate, pH 6.5, and then eluted with a gradient of CHAPS (0–2%; diagonal thin line) in 4 M guanidine hydrochloride/sodium phosphate, pH 6.5 (see the Experimental section). Aliquots of fractions from the gradient elution were separated by SDS/PAGE, and the gel was stained with Coomassie Blue and densitometrically scanned. Totals of 94 % of the hydroxyproline (as a measure of collagen) and 94 % of the galactosamine (as a measure of proteoglycan) of the extract were recovered in the pre-gradient (i.e. unbound) pool. The fractionated proteins are: CMP, —; link protein, ---; albumin, ·····; and a 36 kDa matrix protein, -·-·-

A scheme was devised to determine whether proteins other than CMP are components of any of the four major oligomers. The CMP oligomer pool was labelled with ^{125}I and immunoprecipitated using the mAb 55.9. The immunoprecipitate was washed to remove unbound radioactivity, dissociated in SDS and separated by SDS/PAGE. Autoradiography of the dried gel revealed bands of CMP oligomers and CMP. These bands were

Figure 4 Fractionation of CMP and CMP oligomers by gel filtration on a Sepharose CL-6B column

The CMP oligomer pool (Figure 3, fractions 19–35) was concentrated, applied to a column (2.5 cm $\times$ 200 cm) of Sepharose CL-6B and developed as described in the Experimental section. Aliquots of fractions were separated by SDS/PAGE, and protein bands were localized by staining with Coomassie Blue. A partial fractionation of CMP oligomers (labelled 1–4) from CMP is seen (fractions 58–82). Fractions 60–66, containing the CMP oligomers, and excluding much of the CMP, were pooled. (The faint band seen above 'oligomer 1' was insufficient for ^{125}I labelling in the subsequent experiment of Figure 5.) The positions of two molecular-mass markers are shown on the left (kDa).

Figure 5 SDS/PAGE of immunoprecipitated ^{125}I -labelled CMP oligomers

An aliquot of the CMP oligomer pool (see the legend to Figure 4) was labelled using Na^{125}I . The ^{125}I -labelled CMP oligomers were recovered after immunoprecipitation as described in the Experimental section and were separated by SDS/PAGE. ^{125}I -labelled CMP oligomer bands, localized in the dried gel by autoradiography, were separately excised, reduced with DTT and again fractionated by SDS/PAGE on a 7.5% gel. The ^{125}I -labelled CMP subunit band is the predominant band derived from CMP oligomers 1–4 (lanes 1–4 respectively). Fainter bands at 140 and 110 kDa are seen in lanes 1–3.

excised from the gel and reduced using DTT. Then, upon SDS/PAGE and autoradiography, only one major band, that of CMP subunit, showed in all wells (Figure 5). Two faint bands at 140 and 110 kDa amounted to less than 9% of the CMP subunit band, as determined by densitometric scanning.

DISCUSSION

Evidence has been provided in this study for the presence of some CMP, not as trimers, but as higher oligomers. Based upon their size range of 270–510 kDa, as determined by SDS/PAGE on 5% gels, 5–10 CMP subunits may oligomerize respectively. Oligomerization is through disulphide bonding, as CMP subunits were the products of reducing CMP oligomers with DTT. A preparation of CMP oligomers, after ^{125}I labelling and purification by immunoprecipitation, contained less than 9% of two unidentified 110 and 140 kDa protein bands. At this low level it is probable that these two diffuse bands are extraneous and that only CMP subunits are essential components of the CMP oligomers.

Why have these larger forms of CMP not been evident from the work of others? The methods used for CMP isolation appear to make the difference: application of fractionation steps based on size (column chromatography on Sephadex G-200 and Sepharose CL-6B), which are part of the standard protocol [2], favour isolation of the trimeric form of CMP. Also, in the present work, identification of CMP oligomers, CMP and CMP subunits has been aided by immunodetection with a mouse anti-bovine CMP mAb, 55.9. Some isolated CMP was seen by electron microscopy to assume the form of oligomeric chains [19]. In the present work, oligomeric CMP was still found when both extraction and isolation pHs are maintained at 6.5, and in

the presence or absence of *N*-ethylmaleimide. Therefore, it appears unlikely that CMP oligomers are an artifact of isolation or of non-specific disulphide exchange. Two cysteine residues in the C-terminal segment of the CMP subunit appear to be involved in the inter-subunit disulphide bonds of the CMP trimer [4]. Whether the same two residues are involved in the disulphide-bond formation required for CMP oligomer formation remains to be demonstrated.

CMP oligomer formation may be an intermediate stage in the formation of tissue-bound (i.e. guanidine-insoluble) CMP [12]. If so, an increase in CMP oligomers with age may be predicted. At present, it is not known what physiological variables affect the level of CMP oligomers in cartilage.

The physiological roles of CMP are poorly understood. By immuno-electron microscopy of the matrix surrounding embryonic chick chondrocytes, CMP has been seen closely associated with collagen fibrils [6,7]. The matrix synthesized by embryonic chick chondrocytes also contains a network of collagen-independent CMP filaments. Disulphide-bond formation is not involved in the genesis of these filaments [8]. In addition, there is evidence that aggrecan interacts with CMP *in vitro* [5]. Based upon these findings, it is clear that CMP serves a structural role in the cartilage matrix. However, it must be stressed again that this role is limited at least to non-articular cartilages. Further understanding of the structural complexities of CMP and its oligomeric forms, and of improved means for isolating CMP in its native form [20], are important steps towards defining the physiological roles of CMP.

We thank the Hybridoma Core Facility of the Multipurpose Arthritis Center at the University of Alabama at Birmingham (Dr. Mary Ann Accavitti and Ms. Fran Allen) for their assistance in raising mAbs to bovine CMP. We are indebted to Ms. Kelly Morrison for amino acid and hexosamine analyses, and for help with the Figures. This investigation was supported by a grant (AG-10070) from the National Institutes of Health.

REFERENCES

- Paulsson, M. and Heinegård, D. (1979) *Biochem. J.* **183**, 539–545
- Paulsson, M. and Heinegård, D. (1981) *Biochem. J.* **197**, 367–375
- Kiss, I., Deák, F., Holloway, R. G., Delius, H., Mebust, K. A., Frimberger, E., Argraves, W. S., Tsonis, P. A., Winterbottom, N. and Goetinck, P. F. (1989) *J. Biol. Chem.* **264**, 8126–8134
- Haudenschild, D. R., Tondravi, M. M., Hofer, U., Chen, Q. and Goetinck, P. F. (1995) *J. Biol. Chem.* **270**, 23150–23154
- Dang Lord, N., Baker, J. R. and Christner, J. E. (1984) *Fed. Proc.* **42**, 1626
- Winterbottom, N., Tondravi, M. M., Harrington, T. L., Klier, F. G., Vertel, B. M. and Goetinck, P. F. (1992) *Dev. Dyn.* **193**, 266–276
- Tondravi, M. M., Winterbottom, N., Haudenschild, D. R. and Goetinck, P. F. (1993) *Prog. Clin. Biol. Res.* **383B**, 515–522
- Chen, Q., Johnson, D. M., Haudenschild, D. R., Tondravi, M. M. and Goetinck, P. F. (1995) *Mol. Biol. Cell* **6**, 1743–1753
- Paulsson, M. and Heinegård, D. (1982) *Biochem. J.* **207**, 207–213
- Lohmander, L. S., Lark, M. W., Dahlberg, L., Walakovits, L. A. and Roos, H. (1992) *Clin. Biochem.* **25**, 167–174
- Saxne, T. and Heinegård, D. (1989) *Arthritis Rheum.* **32**, 1080–1086
- Paulsson, M., Inerot, S. and Heinegård, D. (1984) *Biochem. J.* **221**, 623–630
- Baker, J. R., Walker, T., Morrison, K., Neame, P. and Christner, J. (1989) *Matrix* **9**, 92–98
- Choi, H. U., Johnson, T. L., Pal, S., Tang, L.-H., Rosenberg, L. and Neame, P. J. (1989) *J. Biol. Chem.* **264**, 2876–2884
- Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. and Lowry, P. J. (1981) *Anal. Biochem.* **117**, 136–146
- Baker, J. R., Caterson, B. and Christner, J. E. (1982) *Methods Enzymol.* **83**, 216–235
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
- Heinegård, D. and Oldberg, Å. (1989) *FASEB J.* **3**, 2042–2051
- Hauser, N. and Paulsson, M. (1994) *J. Biol. Chem.* **269**, 25747–25753