Structural Characterization of Inter-α-inhibitor

EVIDENCE FOR AN EXTENDED SHAPE*

(Received for publication, May 4, 1998, and in revised form, October 21, 1998)

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Inter-α-inhibitor (IαI) is a 180-kDa serum protein consisting of three polypeptides. Two of these, the heavy chains 1 and 2 (H1 and H2), are of 75–80 kDa and have similar amino acid sequences. The third polypeptide, bikunin, has a molecular mass of 25 kDa and contains a 7-kDa chondroitin sulfate chain that is covalently linked to the C-terminal amino acid residues of H1 and H2. IαI has been shown to be required for the formation of the hyaluronan-containing extracellular matrix of certain cell types. How IαI exerts this function is not known, but it appears that upon interaction with cells, the heavy chains are released and become covalently linked to hyaluronan. Our results indicate that IαI and its heavy chains are extended molecules; thus, upon electron microscopy, IαI appeared to consist of two globular domains connected by a thin structure 31-nm long and the isolated heavy chains of a globular domain and a “tail” about 15-nm long. Analysis of the heavy chains by partial proteolysis showed that the C-terminal halves are particularly sensitive to hydrolysis indicating that they are loosely folded. Furthermore, electron microscopy showed that partially degraded heavy chains lacked the extended regions. Taken together, these results suggest that the N-terminal half of the heavy chains forms a globular domain, whereas the other half has an extended and loosely folded structure.

Although IαI was isolated more than 30 years ago (11), its physiological role is still unclear (for a review see Ref. 3). Recent findings indicate that this protein is required for the maturation of oocytes; in vitro experiments have shown that IαI stabilizes the hyaluronan-containing extracellular matrix that is formed by the cells surrounding the oocytes (12). The observation that the capillaries near the follicles become leaky to plasma proteins during ovulation supports the idea that the observed effect occurs in vivo (13). IαI has also been shown to be required for the formation of the hyaluronan-containing structure that surrounds fibroblasts and mesothelial cells (14). The physiological role of these coats is unclear, but it has been suggested that they may protect against compression (15), promote cell migration (16), and help to keep cells separated (15). It is not clear in what way IαI stabilizes this extracellular structure, but it appears that when the bikunin-containing proteins interact with cells, the bikunin moiety is displaced by hyaluronan molecules that become covalently linked to the heavy chains (17, 18).

Recent observations have indicated that IαI may also play a role in inflammation. Thus hyaluronan isolated from the synovial fluid of patients with rheumatoid arthritis has been found to contain covalently linked heavy chains (19, 20). Furthermore, upon inflammation, fibroblasts and monocytes secrete a protein named TSG-6, which reacts with IαI by displacing H1 and forming a covalent link with the chondroitin sulfate of bikunin (21). Upon this reaction, the ability of bikunin to inhibit plasmin is enhanced, and the formed complex appears to have a strong anti-inflammatory capacity (22). The molecular details of the interactions of IαI and TSG-6 are still unknown.

For further functional studies of IαI, detailed structural information will be helpful. The amino acid sequences of the heavy chains and bikunin are known, as are the structures of their glycans and disulfide arrangements (1, 23). In this study, we have investigated the conformation of IαI and its isolated heavy chains with various techniques.

MATERIALS AND METHODS

Enzymes

Chymotrypsin and elastase from pig pancreas were purchased from Boehringer-Mannheim. Thermolysin (type X) was from Sigma, hyaluronidase from ovine testes from Calbiochem, and chondroitinase AC II Arthro from Seikagaku Corp., Tokyo, Japan.

Purification of the Heavy Chains of Human IαI

For the isolation of IαI, a side fraction from the commercial production of factor IX was used (kindly provided by I.-M. Löföf; Pharmacia-Upps, Stockholm, Sweden). After dialysis against phosphate-buffered saline (PBS) and removal of insoluble protein, this material was subjected to gel filtration on Sephacry S-400, which yielded pure IαI. For the release of the heavy chains, 2 M NaOH was added to a solution of IαI (1 mg/ml in PBS) to give a final concentration of 0.05 M (24). After 15 min at room temperature, Tris-HCl, pH 8.0, was added to yield a final concentration of 0.25 M. Alternatively, one volume of IαI (1 mg/ml in
PBS) was mixed with two volumes of 0.4 M sodium acetate, pH 6.0, followed by the addition of chondroitinase and phenylmethanesulfonyl fluoride to yield the final concentrations 20 milliunits/ml and 0.2 mM, respectively (25). The mixture was incubated for 20 h at 37 °C. The sample was then dialyzed against 20 mM sodium phosphate, pH 7.6, and equilibrated with the same buffer. The proteins were eluted at 4 °C at a linear flow rate of 20 cm x h⁻¹ with 100 ml of a gradient from 0 to 0.7 M NaCl in 20 mM sodium phosphate, pH 7.6 (26). The fractions were analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Unless specified otherwise, protein concentrations were determined by UV measurements. The absorbance coefficients for the protein moieties of IoI, H1, and H2 were calculated from the amino acid compositions (Lasergene, DNASTAR Inc.). The published carbohydrate contents were then used for the calculation of the corresponding values for the whole proteins: 0.60, 0.47, and 0.72 mg⁻¹ ml⁻¹, respectively. The protein solutions were stored at −20 °C until they were used for experiments.

Gel Filtration

The proteins were applied to a Superdex 200 gel (3.2/30; Pharmacia Biotech) equilibrated with PBS at a flow rate of 50 μl/min; the absorbances at 280, 254, and 214 nm of the eluate were continuously measured. Thymidine and blue dextran were used as markers for flow-through. Thymidine absorption at 280, 254, and 214 nm of the eluate were continuously measured. Thymidine and blue dextran were used as a markers for flow-through. The absorbance coefficients for the proteins were determined by UV measurements. The absorbance coefficients for the proteins were calculated from the amino acid compositions (Lasergene, DNASTAR Inc.). Published carbohydrate contents were then used for the calculation of the corresponding values for the whole proteins: 0.60, 0.47, and 0.72 mg⁻¹ ml⁻¹, respectively. The protein solutions were stored at −20 °C until they were used for experiments.

Electron Microscopy

After thawing the protein samples, proteinase inhibitors were added: 0.1 M 6-aminohexanoic acid, 5 mM N-ethylmaleimide, 5 mM benzamidine hydrochloride, and 0.5 mM phenylmethanesulfonyl fluoride. The solutions were then dialyzed at 4 °C overnight against 0.2 M NH₄HCO₃, pH 7.9. Glycerol spraying and rotary shadowing were performed as described previously (27–30). Briefly, buffer and glycerol were added to make the final concentrations of protein and glycerol 5 and 400 mM/liter, respectively. The contrast was increased by the addition of polyethylene glycol 1500 (0.01 g/liter). The samples were sprayed onto freshly cleaved mica pieces, dried in vacuo and rotary shadowed at 9° with carbon/platinum through electron bombardment heating in a Balzers BAF 400 D freeze etching device. The samples were observed in a Jeol 1200 EX electron microscope operated at 60 kV accelerating voltage. Evaluation of the data from the electron micrographs was done as described previously.

For the selective removal of bikunin from IoI, the procedure of Enghild et al. (24) was used: a sample containing 0.9 mg/ml IoI and 6 μg/ml hylauronidase in PBS was incubated for 2.5 h at 37 °C. H2 was selectively removed by elastase digestion essentially as described by Balducky et al. (26) using an enzyme to protein ratio of 1:1,000 with the conditions described below for digestion of the isolated heavy chains. Limited chymotrypsin digestion of H1 was done as described below with an enzyme to protein ratio of 1:50. Subsequent electrophoretic analysis of the different protein samples showed bands with the expected apparent molecular masses.

Limited Proteolysis of the Heavy Chains

Chymotrypsin—2 μl of a solution with different concentrations of enzyme was added to 18 μl of a solution containing heavy chain (0.4 mg/ml) in 100 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 1 mM CaCl₂ to give various molar ratios of enzyme to heavy chain. After 150 min of incubation at 37 °C, the reaction was stopped by the addition of phenylmethanesulfonyl fluoride to a final concentration of 2 μM.

Trypsin—The reaction was performed as for chymotrypsin, except the buffer was 100 mM Tris-HCl, pH 7.4, with 50 mM NaCl and 20 mM CaCl₂.

Elastase—The reaction was performed as for chymotrypsin, except the buffer was 100 mM Tris-HCl, pH 7.4, and 50 mM NaCl.

Thromolysin—The reaction was performed as for elastase but was terminated by the addition of EDTA to a final concentration of 10 μM.

After proteolysis, the proteins were separated by electrophoresis in 15% polyacrylamide gels in Tricine buffer (31). The proteins were stained or transferred by electrophoresis onto a nitrocellulose membrane (Problott, Applied Biosystems; Foster City, California). The membrane was stained, and the appropriate bands excised and subjected to N-terminal sequencing in a 476 A amino acid sequenator (Applied Biosystems).

Circular Dichroism

Samples containing 0.1–0.3 mg/ml protein were dialyzed against 25 mM sodium phosphate buffer (pH 7.6), and the protein concentrations were determined by quantitative amino acid analysis. Circular dichroism spectra were obtained at room temperature on a JASCO-720 spectropolarimeter with a 0.1 cm cell. Data points were collected from 250 to 190 nm in 0.5 nm intervals at a rate of 50 nm/min; for each spectrum, 10 scans were performed and added. The percentages of secondary structural elements were estimated with the method of variable selection using a data base of 22 proteins with known secondary structure (32, 33).

RESULTS

Gel Filtration

Analysis of IoI, H1, and H2 by gel filtration (Fig. 1A) showed that more than 90% of the applied material eluted as one peak. Based on their amino acid sequences and known carbohydrate contents, the molecular masses of the constituents of IoI (H1, H2, and bikunin) are 77, 78 (1), and 25 kDa (1, 2), respectively, yielding a total mass of 180 kDa for the whole protein. The gel filtration experiments showed that IoI, H1, and H2 behave like globular proteins of 350 ± 7, 100 ± 4, and 140 ± 1 kDa (Fig. 1B, C; three determinations) indicating that they have extended structures. The corresponding Stokes radii are 6.4 ± 0.2, 3.8 ± 0.2, and 4.5 ± 0.1 nm, respectively (Fig. 1B, C). The ratios between these radii and those of spherical proteins of the same masses, the frictional ratios, are 1.7, 1.4, and 1.6, respectively (34).

Electron Microscopy

IoI molecules visualized by rotary shadowing (Fig. 2, A and B) showed particles with a diameter of 11 ± 2 nm. Most of these (63%) occurred pairwise joined by a thin strand. The average distance between the centers of the particles measured along the strand (Fig. 3A, shaded bars) was 42 ± 8 nm (Fig. 3B) implying that the strand was about 31-nm long. In the middle of the strand, there was a small globular structure the size of which was at the limit of the resolution of the method (Fig. 2B, arrowheads). To test the idea that this globule was bikunin, IoI was subjected to limited hylauronidase treatment, which has previously been shown to selectively remove bikunin (24). As shown in Fig. 2C, the treated molecules lacked the globule, 95% of the total number. To further test the identity of the structures seen by electron microscopy, H2 was specifically released from IoI by limited elastase digestion (26). Fig. 2D shows that this treatment leads to the disappearance of one of the large globular domains, whereas the small globule remained (indicated by arrowheads). Isolated H1 studied with the same technique (Fig. 2, E and F) displayed a globular domain (diameter, 11 ± 2 nm) with a thin tail; the average length of the molecule (Fig. 3A, open bars) was 20 ± 5 nm (Fig. 3B), the tail being about 15 nm. Mild proteolytic treatment of H1 (Fig. 2G) resulted in removal of the tail, whereas the size of the globular domain seemed unaffected. Apparently identical results were obtained with H2 (data not shown). The extended structure of IoI and its isolated heavy chains was also seen with negatively stained material (data not shown).

Partial Proteolysis

Flexible regions of proteins are generally more sensitive to proteolytic cleavage than the tightly folded ones (35). Partial proteolysis can therefore be used for the structural analysis of proteins. Fig. 4A shows the effect of incubating H1 with increasing amounts of different proteinases as judged by SDS-PAGE followed by staining with Coomassie Brilliant Blue.
peachment of $t_1$, $t_2$, and $t_3$ (and other, larger fragments) and the appearance of three major bands of 27, 18, and 10 kDa (denoted $t_4$, $t_5$, and $t_6$). Sequence analysis showed that $t_6$ was an N-terminal fragment and that $t_4$ and $t_5$ were the C- and N-terminal halves of $t_6$ (Fig. 4, B and C). Further increases in the enzyme concentration lead to the disappearance of $t_3$ and a simultaneous increase of $t_4$ and $t_5$ (lanes 5 and 6). As judged by its apparent molecular mass on SDS-PAGE, $t_4$ appears to extend beyond the C terminus of $t_5$ (Fig. 4C). Whether this is actually the case or whether $t_4$ behaves anomalously upon electrophoresis remains to be clarified.

Treatment of H1 with chymotrypsin (lanes 8–12) yielded fragments with apparent molecular masses close to those obtained with thermolysin (bands c1–c4). Their positions within the molecule were essentially the same as those found for thermolysin (Fig. 4, B and C). The fragments generated by treating H1 with elastase (lanes 14–18) or trypsin (not shown) were similar in size to those obtained with the two other enzymes; their N-terminal amino acid sequences were not determined.

We also used partial proteolysis to probe the structure of H2 (Fig. 5A). At a thermolysin/H2 ratio of 1:10,000, a number of bands in the range of 80–40 kDa were formed (lane 2). Some of these (collectively denoted $t_1$) were analyzed by amino acid sequencing and found to have the N terminus of the intact polypeptide (Fig. 5, B and C). It should be noted that this degradation occurred at a proteinase concentration ten times lower than that required to achieve a similar effect on H1. When the concentration of enzyme was increased (lanes 3–5), new bands appeared ($t_2$–$t_4$) apparently through truncation of the C-terminal end. At the highest proteinase concentrations used (lane 5), cleavage near the N terminus also took place (band $t_3$). Similar results were obtained with chymotrypsin (lanes 8–10) and elastase (lanes 13–15).

**Circular Dichroism**

The relative content of secondary structures in the heavy chains was assessed by circular dichroism measurements. The spectra of the two proteins were essentially identical (Fig. 6). The relative occurrence of different secondary structures were obtained by fitting the spectra to those of proteins with known secondary structure (32, 33). Three determinations were performed yielding values varying less than 2%. The estimated uncertainty in the procedure was less than 7% for each type of structure. This analysis yielded the following results: 34% $\alpha$-helix, 11% anti-parallel $\beta$-sheets, 8% parallel $\beta$-sheets, 16% turns, and 32% unordered structures; similar compositions have been obtained for globular proteins (36).

**DISCUSSION**

In this study, we have found that IαI and its isolated heavy chains behave as extended molecules in free solution; their Stokes radii as estimated by gel filtration are 40–70% larger than those of globular proteins of the same mass. These results are supported by electron microscopy, which shows that IαI consists of two globular domains connected by a thin flexible structure (Fig. 2, A and B). With the same technique, the isolated heavy chains seem to consist of a globular domain of the same size as those in IαI and of a thin tail whose length is about half that of the strand connecting the globular domains in the whole molecule (Fig. 2, E and F). These tails appear to be the C-terminal ends of the polypeptides, because upon treatment of the heavy chains with low concentrations of proteinases, the C-terminal parts of the polypeptides are degraded (Figs. 4C and 5C) and the extended regions seen by electron microscopy removed (Fig. 2G). Biochemical analysis has shown that the C-terminal ends of the two heavy chains in IαI are
covalently linked to the chondroitin sulfate chain of bikunin (6).

In the electron micrographs of IαI, a small globule can be seen in the middle of the strand connecting the larger globular domains (Fig. 2B, arrowheads). This structure appears to be bikunin, because it is not present in molecules subjected to mild hyaluronidase digestion (Fig. 2C), a treatment selectively releasing the bikunin moiety (24).

It is possible that the chondroitin sulfate chain of bikunin constitutes part of the thread-like structure between the globular domains in IαI. However, the finding that the sizes of the

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**Fig. 2.** Electron micrographs of IαI and H1 after glycerol spraying/rotary shadowing. Panels A and B, IαI shown at two different magnifications; the molecules appear as two globular domains connected by a thin flexible strand. The arrowheads in panel B indicate a small globular structure in the middle of the strand. Panel C, IαI after limited treatment with hyaluronidase, which releases bikunin; note the absence of the small globular structure seen in panel B. Panel D, IαI subjected to mild proteolytic digestion, which selectively releases H2; the arrowheads indicate the small globule at the end of the extended region. Panels E and F, isolated H1 shown at two different magnifications; note the tail on the molecules. Panel G, H1 subjected to mild proteolytic digestion; note the absence of the extended structure. The magnifications in Panels A and E were the same, as were those in panels B, C, D, F, and G; the bars show 100 and 30 nm, respectively.

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**Fig. 3.** Size distribution of molecular structures seen by electron microscopy. A, the distances between the centers of the two globules in 100 IαI molecules measured along the connecting strand (shaded bars). (Structures containing only one globular domain were not included in the calculations.) Also shown are the distances from the middle of the globular region to the end of the tail in 100 H1 molecules (open bars). B, schematic representation of IαI and H1 as seen by electron microscopy. For the sake of simplicity, the molecules are shown as straight, extended structures. The numbers show the average lengths (±S.D.) as obtained by measurements on the micrographs.
isolated heavy chains are about half of the whole molecule indicates that the contribution of the polysaccharide is small; the fact that the bikunin polypeptide is selectively released upon mild hyaluronidase treatment might be because of the heavy chains being linked to the chondroitin sulfate chain at relatively closely located sites (24). An alternative explanation for our results is that the release of the heavy chains from Iα was incomplete and that the tails are bikunin. However, the observation that the heavy chains ran as homogeneous bands upon SDS-PAGE with an apparent molecular mass of the expected value argues against this possibility. In this study, we used two procedures for releasing the heavy chains from Iα: short exposure to high pH and chondroitinase digestion. The former method is the simpler to perform but could possibly bring about conformational changes. However, we have not been able to detect any such differences between heavy chains prepared by either procedure.

Our results suggest that the heavy chains of Iα have a club-like structure. There are numerous examples of proteins of this kind. For example, the subunit of the cartilage oligo-

![Graph 1](image1.png)

**FIG. 4.** Partial proteolytic degradation of H1. A, the isolated polypeptide (10 μg) was mixed with different amounts of proteolytic enzyme and incubated for 2 h at 37°C. The resulting fragments were detected by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Lane 1 shows untreated H1, lanes 7, 13, and 19 show enzyme alone at the highest concentration. The ratios of enzyme:H1 in lanes 2–6 were 1:1,000, 1:300, 1:100, 1:10, and 1:5, respectively. The ratios for lanes 8–12 and 14–18 were 1:1,000, 1:100, 1:30, 1:10, and 1:5, respectively. The positions of marker proteins with their masses in kDa are shown on the right. The proteolytic fragments in a gel run in parallel were transferred to a membrane, and the bands indicated were subjected to amino acid sequencing. B, the N-terminal amino acid sequences of the different proteolytic fragments; the position of the initial amino acid residue within the intact protein is indicated as a superscript. C, the alignment of the different proteolytic fragments on the basis of their N-terminal amino acid sequence and molecular mass as estimated by SDS-PAGE. The horizontal axis shows the numbering of the amino acid residues starting from the N terminus.

![Graph 2](image2.png)

**FIG. 5.** Partial proteolytic degradation of H2. A, the isolated polypeptide was digested as described in Fig. 4. Lane 1 shows untreated H2, lanes 6, 11, and 16 show enzyme only. The ratios enzyme:H2 in lanes 2–5, 7–10, and 12–15 were 1:10,000, 1:1,000, 1:100, and 1:10, respectively. B, the N-terminal amino acid sequences of the indicated fragments. C, the alignment of the fragments.
are indicated with \( \text{S-S} \) characteristics obtained from these spectra are described in the text. The structural characteristics of H1 and H2, respectively. The hatched areas shown as (adapted from Ref. 1). The polypeptide regions resistant to intermediate X and \( \text{O} \). Furthermore, there is a high frequency of the structure-breakage in this region of the protein occurs at many closely spaced sites. The extended part of the heavy chains contains amino acid proline near the C termini of H1 and H2 (39). The fact that the proteinase-sensitive parts of the heavy chains constitute approximately half the polypeptides (see Fig. 7) indicates that there are regions other than the tails that contain flexible structures. However, as judged by electron microscopy (Fig. 2G) and gel filtration, the sizes of the globular domains were not affected by partial proteolytic digestion. These findings indicate that the fragments formed from the globular domains were not released.

It is possible that the structures of Iol and its isolated heavy chains were altered during the preparation for electron microscopy. The fact that this technique did not reveal any difference in the sizes between H1 and H2, whereas gel filtration showed that H1 was less extended than H2, suggests that this is the case. Possibly, electron microscopy of unstained, vitrified samples could provide the sizes at more physiological conditions.

The experiments with partial proteolytic cleavage described in this paper is an extension of an earlier study in which it was shown that upon treatment of intact Iol with elastase, fragments of H1 and H2 were released by C-terminal cleavages (26). Similar results were also obtained upon incubation of Iol with tumor cells (40). Whether this cleavage process is of physiological significance remains to be seen. It is still unclear how Iol supports the formation of the hyaluronan-containing pericellular coat on various cell types. It has been suggested that the heavy chains bind to the cell surface and then become covalently linked to hyaluronan molecules through their C-terminal ends (41). If this is the case, it would make sense for the globular domains to be close to the cell surface and the flexible C-terminal parts to extend out of the glycocalyx (38).

Acknowledgments—We thank Å. Engström for help with the gel filtration experiments and T. C. Laurent for valuable suggestions.

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