The Low pH in Trans-Golgi Triggers Autocatalytic Cleavage of Pre-α-inhibitor Heavy Chain Precursor*

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Pre-α-inhibitor is a plasma protein whose physiological function is still unknown, but in vitro studies suggest that it might be involved in inflammatory reactions. Pre-α-inhibitor consists of a 25- and a 75-kDa polypeptide: bikunin and heavy chain 3 (H3), respectively. H3 is synthesized with a 30-kDa C-terminal extension, which is released in the Golgi complex through cleavage between an Asp and a Pro residue. We now provide evidence that this cleavage is triggered by the low pH in the late Golgi and occurs through an intramolecular process. First, incubation in vitro of the H3 precursor (proH3) at pH 6.0 or lower results in rapid cleavage of the protein. Second, the rate of the cleavage reaction does not depend on the concentration of proH3 and is not affected by the presence of various protease inhibitors. Third, raising the pH in organelles of cells producing proH3 abolishes cleavage during secretion. The amino acid residues near the cleavage site of proH3 differ from those of previously described self-cleaving proteins, indicating that the mechanisms of scission are different.

During their transport to the cell surface via the Golgi complex, secretory proteins are extensively modified. A common modification is proteolytic cleavage, which usually occurs late during intracellular transport: in the trans-Golgi network and/or in the secretory vesicles (1). The cleavage typically occurs next to a dibasic sequence, and a number of enzymes have recently been identified that mediate this process (2).

The ionic conditions along the secretory pathway vary: for example, the pH in the endoplasmic reticulum is around 7.0, approximately half a pH unit lower in the trans-Golgi, and slightly below 6.0 in the trans-Golgi network (3, 4). Some of the reactions occurring in the latter compartment, such as sorting of secreted proteins and cleavage of prohormones and proenzymes, have been shown to depend on low pH (1, 5–7).

We are studying the structure and function of bikunin-containing proteins (for a review, see Ref. 8). Bikunin is a 25-kDa protease inhibitor with a chondroitin-sulfate chain. In plasma, most of the bikunin is complexed with one or two polypeptides of about 75 kDa. There are three such polypeptides, named heavy chain 1, 2, and 3 (H1, H2, and H3), having sequence similarity of 38–55% within one species (9). Bikunin and the heavy chains are all synthesized by hepatocytes, and during their passage through the Golgi complex they become linked through an ester bond between a hydroxyl group of an internal N-acetylgalactosamine residue of the chondroitin sulfate chain and the a-carboxyl group of the C-terminal aspartic acid residue of the heavy chains (10, 11). In this fashion, pre-α-inhibitor (PαI) is formed from one bikunin and one heavy chain (H3) and inter-α-inhibitor (IαI) from one bikunin and two heavy chains (H1 and H2) (10, 12). The physiological function of PαI and IαI is not clear, but it has been shown that hyaluronan in inflamed tissue contains covalently linked heavy chains; apparently hyaluronan can displace the chondroitin sulfate chain in the bikunin-containing proteins (13). Furthermore, IαI has been found to interact with TSG-6, a hyaluronan-binding and inflammation-related protein (14).

The heavy chains are synthesized as precursors with C-terminal extensions of about 30 kDa (15–17). These fragments are released in the Golgi complex by cleavage between an Asp and a Pro residue (9). Using transfected COS-1 cells, we have previously shown that these two residues are absolutely essential for cleavage (18). The fact that there is no known protease that specifically cleaves at an Asp-Pro site and that the cleavage of the heavy chain precursors occurs earlier than the cleavage of proproteins with dibasic sequences (19) prompted us to study this reaction further. In the present paper we present evidence that the cleavage of the H3 precursor is autocatalytic.

**EXPERIMENTAL PROCEDURES**

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1 The abbreviations used are: H1, H2, and H3, heavy chains 1, 2, and 3; PαI, pre-α-inhibitor; IαI, inter-α-inhibitor; proH3, precursor of H3; PAGE, polyacrylamide gel electrophoresis; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; E-64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64); bestatin, leupeptin, and aprotinin was from Sigma. The expression vector pXM was obtained from Genetics Institute Inc. (Cambridge, MA), oligonucleotides from DNA Technology (Aarhus, Denmark), and Tran35S-label (1000 Ci/mmol) from ICN. P35S was purified from rat plasma as described previously (12); an antiserum to this protein was obtained by intramuscular injection of the protein into a rabbit.

2 Construction of Expression Vector and Mutagenesis—The cDNA coding for rat H3 (12) was subcloned into the eukaryotic expression vector pX3M, which provides adenovirus late promotor-driven expression of introduced cDNA. Truncations of the C-terminal part of H3 were made by conversion of the codons corresponding to Cys662 and Pro663 to stop codons. Substitutions of amino acid residues as well as truncations were made with the unique site elimination procedure through the use of the
Secreted ProH3 Is Cleaved at Low pH—We wanted to analyze the process by which the precursor of heavy chain 3 (proH3) is cleaved. We therefore sought to obtain the cleavage in a cell-free system. As a substrate we used the partially cleaved proH3 produced in COS-1 cells (18). This recombinant protein was incubated with solubilized Golgi membranes isolated from rat liver (prepared as described in Ref. 20). The pH in the trans-Golgi network has been reported to be around 6.0 (3), and this value was therefore chosen for the pH of the incubation mixture. As a control in this experiment a sample lacking the membranes was used. Fig. 1A shows the electrophoretic analysis of the reaction: upon incubation the amount of proH3 (113 kDa) and of H3 (80 kDa) decreased and increased, respectively. To our surprise we found that cleavage occurred at the same rate whether Golgi membranes were present or not. Fig. 1B shows a quantitative analysis of the time course for the formation of H3 upon incubation. The amount increased rapidly during the first 60–100 min and then leveled off, yielding a maximum value of 50–53%; half-maximal cleavage occurred within 20–30 min. Further analysis showed that the pH optimum of the reaction was between 3.5 and 4.5 (Fig. 2).}

Characterization of the Cleavage Process—In the experiments described above, medium from cells expressing proH3 was used without previous fractionation. To see whether cleavage was caused by a soluble factor present in the medium, we isolated proH3 by immunoprecipitation and then incubated the protein at low pH: the degree of cleavage was the same as that obtained with unfraccionated medium (Fig. 3, cf. bars 1 and 2).
cysteine, and aspartic proteases (described under "Experimental Procedures") was added; no inhibition of the processing could be detected (Fig. 3, bar 5). The presence of the metal ion chelator EDTA was also without effect (bar 6). In addition, analysis of proH3 by gel filtration showed that it occurred as a monomer (data not shown), ruling out the possibility that the cleavage reaction was intermolecular.

To see if the native structure of proH3 was essential for cleavage, we incubated the protein in the presence of SDS: very little cleavage occurred (Fig. 3B, bar 3). Treatment of H3 with dithiothreitol did not significantly affect cleavage (bar 2), indicating that disruption of its cysteine bridges does not induce a major conformational change.

Effects of Weak Amine on Processing—We then wanted to see if cleavage of proH3 during intracellular transport also requires low pH. It has previously been shown that treatment of cells with weak amines elevates the internal pH of the cellular organelles (21). We therefore added methylamine to the medium of COS-1 cells expressing proH3 to a final concentration of 5 or 10 mM and determined the degree of cleavage. As shown in Fig. 4, cleavage decreased by approximately 45 and 70%, respectively.

Mutations in C-terminal Extension Affect Cleavage—Using COS-1 cells transfected with proH3 cDNA, we have previously shown that the C-terminal extension is required for the coupling of H3 to bikunin (18). To see whether it also has a role in the cleavage reaction, we introduced various mutations in this part of proH3 and assessed their effect on cleavage. Deletion of the C-terminal extension, except for the 14 amino acid residues nearest the cleavage site, abolished cleavage, as did deletion of the last 65 amino acid residues (Fig. 5B, lanes 2 and 3). In heavy chains 1 and 3 a segment of the C-terminal propeptide has been found to have sequence similarity with the multicopper-binding domain of multicopper oxidase proteins (9), marked with a dashed line in Fig. 5B. Substitution of Ser for Gly728 or Phe or His for Tyr731 within this domain abolished cleavage (<1% cleaved) or reduced it by approximately 75%, respectively (lanes 6 and 7). Mutation of another conserved Gly further upstream (Gly707 to Ser) decreased the cleavage by approximately 50% (lane 4). The possible function of three clustered His residues near the multicopper oxidase region was also studied; mutation of His776, conserved within all three heavy chains, as well as His782 and His785, conserved between H2/H3 and H1/H3, respectively, to Asn decreased cleavage by approximately 95% (lane 5).

DISCUSSION

Earlier studies have shown that the heavy chains of pre- and inter-α-inhibitor are synthesized as precursors with C-terminal extensions, which are cleaved off during transport to the cell surface; for all of these polypeptides cleavage seems to occur between an Asp and a Pro residue (15, 16). In the present study we found that incubation at low pH of the precursor of pre-α-inhibitor heavy chain (proH3) induced cleavage of its C-terminal extension. It has long been known that the bond between Asp and Pro residues is sensitive to acid treatment (22). Thus, incubation in 75% formic acid for 24 h has often been used for the generation of specific fragments of polypeptides (23). However, the rates reported for such cleavages are at least 100 times lower than those we obtained with proH3 under considerably less acidic conditions. Furthermore, we found that cleavage was optimal around pH 4 (Fig. 2) and that denaturation, which has been shown to improve the cleavage of other proteins (23), abolished that of proH3 (Fig. 3B, bar 3). These findings, together with the observation that the cleavage reaction was dilution independent and unaffected by immobilization of the

![Fig. 4. Cellular cleavage of proH3 depends on low pH. Methylamine was added to the medium of COS-1 cells expressing proH3 to a final concentration of 5 or 10 mM. The cells were then labeled with [35S]Met for 4 h, and the cleavage of secreted H3 was assessed as described in the legend to Fig. 1. The relative amount of cleaved precursor (average of two experiments) is shown as bars in the lower panel.](image)
protein (Fig. 3A, bars 2–4), strongly suggest that proH3 is cleaved through an intramolecular reaction. To explain the acid lability of Asp-Pro bonds, it has been proposed that at low pH, the β-carboxyl group of Asp initiates a nucleophilic attack on the α-carbonyl carbon, giving rise to an unstable structure containing a five-membered ring (within square brackets). Due to the presence of a protonated amide nitrogen, the formed intermediate may dissociate leading to cleavage of the polypeptide chain.

C-terminal extension is inferred from the fact that it contains two cysteine residues and that its electrophoretic mobility is sensitive to reduction (12).

FIG. 5. Mutations in C-terminal extension reduce cellular cleavage. A, proH3 with different mutations was expressed in COS-1 cells, and cleavage was assessed as described in the legend to Fig. 1. B, the amino acid sequence of the C-terminal extension of rat proH3 starting 12 amino acid residues upstreams of the Asp-Pro cleavage site (indicated with an arrowhead). The residues that are identical in proH3 of rat, human, and mice as well as in heavy chains 1 and 2 of mice are printed in reverse. Amino acid residues mutated in this and a previous study are indicated with an asterisk, a region showing sequence similarity with a multicopper oxidase domain with a hatched line, and deletions with a △. C, proH3 is shown as a box with the C-terminal extension hatched and with the mutated amino acids indicated; those marked with a + were found to be absolutely required (<1% cleaved) and those marked with (+) partially required for processing (5–30% cleaved). Mutation of those marked with * was without effect. Muta
tional analysis of the amino acid residues near the cleavage site has been described previously (18). The presence of a disulfide bridge in the protein (Fig. 3A, bars 2–4), strongly suggest that proH3 is cleaved through an intramolecular reaction. To explain the acid lability of Asp-Pro bonds, it has been proposed that at low pH, the β-carboxyl group of Asp initiates a nucleophilic attack on the α-carbonyl carbon, giving rise to an unstable structure containing a five-membered ring (within square brackets). Due to the presence of a protonated amide nitrogen, the formed intermediate may dissociate leading to cleavage of the polypeptide chain.

FIG. 6. Proposed mechanism for autocatalytic cleavage of proH3. At low pH, the β-carboxyl group of Asp initiates a nucleophilic attack on its α-carbonyl carbon, giving rise to an unstable structure containing a five-membered ring (within square brackets). Due to the presence of a protonated amide nitrogen, the formed intermediate may dissociate leading to cleavage of the polypeptide chain. This reaction requires the presence of an adjacent protonated amide nitrogen. The degree of protonation of the amide nitrogen of a Pro residue is higher than those of all other amino acid residues, which could account for the acid sensitivity of a Asp-Pro bond. The simplest interpretation of our results is that this type of reaction occurs in the H3 precursor and that the rate is enhanced through the influence of the adjacent polypeptide structures. An observation that supports this model is that substitution of Glu for Asp, which precludes the formation of the pentagonal intermediate structure, completely abolishes cleavage (18).
Bikunin is synthesized as a precursor also containing α₁-microglobulin (25). The two proteins are separated by a dibasic sequence and pulse-chase experiments with isolated rat hepatocytes have shown that the precursor is cleaved just before it reaches the cell surface (19). The same type of experiment has also shown that the cleavage of proH3 (which is a prerequisite for the coupling of H3 to bikunin) occurs about 10 min before the bikunin precursor is cleaved (19). These observations suggest that the bikunin and H3 precursors are cleaved in different compartments: the secretory vesicles and the trans-Golgi/trans-Golgi network, respectively. The residence time of a secretory protein in the Golgi complex and the secretory vesicles is 5–20 min (26), indicating that the protein spends <10 min in the trans-Golgi network. As judged by our in vitro experiments, this time would not be sufficient for efficient cleavage of proH3, given a pH in this part of the Golgi of about 6.0. It therefore seems that there must exist other factors that enhance the reaction. The Golgi is known to have a high free Ca²⁺ concentration (27), but we have not seen any effect of this ion on the in vitro cleavage reaction. Another possibility is that the pH is lower locally than what has been measured so far. In this context it is interesting to note that different cell lines differ in their capacity to cleave proH3 upon transfection (12, 17).

If the different components of the proposed cleavage reaction (Fig. 6) would be in equilibrium, cleavage of the polypeptide would not be complete, as is the case in our system (see Fig. 1B). However, if one of the final products would react irreversibly with another compound, the reaction would go to completion. Since anhydrides may react readily with carbohydrates, it is possible that the complete cleavage of the heavy chains seen in hepatocytes is due to their coupling to the chondroitin sulfate chain of bikunin. This hypothesis is supported by the observation that heavy chain 1 and 2 expressed in COS cells are cleaved only when coexpressed with bikunin (24). We have previously shown that after cleavage of proH3, the C-terminal extension (in the absence of bikunin) remains bound to the heavy chain (18). It is conceivable that one of the functions of this part of proH3 is to prevent molecules other than chondroitin sulfate to become coupled.

We found that when H3 was expressed with a truncated C-terminal extension, cleavage did not take place, suggesting that the deleted part is essential for processing. A region of the C-terminal extension has been found to have sequence similarity with multicopper oxidases (9) (Fig. 5). When two conserved amino acid residues in this region were mutated, cleavage of the resulting protein was drastically reduced, implying a specific role for these residues in the reaction. However, truncation of the protein downstream of this region also abolished cleavage, indicating that the general structure of the C-terminal extension is essential for activity.

A number of proteins have been shown to have the capacity to catalyze their own cleavage, such as glycosylasparaginases, the pyrovoyl enzyme precursor, and hedgehog proteins (reviewed in Ref. 28). A common feature of these reactions is an N-O or N-S acyl rearrangement of a serine, threonine, or cysteine residue at the C-terminal side of the cleavage site, yielding an unstable ester-containing intermediate. Since there is a Pro residue in the corresponding position in proH3, it seems that the cleavage mechanism of H3 is different from those previously described.

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