FOR THE RECORD

Disulfide structure of alfineprase: A recombinant analog of fibrolase

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Abstract

The disulfide structure of alfineprase, a recombinant analog of fibrolase, was experimentally determined by a combination of peptide mapping, Edman degradation, and mass spectrometry. The three disulfide bonds were determined to be Cys-116/196, Cys-156 /180, and Cys-158/163 with the residue number system of alfineprase.

Keywords: Fibrolase; alfineprase; disulfide bond; Edman degradation; mass spectrometry

Fibrolase is a zinc-containing metalloproteinase isolated from the venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*). It is a small protein that contains 203 residues (Randolph et al. 1992). Alfineprase (3–203 Fibrolase [3-Ser], CAS registry number 259074–76–5) is a recombinant analog of fibrolase. Soluble alfineprase is secreted by *Pichia pastoris* and a series of chromatography steps has been developed to generate pure material at Amgen. Alfineprase differs from fibrolase in the amino-terminal region, where the amino-terminal three residues of fibrolase (Glu-Gln-Arg) were replaced with a single serine residue. The truncated analog was designed to prevent the formation of cyclized glutamine (pyroglutamic acid). Fibrolase and alfineprase are similar with respect to thrombolytic activity (Markland et al. 1994) and are irreversibly bound by α_2 -macroglobulin (Ahmed et al. 1990). The enzymes exhibit direct fibrinolytic activity without the activation of plasminogen, thus little hemorrhagic activity is observed (Retzios and Markland 1988; Ahmed et al. 1990). Because of their thrombolytic activity, low hemorrhagic response, and α_2 -macroglobulin binding, these enzymes have a high therapeutic potential for treating thrombotic disease.

Alfineprase is in a clinical trial for the treatment of peripheral arterial occlusion. Figure 1 shows the sequence of alfineprase with all six cysteine residues indicated. The number system used in this report is based on the amino acid sequence of alfineprase, which is two amino acids shorter than fibrolase.

Fibrolase contains six cysteine residues that form three disulfide bonds. It was experimentally determined that Cys-118 is disulfide linked to Cys-198 (Randolph et al. 1992). However, the remaining two disulfide bonds, which involve Cys-158, Cys-160, Cys-165, and Cys-182, have not been experimentally determined because of the difficulty in digesting the portion of protein containing these cysteine residues. Predictions of the remaining two disulfide bonds have been made on the basis of homology modeling. For example, Randolph et al. (1992) predicted the 158/165 and 160/182 pattern and Manning (1995) predicted the 158/182 and 160/165 pattern. No experimental evidence has been reported to support either configuration. This report describes the experimental results that lead to the determination of disulfide bond structure in alfineprase, the recombinant analog of fibrolase.

Results and Discussion

On the basis of the sequence of alfineprase and the specificities of five commonly used proteases (endoproteinases Asp-N, Glu-C, Lys-C, Arg-C, and trypsin), endoproteinase

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      10          20          30
SFPQRYVQLV IVADHRMNTK YNGDSDKIRQ
      40          50          60
WVHQIVNTIN EIYRPLNIQF TLVGLEIWSN
      70          80          90
QDLITVTSVS HDTLASFGNW RETDLLRRQR
      100         110         116        120
HDNAQLLTAI DFDGDTVGLA YVGGMCQLKH
      130         140         150
STGVIQDHSA INLLVALTMA HELGHNLGMN
      156 158 160 163 170 180
HDGNQCHCGA NSVMAAMLS DQPSKLFSDC
      190         196        200
SKKDYQTFLT VNNPQILNK P

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Fig. 1. Sequence of alfineprase with cysteine residues marked. Alfineprase differs from fibrolase in that the amino-terminal three residues (EQR) in fibrolase are replaced by a single residue S; therefore, the residue numbers of alfineprase differ from those of fibrolase by two.

Asp-N is the only protease that may generate a reasonable number of small peptide fragments. It was found that alfineprase is very resistant to proteolytic digestion under native conditions. Alfineprase is a zinc metalloproteinase, and zinc is required to maintain the conformation and protease activity of alfineprase (Ahmed et al. 1990; Pretzer et al. 1992). To make alfineprase more susceptible to enzymatic digestion and to eliminate the enzymatic activity of alfineprase, EDTA was added to the digestion buffer. However, Asp-N is also a metalloproteinase and thus inhibited by EDTA, although to a lesser extent. It was found that 2 mM of EDTA is required to completely eliminate the autodigestion of alfineprase and unfold alfineprase to an extent that can be readily digested, while keeping the activity of Asp-N.

Endoproteinase Asp-N-digested alfineprase was subjected to liquid chromatography/mass spectrometry (LC/MS) analysis. Figure 2 shows the UV chromatograms of Asp-N-digested alfineprase with disulfide bonds intact and reduced. It is seen from Figure 2 that chromatographic peaks A-E in the nonreduced map are not present in the reduced map, and chromatographic peaks 1-4 in the reduced map are not present in the nonreduced map. Identities of peaks 1-4 were deduced from their determined masses in combination with the specificity of Asp-N and then confirmed by amino-terminal sequencing. Identities of peaks A-E were deduced from their determined masses in combination with the specificity of Asp-N as well as peptides already identified in peaks 1-4. Table 1 shows the assignments of these chromatographic peaks. Alfineprase has six cysteine residues (at positions 116, 156, 158, 163, 180, and 196). From peaks C, D, and E, it can be concluded that Cys-116 is linked to the Cys-196 because only these two cysteine residues are present in peaks C, D, and E. However, all of the remaining four cysteine residues (Cys-156,

Fig. 2. Comparison of peptide maps of Asp-N-digested alfineprase with disulfide bonds intact (*top trace*) and reduced (*bottom trace*). Peaks labeled A-E in the nonreduced map are not present in the reduced map and peaks labeled 1-5 in the reduced map are not present in the nonreduced map.

158, 163, and 180) are present in both peaks A and B, which makes the assignments of these disulfide bonds difficult. From the masses of peak A and B, it is known that all of the four cysteine residues are disulfide linked.

To determine the disulfide linkages between cysteine residues at positions 156, 158, 163, and 180, the compounds represented by peaks A and B were collected and subjected to five cycles of Edman degradation before being analyzed by mass spectrometry. After five cycles of Edman degradation, Cys-156 will be removed from the peptide chain. Figure 3 shows the mass spectra of the two compounds represented by peaks A and B after five cycles of Edman degradation. Both spectra show a main mass spectral peak with monoisotopic mass at 1391.5 Da, which corresponds to the mass of fragment $H^{157}-S^{170}$, with Cys-158 and Cys-163 disulfide linked (theoretical mass 1391.55 Da). Besides the

Table 1. Assignments of peaks 1-5 in the reduced peptide map and peaks A-E in the nonreduced peptide map of alfineprase^a

Peak	Peptide	Determined mass	Theoretical mass
Reduced			
1	$D^{171} - (C^{180}) - K^{183}$	1481.6	1481.7
2	$D^{152} - (C^{156} - C^{158} - C^{163}) - S^{170}$	1910.4	1910.7
3	$D^{184} - (C^{196}) - P^{201}$	2106.8	2107.0
4	$D^{92} - (C^{116}) - Q^{126}$	3651.7 ^b	3652.1 ^b
5	$D^{92} - (C^{116}) - H^{151}$	6342.7 ^b	6343.2 ^b
Nonreduced			
A	$(D^{152} - S^{170})/(D^{179} - K^{183})$	2485.8	2486.0
B	$(D^{152} - S^{170})/(D^{171} - K^{183})$	3388.5	3388.4
C	$(D^{105} - Q^{126})/(D^{184} - P^{201})$	4383.8 ^b	4384.0 ^b
D	$(D^{92} - Q^{126})/(D^{184} - P^{201})$	5758.2 ^b	5758.5 ^b
E	$(D^{92} - H^{151})/(D^{184} - P^{201})$	8449.5 ^b	8449.6 ^b

^a The positions of cysteine residues are also indicated for peptides 1-5. All masses are monoisotopic unless otherwise noted.

^b Average mass as opposed to monoisotopic mass.

Fig. 3. Mass spectra of the two compounds in fraction A (top) and fraction B (bottom) in the nonreduced Asp-N peptide map after five cycles of Edman degradation. The insets show zoom-scan spectra of the singly charged ion of fraction A and doubly charged ion of fraction B. The monoisotopic masses are determined from the zoom-scan spectra.

major ions that correspond to peptide $H^{157}-S^{170}$, some other ions were also observed in Figure 3. These ions were not present when the same procedure was repeated on a new Asp-N digest of alfineprase (spectra not shown), indicating that these ions are due to contamination in the process. The fact that both peptide chain $D^{179}-K^{183}$ in fraction A and chain $D^{171}-K^{183}$ in fraction B were removed by five cycles of Edman degradation is consistent with Cys-156 being linked to Cys-180 and Cys-158 being linked to Cys-163. These disulfide linkages also are consistent with linkages in various members of the matrix metalloproteinase/snake toxin superfamily (Manning 1995).

Materials and methods

Materials

Purified, recombinant alfineprase (CAS registry number 259074-76-5) is from Amgen. Endoproteinase Asp-N was purchased from Roche Diagnostics Corporation. Dithiothreitol (DTT) was purchased from Calbiochem. Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from Sigma Chemical Co. Sodium phosphate monobasic and sodium phosphate dibasic were

purchased from J.T. Baker. Acetonitrile was purchased from Burdick Jackson. Trifluoroacetic acid (TFA) was purchased from Pierce. Water was purified with a Milli-Q water purification system.

Endoproteinase Asp-N digestion and disulfide reduction

Alfineprase was diluted to a final concentration of 0.2 mg/mL into a 0.1 M phosphate buffer (pH 7.1) containing 2 mM EDTA. A 200- μ L portion of the above alfineprase solution was digested with 2 μ g of endoproteinase Asp-N at 37°C for 4.5 h. After digestion, 2.5 μ L of DTT solution (at 33 mg/mL) was added into a 100- μ L portion of the digestion solution and the sample was incubated at room temperature for 30 min for reduction of disulfide bonds. Both the nonreduced and reduced Asp-N digests of alfineprase were analyzed on the LC/MS system.

LC/MS

The LC/MS system consisted of an Agilent HP1100 HPLC system directly connected to a Finnigan-MAT LCQ electrospray-ion trap mass spectrometer equipped with an electrospray interface. Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA and 90% acetonitrile in water. The column used was a YMC C-8 column with 300- $\text{\AA}$ pore, 5- μ m particle size, and 2 mm id $\times$ 250 mm length. The column temperature was set at 30°C. The protein digest was resolved with gradient elution (2–32% B in 20 min, then to 60% B in 42 min) with a flow rate of 0.2 mL/min. The chromatogram was monitored by both UV (set at 215 nm wavelength) and mass spectrometry. Mass spectrometric detection included full scan in positive mode, as well as data-dependent zoom scan and MS/MS of the most intense ion. Monoisotopic masses were determined from zoom scans and average masses were determined using "MagTran," a custom charge deconvolution software written according to the algorithm described by Zhang and Marshall (Zhang and Marshall 1998; Zhang 1998).

Amino-terminal sequencing

To confirm the identity of some chromatographic fractions, these fractions were collected and subjected to amino-terminal sequence analyses. Amino-terminal sequence analyses were performed on an Agilent G1000A series protein sequencer.

Edman degradation and mass spectrometry

To determine the disulfide linkages among four cysteine residues within one proteolytic fragment in the nonreduced Asp-N digest, the peptide was first subjected to a specific number of cycles of Edman degradation, followed by mass spectrometric analysis. Edman degradation was performed on an ABI Procise protein sequencer (PE Biosystems) on sample applied to a PVDF membrane. After the specified number of Edman degradation cycles, the sample was then eluted from the PVDF membrane by vortex mixing in a solution of 0.1% TFA in 50% acetonitrile and analyzed by mass spectrometry. Mass spectrometric analyses were performed on a Finnigan-MAT LCQ quadrupole ion-trap mass spectrometer using a custom nano-electrospray interface built at Amgen. Glass capillary nanospray needles (New Objectives) with $\sim$ 2- μ m i.d. tips were used, and a needle voltage of 0.75 kV was supplied. The

resulting flow rate for this nanospray system was estimated to be 50–100 nL/min.

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References

- Ahmed, N.K., Tennant, K.D., Markland, F.S., and Lacz, J.P. 1990. Biochemical characteristics of fibrolase, a fibrinolytic protease from snake venom. *Hae-mostasis* **20**:147–154.
- Manning, M.C. 1995. Sequence analysis of fibrolase, a fibrinolytic metalloproteinase from agkistrodon contortrix contortrix. *Toxicon* **33**:1189–1200.
- Markland, F.S., Friedrichs, G.S., Pewitt, S.R., and Lucchesi, B.R. 1994. Thrombolytic effects of recombinant fibrolase or apsa in a canine model of carotid artery thrombosis. *Circulation* **90**:2448–2456.
- Pretzer, D., Schulteis, B., Vander, V.D.G., Smith, C.D., Mitchell, J.W., and Manning, M.C. 1992. Effect of zinc binding on the structure and stability of fibrolase, a fibrinolytic protein from snake venom. *Pharm. Res.* **9**: 870–877.
- Randolph, A., Chamberlain, S.H., Chu, H., Retzios, A.D., Markland, F.S. Jr., and Masiarz, F.R. 1992. Amino acid sequence of fibrolase, a direct-acting fibrinolytic enzyme from agkistrodon contortrix contortrix venom. *Protein Sci.* **1**: 590–600.
- Retzios, A.D., and Markland, F.S. Jr. 1988. A direct-acting fibrinolytic enzyme from the venom of agkistrodon contortrix contortrix: Effects on various components of the human blood coagulation and fibrinolysis systems. *Thromb. Res.* **52**: 541–552.
- Zhang, Z. 1998. *A computer program for automated charge state deconvolution based on ZSCORE algorithm*. Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics, Orlando, Florida, May 31–June 4, 1998. American Society for Mass Spectrometry, Santa Fe, NM.
- Zhang, Z., and Marshall, A.G. 1998. A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. *J. Am. Soc. Mass Spectrom* **9**: 225–233.