The Heparin-Binding Site of Human Xanthine Oxidase

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The enzyme xanthine oxidase (XOD) has an affinity for heparin and can bind to cultured porcine aortic endothelial cells. We have reported that the exposure of human XOD (h-XOD) to the lysine-specific reagent trinitrobenzenesulfonic acid or the arginine-specific reagent phenylglyoxal caused it to lose its affinity for heparin-Sepharose. The heparin-binding sites in h-XOD are further studied in the present article. From a chymotryptic digest of cyanogen bromide fragmented h-XOD, two peptides with an affinity for heparin-HPLC, A-1 and A-2, were isolated. Amino acid sequence analysis showed that both peptides had lysine and/or arginine residues. The A-1 region may direct its charged side chains toward the solvent while burying its hydrophobic side chains against the hydrophobic inside, because the A-1 peptide forms a highly amphipathic structure. Peptide A-2 contains triple lysine residues and constitutes a hydrophilic region.

Keywords xanthine oxidase; heparin; free radical

Active oxygen metabolites have been implicated in a number of disease processes.1,2 Endothelial cells have been proposed as the initial site of tissue injury by the active oxygens in the vascular system because they are ubiquitous and are located at the blood-tissue barrier. Moreover, endothelial cells are a rich source of superoxide-generating enzyme xanthine oxidase (XOD) [EC 1.2.3.2].3 XOD has a primary biochemical role in purine catabolism, which is the hydroxylation of hypoxanthine and xanthine to form uric acid. XOD is a dimer of identical subunits with a molecular mass of about 145 kDa containing one flavin-adenine dinucleotide (FAD), one molybdopterin and two iron-sulfur centers per subunit as cofactors.3 Mammalian XOD predominantly exists as a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase (XDH) [EC 1.1.1.204] type in vivo. This enzyme can be readily converted to an oxygen-dependent, NAD-independent form (XOD form). XDH-to-XOD conversion can occur reversibly through limited proteolysis or reversibly through cysteine oxidation.4 Conversion may be clinically significant because a XOD type enzyme produces reactive oxygen metabolites. XOD has been demonstrated to be a main source enzyme for active oxygens, because marked inhibition of radical generation has been observed in the presence of potent XOD inhibitors, allopurinol and oxypurinol.5,6 In a number of tissues, including the mammary gland, liver, heart, lung and intestine, it has been demonstrated that XOD is not only a cytoplasmic enzyme, but that it is also localized on the outside surface of the endothelial cell plasma membrane.7 Recently, we have reported that purified human XOD (h-XOD) has an affinity for heparin and can bind to cultured porcine aortic endothelial cells. Studies with amino acid-specific reagents indicated that both lysine and arginine residues seemed to contribute to heparin-binding.8

In the present study, the heparin-binding regions of h-XOD are explored by the determination of amino acid sequences of chymotryptic peptides which have an affinity for a heparin-HPLC column.

MATERIALS AND METHODS

Materials h-XOD was purified by the method described previously.40 TLCK-chymotrypsin was purchased from Sigma, St. Louis, MO, U.S.A. TSKgel heparin-5PW HPLC column was purchased from Tosoh Co., Tokyo, Japan. An Ultron 300 C4 reverse-phase HPLC column was purchased from Shinwa Chemical Ind., Ltd., Kyoto, Japan.

S-Carboxymethylation and Cyanogen Bromide Fragmentation One milligram of h-XOD was dissolved in 1 ml of 0.5 M Tris–HCl, pH 8.5, containing 7 m guanidine HCl and 10 mM EDTA-2Na, and flushed with nitrogen. This sample was reduced with 10 mg of dithiothreitol, flushed with nitrogen again, and incubated for 2 h at room temperature. After the addition of 25 mg iodoacetic acid, the reaction mixture was incubated in the dark for 30 min, followed by extensive dialysis against distilled water. After rotary evaporation, the residue was dissolved in 100 μl of 70% formic acid containing 2 mg of cyanogen bromide, and it was then left to stand overnight at room temperature. The reaction mixture was diluted 10-fold with distilled water and then rotary evaporated.

Chymotrypsin Digestion A cyanogen bromide cleaved sample was dissolved in 150 μl of 8 M urea by warming at 37 °C for 15 min, and followed by the addition of 450 μl of 0.1 M ammonium carbonate. It was digested with 8 μl of TLCK-chymotrypsin (0.1 mg/ml) at room temperature for 20 min.

Isolation of Heparin-Binding Peptides The chymotryptic digest of h-XOD was applied to a heparin-HPLC column (7.5 × 75 mm) equilibrated with 25 mM sodium phosphate, pH 6.5. The column chromatography was operated at a flow rate of 0.5 ml/min. The column was washed with the same buffer for 20 min and the buffer containing 0.15 M NaCl for 20 min. Then, a programmed gradient was run from 0.15 to 1.0 M NaCl for 10 min, and eluted peptides were pooled.

These peptides were separated by C4 reverse-phase HPLC (4.6 × 150 mm) operated at a flow rate of 0.7 ml/min. A gradient system formed between solvent A (0.1% trifluoroacetic acid in Milli Q water) and solvent B

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(0.1% trifluoroacetic acid in acetonitrile) was used. Peptides were eluted with a programmed gradient (0% B for 10 min, 0-60%B for 60 min and 60-100%B for 10 min). The peptides were detected on the basis of the absorbance at 210 nm.

**Amino Acid Sequence Determination** Amino acid sequences were determined with an Applied Biosystems Protein Sequencer Model 473A (Foster City, CA).

**RESULTS AND DISCUSSION**

The chymotryptic digestion of h-XOD was applied on a heparin-HPLC column. As shown in Fig. 1, most of the peptides appeared in the fractions that passed through or were eluted from the column with the buffer containing 0.15 M NaCl. These peptides have either no affinity or only a weak affinity for heparin, and seem not to contribute to binding with glycosaminoglycans *in vivo*. Peptides which had a high affinity for the column were eluted with a gradient of NaCl up to 1 M and were separated by reverse-phase HPLC. Two peptides, termed A-1 and A-2, were recovered as shown in Fig. 2, and their amino acid sequences were assayed.

In accord with the amino acid sequence deduced from the h-XOD cDNA, peptides A-1 and A-2 corresponded to residues Leu781–Met795 and Lys1106–Tyr1122, respectively. The two peptides were highly homologous to that of Leu783–Met797 in mouse XOD and Leu768–Met782 in rat XOD (11) (A-1), and Lys1108–Tyr1124 in mouse XOD (10) and Lys1093–Tyr1109 in rat XOD (11) (A-2), respectively. However, the sequences of both A-1 and A-2 were not homologous to the corresponding sequences in h-XOD reported by Wright, et al. (12) (Fig. 3).

Whereas the amino acid sequence of human XOD reported by Ichida, et al. (9) is highly homologous to those of mouse (10) and rat, (11) with over 90% identity, the sequence reported by Wright, et al. (12) shows only half identities between each of the above sequences.

It was predicted that the Arg7–Arg14 in peptide A-1 folded as an α-helix or β-sheet as judged by secondary structure algorithms. When represented on an α-helical wheel, residue 7–14 in A-1 formed a highly amphipathic structure with only hydrophobic residues on one side and charged residues except Val10 on the other (Fig. 4). The region formed peptide A-1 may direct its charged side chains toward the solvent while burying its hydrophobic side chains against a hydrophobic inside. We have shown previously that the modifications of lysine residues by trinitrobenzenesulfonic acid or arginine residues by phenylglyoxal caused a loss of heparin-affinity of h-XOD. From these results, the basic amino acid residues in the A-1 peptide may draw negatively charged residues in heparin and contribute to binding with heparin. The A-2 peptide contains triple lysine residues and constitutes a hydrophilic region. This region should extend into the peptide A-1

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\text{LGVPANRIVRKVRKRM}
\]

human XOD

\[
\text{LGVPANRIVRKVRKRM}
\]

mouse XOD

\[
\text{LGVPARDNIRKVRKRM}
\]

rat XOD

\[
\text{LGVPARDNIRKVRKRM}
\]

peptide A-2

\[
\text{KKNPSGSWEDVTAAAY}
\]

human XOD

\[
\text{KKNPSGSWEDVTAAAY}
\]

mouse XOD

\[
\text{KKNPSGSWEDVTAAAY}
\]

rat XOD

\[
\text{KKNPSGSWEDVTAAAY}
\]

Fig. 3. Amino Acid Sequences for the Heparin-Binding Sites of Human Xanthine Oxidase

Amino acid sequences for XOD from human, mouse and rat were aligned with the amino acid sequences of peptides A-1 and A-2, determined here. Identical amino acids have been boxed. a) Data from ref. 9. b) Data from ref. 10. c) Data from ref. 10. d) Data from ref. 11.

**Fig. 4. Helical Projection of Residues 7–14 of Peptide A-1**

Hydrophobic residues are represented by stippled circles. Basic amino acid residues are represented plus (+) within the circles.
solvent. The sequence Lys–Lys–Lys is homologous to the heparin-binding domain of proteins such as fibronectin,\textsuperscript{13} selectin\textsuperscript{14} and extracellular-superoxide dismutase (EC-SOD) [EC 1.15.1.1].\textsuperscript{13}

The heparin-Sepharose column chromatography showed that h-XOD bound heparin at physiological ionic strength (0.15 M) and was eluted with the buffer containing 0.3—0.4 M NaCl.\textsuperscript{9} This heparin-affinity is relatively weaker than that of EC-SOD, which requires 0.6 M NaCl to elute,\textsuperscript{16,17} or than that of antithrombin III (ATIII), which requires more than 1 M NaCl to elute from a heparin-column.\textsuperscript{18} The possible heparin-binding site of EC-SOD is a cluster of basic amino acid residues at the C-terminal end, Arg–Lys–Lys–Arg–Arg–Arg (amino acids 210—215).\textsuperscript{17} Moreover, human EC-SOD has a Gly196–Pro197–Gly198 sequence located at the N-terminal of the heparin-binding site. This sequence forms a flexible turn in protein structure and probably exposes the heparin-binding domain to form optimal interactions with heparin.\textsuperscript{19,20} ATIII has two separate heparin-binding regions, and basic residues in these regions form a band of positive charge of appropriate size for interaction with an ATIII-binding sequence in heparin.\textsuperscript{21} In this article, we have shown two predominant heparin-binding sites in h-XOD. Although both regions have basic amino acid residues, the heparin-affinity of h-XOD is lower than those of EC-SOD and ATIII. First, the density or disposition of these residues may be less suitable for interaction with heparin. Second, the fact that h-XOD is a bulky protein with a high molecular mass of about 290 kDa may weaken the affinity for heparin through sterie and/or electrostatic hindrances.

The experiments presented here demonstrate that h-XOD can bind to the surface of endothelial cells by the interaction of its specific region and extracellular glycosaminoglycans. Heparin binding to h-XOD was found to not inhibit the enzymic activity (data not shown). This indicated that h-XOD in vivo, associated with glycosaminoglycans, should retain its enzymic activity. The redox centers of XOD, iron-sulfur centers, FAD and molybdenopterin are known to be located in the 20 kDa domain, 40 kDa domain and 85 kDa domain, respectively.\textsuperscript{22} However our proposed heparin-binding sites are found in the 85 kDa domain, they does not overlap the molybdenopterin-binding site.\textsuperscript{9} No effect of the heparin treatment of h-XOD on its enzymic activity has been consistent with the above result.

REFERENCES