Irreversible conversion of xanthine dehydrogenase into xanthine oxidase by a mitochondrial protease

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Abstract Irreversible conversion of xanthine dehydrogenase (XDH) to its oxygen free radical producing oxidase (XO) form occurs through an uncharacterized proteolytic process, which was studied in human liver. Upon incubation of fresh unfrozen liver cytosol, XDH remained intact. When recombinant human XDH was coincubated with subcellular fractions of human liver, the mitochondrial intermembrane space was shown to contain a heat-labile activity that converted XDH irreversibly to XO. This activity is resistant to inhibitors of all major groups of proteases. We postulate that this novel type of proteolytic enzyme is released into the cytosol upon mitochondrial damage.

Key words: Ischemia; Reperfusion; Reactive oxygen species

1. Introduction

Xanthine dehydrogenase/oxidase (EC 1.1.1.204/1.2.3.22) catalyzes the last two steps of purine catabolism in man, formation of the end product uric acid from hypoxanthine and xanthine. The mammalian enzyme is synthesized as a dehydrogenase (XDH), which uses NAD^+ as the electron acceptor, but it can be converted into an oxidase (XO) both in vivo and in vitro. The oxidase form uses molecular oxygen as the electron acceptor and releases substantial amounts of superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) under appropriate conditions, e.g. during tissue reperfusion following ischemia, thus potentially contributing to tissue injury (for reviews, see [1,2]).

Xanthine dehydrogenase is a homodimer with a subunit molecular mass of approximately 150 kDa. Conversion into xanthine oxidase (XO) may occur through sulfhydryl group oxidation, which can be reversed with thiol reagents, e.g. dithiothreitol (DTT) [3]. Reversible XDH-XO conversion has been attained experimentally by glutathione depletion [4], ischemia [5–7], ischemia-reperfusion and oxidizing agents, such as hydrogen peroxide [8].

Irreversible conversion occurs during purification procedures, unless the tissue samples to be analyzed are prepared rapidly and with a minimum of manipulation prior to measuring the activities [9]. Even if such precautions are taken, tissue preparations consistently contain a minimum of 10–15% of total XDH+XO activity in the oxidase form [5].

A model for the XDH tertiary structure has been proposed [10,11]. According to this model, the 150 kDa subunit consists of three relatively protease-resistant globular domains with approximate molecular masses of 20, 36–40 and 85–92 kDa, held together by protease-sensitive peptide 'hinges'. Proteolysis at these sites can be attained in vitro by partial digestion with a variety of proteases, including trypsin, α-chymotrypsin, thermolysin and subtilisin [9–12]. The observation that the proteolytically modified XDH comigrates with the non-digested native polypeptide in non-reducing gels and gel filtration experiments suggests that these domains remain associated with each other despite limited proteolysis [11]. When the modified peptide is run on a denaturing gel, the fragments dissociate giving rise to three bands of the above-mentioned sizes.

Proteolytic processing of XDH during tissue ischemia takes place through cleavage of an approximately 15–20 kDa fragment from each subunit [13,14], and is believed to occur subsequent to the reversible conversion [5,6]. The proteolytic conversion has been hypothesized to be modulated by intracellular calcium levels [5,15], though apparently not through a calcium-dependent protease (calpain) [16]. Calcium-dependent proteolytic XDH-XO conversion has been shown to be sensitive to calmodulin inhibitors [17,18], but the enzyme responsible for the conversion is unknown.

In this study, we demonstrate a proteolytic activity found in human liver that converts XDH irreversibly to XO. This soluble activity has been localized to the mitochondrial intermembrane space and is resistant to conventional antiproteases.

2. Materials and methods

2.1. Human liver tissue samples

Fresh human liver samples were obtained from partial orthotopic liver transplantations at the Hospital for Children and Adolescents, University of Helsinki. The donor livers had been perfused with and preserved in ice-cold University of Wisconsin solution for up to 4 h prior to tissue homogenization. The liver samples were obtained after approval of the local ethical committee and guidelines for discarded human tissue were followed.

Mitochondria were purified from fresh liver tissue as described below. Warm ischemia/hypoxia was simulated by incubating ≤100 mg tissue pieces under 50 mM potassium phosphate buffer (pH 7.8) at 37°C for up to 2 h.

2.2. Purification and sequencing of XDH cleavage products

Xanthine oxidase was purified from human milk as described [19], and from human liver using an immunoaffinity column with rabbit polyclonal anti-XO antiserum coupled to Sepharose [19]. The purified enzyme preparations were electrophoresed on a 5% denaturing polyacrylamide gel and electroblotted onto a polyvinylidene difluoride filter (Immobilon-P, Millipore). Staining of the filter with 0.25% Coomassie blue showed three clear bands corresponding to the molecular weights of 150, 130 and 85 kDa. These bands were cut out using a scalpel and loaded into an automated gas-phase protein sequencer (Applied Biosystems model 477A) with a phenylthiohydantoin analyzer.

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Abbreviations: XDH, xanthine dehydrogenase; XO, xanthine oxidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MPT, mitochondrial permeability transition

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2.3. Purification and subfractionation of liver mitochondria

The mitochondria were purified and subfractionated from human liver by minor modifications of a method described by Schnaitman and Greenawalt [20]. Briefly, liver tissue (250 g) was homogenized in ice-cold 10 mM potassium phosphate/0.3 M sucrose/1 mM EDTA/0.5 mM DTT buffer (pH 7.4) by mincing with scissors and by 5 strokes in a loose-fitting Potter-Elvehjem homogenizer. The homogenate was centrifuged at $1000\times g$ for 10 min to pellet the nuclei and residual tissue. The supernatant was centrifuged twice at $10\,000\times g$ for 10 min to pellet the mitochondria with intermediate washing with one pellet volume of homogenization buffer. The pellet was resuspended to a protein concentration of approximately 100 mg/ml in the same buffer.

The suspension was incubated with digitonin (1.2 mg/10 mg protein) for 15 min on ice with stirring to lyse the mitochondrial outer membranes, diluted 1:4 with homogenization buffer, and centrifuged for 10 min at $15\,000\times g$ to pellet the mitoplasts (inner membrane+matrix). The supernatant was further centrifuged for 20 min at $144\,000\times g$ to pellet the outer membranes. This second supernatant contains the intermembrane proteins. The mitoplasts were treated with lubrol (0.16 mg/mg of mitoplasts) for 10 min on ice with stirring, diluted 1:4 with homogenization buffer and centrifuged at $144\,000\times g$ for 50 min to pellet the inner membranes. This supernatant contains the mitochondrial matrix proteins. Each subfraction was adjusted to 3 mg protein/ml with the homogenization buffer and measured for marker enzymes as follows: monoamine oxidase for the outer membrane proteins [21], sulfite oxidase for the intermembrane space [22], succinate dehydrogenase for the inner membrane [23] and malate dehydrogenase for the matrix proteins [24].

To exclude lysosomal proteins, which represent a substantial fraction of total proteolytic activity in these preparations [25], in control experiments the initial mitochondrial pellet was treated with a low concentration of digitonin (5 μg/mg protein for 20 min at 0°C) prior to lysis of the outer membranes [26]. After centrifugation ($10\,000\times g$ for 10 min) and washing, the mitochondria were subfractionated, and the lysosomal marker enzyme acid phosphatase was measured in all fractions according to Stevens and Thomas [27].

2.4. Proteolysis assay

Full length recombinant human XDH was expressed in COS-1 cells as described [28]. $100\,000\times g$ supernatants (S100) from these cell lysates were incubated with samples of human liver, and the proteolysis of XDH was revealed by denaturing polyacrylamide gel electrophoresis followed by Western blotting using polyclonal XDH antiserum as described [28].

The protease inhibitors were from Boehringer Mannheim, except for phenylmethylsulfonyl fluoride (PMSF) from Sigma, *N*-carbobenzoxy-Leu-Leu-Tyr-diazomethyl ketone (N-CBZ-LLY-ZMK) from Molecular Probes, acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) from Bachem, and *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) from Enzyme System Products. The concentrations used were as follows: antipain 74 μM; bestatin 130 μM; chymostatin 33 μM; E-64 5.6 μM; leupeptin 2 μM; pepstatin 1 μM; phosphoramidon 360 μM; Pefabloc SC 1.6 mM; EDTA 1 mM; aprotinin 150 μM; calpain inhibitor I 44 μM; N-CBZ-LLY-ZMK 50 μM; Ac-DEVD-CHO, Ac-YVAD-CMK and Z-VAD-FMK 100 μM; PMSF 1 mM.

2.5. XDH and XO activity measurements

Xanthine dehydrogenase/oxidase activities were measured by a radiochemical assay described earlier [28]. Briefly, the samples were incubated for 60 min in the presence of labelled substrate (45 μM [^{14}C]xanthine, Amersham, specific activity 50–60 mCi/mmol) in 50 mM potassium phosphate buffer pH 7.8 containing 0.5 mM DTT and 1.0 mM EDTA. Total XDH+XO activity was measured in the presence of 0.5 mM NAD^+ in the reaction mixture, and xanthine oxidase activity in parallel reactions without NAD^+. The uric acid produced was separated from xanthine by HPLC and quantified by a flow-cell scintillation counter. The results (mean ± S.D. from triplicate experiments) are expressed as a percentage of xanthine oxidase activity of total XDH+XO activity.

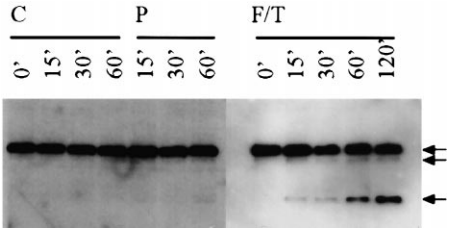

Fig. 1. XDH proteolysis in human liver preparations. Samples from human liver were incubated: (C) as cytosol from unfrozen tissue, (P) as fresh tissue pieces under buffer, and (F/T) as freeze-thawed tissue pieces. After incubation at 37°C for the indicated times, the cytosolic fractions from P and F/T were extracted, and all samples (50 μg protein/lane) were analyzed by Western blotting using rabbit XDH antiserum. The arrows indicate the 150, 130 and 85 kDa peptides.

3. Results

SDS-PAGE analysis of purified xanthine oxidase from human milk and freeze-thawed human liver revealed that in both sources the 150 kDa polypeptide was processed into a fragment of 130 kDa and further to 85 kDa. By sequencing the amino-terminus of the 130 kDa peptide, the primary cleavage was determined to occur between amino acids Gln^{183} and Lys^{184}. No N-terminal sequence could be obtained from the 85 kDa peptide.

In contrast to freeze-thawed liver, cytoplasmic fractions from fresh human liver showed an intact XDH peptide with no signs of proteolysis when incubated for up to 1 h at 37°C (Fig. 1). Accordingly, the enzymatic activity was predominantly in the dehydrogenase form (Table 2).

Warm ischemia was simulated by further incubating fresh ≤100 mg tissue pieces at 37°C, and as in supernatants freshly prepared from unfrozen liver, no signs of XDH proteolysis were seen in the cytoplasmic fractions from these experiments. Strikingly, one freeze-thaw cycle of the tissue pieces prior to incubation at 37°C resulted in rapid progression of XDH proteolysis that could already be observed at 15 min (Fig. 1).

The fact that the cytoplasmic compartment from fresh human liver contained no XDH-XO proteolytic activity led us to examine the insoluble fraction of the homogenate, specifically the mitochondria. Following subfractionation, the marker enzyme distribution of each of these subfractions is shown in Table 1.

Table 1
Marker enzyme activity distribution (submitochondrial localization in parentheses) in the preparations of mitochondrial subfractions

Enzyme	Relative activity (% of total mitochondrial activity)			
	M	IM	IMS	OM
Malate dehydrogenase (M)	67	1	29	2
Succinate dehydrogenase (IM)	14	59	7	20
Sulfite oxidase (IMS)	0	0	96	4
Monoamine oxidase (OM)	0	0	40	60

M = matrix, IM = inner membrane, IMS = intermembrane space, OM = outer membrane.

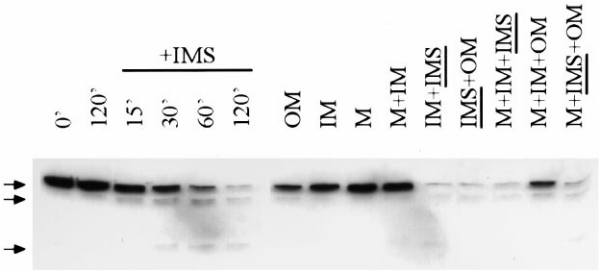

Fig. 2. Degradation of recombinant XDH by mitochondrial intermembrane space proteins. Western blots of COS cell S100 supernatants (1 μg protein) treated at 37°C with specific mitochondrial subfractions (30 μg total protein). Six left lanes: Time course of XDH degradation when incubated either alone or with intermembrane space proteins (IMS). Right lanes: Degradation of XDH when incubated for 120 min with mitochondrial outer membrane proteins (OM), inner membrane proteins (IM), matrix proteins (M) or with the indicated combinations. Arrows as in Fig. 1.

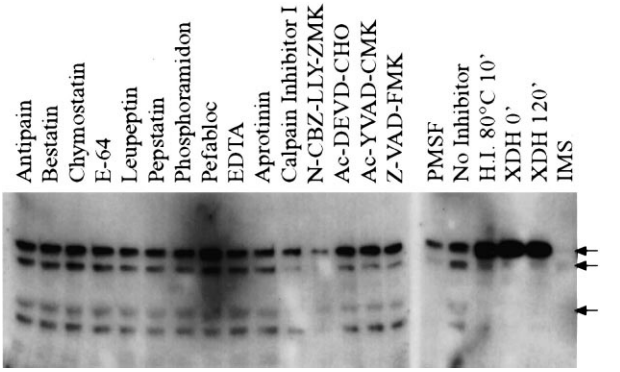

Fig. 3. Effect of various protease inhibitors on XDH proteolysis. Recombinant XDH was incubated with mitochondrial intermembrane space (IMS) proteins for in the presence of the indicated protease inhibitors (see Section 2 for abbreviations and concentrations). The right lanes show heat inactivation (H.I.) of the proteolytic activity, and XDH and IMS proteins incubated separately. Incubation conditions as in Fig. 2.

By coincubating subfractions of the mitochondria with recombinant human XDH, the XDH-XO proteolytic activity was found to be specifically localized in the mitochondrial intermembrane space (Fig. 2). The activity was soluble and could be inactivated by heating (80°C for 10 min). Experiments with a number of antiproteases including inhibitors of serine proteases, cysteine proteases, aspartic proteases, metalloproteases, calpains and caspases showed that the XDH-XO converting activity does not belong to any of these types of proteinolytic enzymes (Fig. 3).

When the cytoplasmic fraction of human liver was coincubated with IMS proteins in the absence of DTT, the proportion of XO increased ca. sevenfold (Table 2). This was mainly due to reversible XDH-XO conversion. In the presence of 0.5 mM DTT, reversible conversion was greatly decreased, and IMS proteins caused an approximately twofold increase in the proportion of XO.

The lysosomal marker enzyme acid phosphatase activity was restricted ($>90\%$) to the cytoplasmic preparation and the supernatant from the low-digitonin wash. Removal of lysosomal proteins by these procedures did not decrease the XDH-XO proteolytic activity of the IMS subfraction of the mitochondrial preparations (data not shown).

4. Discussion

In this study we have shown that the cytoplasmic xanthine dehydrogenase of human liver is irreversibly cleaved to xanthine oxidase by a mitochondrial protease. This finding is in accordance with an early report by Stirpe and Della Corte which suggests that a 'xanthine oxidase activating factor' resides in cellular compartments other than the cytosol [9]. However, since these preliminary experiments the proteolytic activity has eluded further localization and characterization.

Our data support the notion [5,6] that irreversible conversion of XDH to XO occurs more readily if the enzyme is first converted into reversible oxidase in the absence of DTT (Table 2).

The proteolytic conversion occurs by cleavage of the polypeptide chain at position Gln^{183}-Lys^{184} and subsequently at a site downstream, yielding a major cleavage product of approximately 85 kDa. This is in agreement with earlier findings for the rat XDH in in vitro experiments by digestion with trypsin [11].

Reoxygenation of previously anoxic cells has been shown to lead to loss of the mitochondrial membrane potential, followed by uncoupling of oxidative phosphorylation, swelling, and eventual lysis of the mitochondria. Thus mitochondrial proteins are released into the cytoplasm [29]. The loss of membrane potential, also called the mitochondrial permeability transition (MPT), has been shown to occur at the early stages of apoptosis as well as necrosis [30–32].

The different intracellular localization of XDH-XO converting proteolytic activity and the intact XDH peptide also suggests that proteolytic modification of XDH to XO is mainly triggered by reperfusion of ischemic tissue and not by the ischemia itself, as previously speculated [33]. It is possible that the protease has (a) physiological substrate(s) in the mitochondrial intermembrane space and that it only exerts its action on XDH in conditions preceding cell death. The rate of proteolytic XDH to XO conversion during ischemia without reperfusion has been shown to be relatively slow in this study

Table 2
The effect of mitochondrial intermembrane space (IMS) proteins on the proportion of xanthine oxidase activity of total XDH+XO activity in the cytoplasm of human liver

	Xanthine oxidase (%) of total XDH+XO activity		
	Cytoplasm (0 min)	Cytoplasm (120 min)	Cytoplasm+IMS proteins (120 min)
−DTT	8.6 ± 0.2	30.4 ± 0.9	56.5 ± 1.7
+0.5 mM DTT	7.0 ± 0.2	11.9 ± 0.6	17.8 ± 1.0

The incubations were carried out at 37°C with and without dithiothreitol (DTT) to assess the contribution of the reversible and irreversible forms of xanthine oxidase.

and in previous reports [7,34]. The fall of intracellular pH associated with ischemia has been shown to be cytoprotective [35–37], supposedly by inhibition of the MPT [38,39].

As the XDH-XO converting protease activity is not inhibited by Z-VAD-FMK, it appears to be distinct from the 'apoptosis-inducing factor' (AIF), a so far largely uncharacterized proapoptotic 50 kDa protein from the mitochondrial intermembrane space with presumable proteolytic activity [30]. Similarly, a proteolytic activity recently characterized from rat liver mitochondrial intermembrane space was inhibited by leupeptin and E-64, which had no effect in our experiments [40].

Intracellular calcium influx and subsequent mitochondrial Ca^{2+} overload during and after ischemia have been shown to promote the MPT [41]. The role of excess intracellular calcium on XDH-XO conversion is thus likely to be indirect, rather than directly coupled to the activation mechanism of the protease. This would explain the lack of effect of metal chelators and calpain inhibitors on XDH-XO conversion in a cell-free system, shown in this study. The reported inhibitory effect of the calmodulin inhibitor trifluoperazine on the conversion [17] could be also explained by the known ability of trifluoperazine to prevent the MPT [29].

Our findings also demonstrate that if the integrity of the mitochondria is ensured during homogenization of the tissue, almost all XDH-XO activity can be preserved in the dehydrogenase form. The possibility that even the residual oxidase activity is an artefact due to tissue preparation cannot be ruled out. In contrast, in freeze-thawed tissues, in which the integrity of biological membranes and cellular organelles, including the mitochondria, has been affected, the rate of conversion is significantly enhanced. We postulate that this is a consequence of a leak of mitochondrial proteins into the cytoplasm and ensuing XDH proteolysis. Thus isolation of the cytoplasm prior to freezing of tissues to be analyzed is of crucial importance and failure to do so leads to significant background oxidase activity. These findings could account for the controversial reports of the proportion of oxidase in tissues and also of the rate of XDH-XO conversion [6,15,34,42–46].

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