Three Forms of Xanthine: Acceptor Oxidoreductase in Rat Heart

Zbigniew W. Kaminski, Roman Pohorecki, Christian L. Ballast, and Edward F. Domino

The enzyme xanthine: acceptor oxidoreductase found in rat heart equilibrates between three forms differing in electron acceptor specificity. Form D transfers electrons exclusively to NAD$^+$ and accounts for 85% of total oxidoreductase activity. Form O transfers electrons to molecular oxygen and accounts for 8%. The D/O form prefers NAD$^+$, but without NAD$^+$ transfers electrons to oxygen. Interconversion from D to O and O to D forms is catalyzed by sulfhydryl group-modifying reagents: Cd$^{2+}$, Cu$^{2+}$, disulfiram, and heating with dithiothreitol. This suggests that sulfhydryl groups participate in the first stage of enzyme conversion. The NADH/NAD$^+$ concentration ratio may regulate the dehydrogenase activity of xanthine: acceptor oxidoreductase (NAD$^+$-dependent activity of D and D/O forms). Accumulating NADH inhibits hypoxanthine hydroxylation. The amount of form O increases during cardiac ischemia, facilitating superoxide radical-ion generation. Also, NADH/NAD$^+$ does not regulate form O, promoting adenylate nucleotide pool depletion, especially in the heart which has low de novo purine nucleotide synthesis. (Circulation Research 1986;59:628–632)

In recent years, there has been much interest in the biochemical mechanisms of cardiac tissue injury. Schoutsen et al. and Chambers et al. found a NAD$^+$-dependent xanthine oxidoreductase in rat heart. However, this enzyme has not been well characterized. Xanthine: acceptor oxidoreductase catalyzes two consecutive hydroxylation reactions: hypoxanthine to xanthine, and xanthine to uric acid, the end product of human purine catabolism. There are two well-known classes of oxidoreductases. The first uses molecular oxygen as an electron acceptor; the second uses NAD$^+$. Xanthine: oxygen oxidoreductase (trivial name xanthine oxidase; current E.C. 1.1.3.22) has been extensively investigated in the last 80 years. The NAD$^+$-dependent enzyme (xanthine: NAD$^+$ oxidoreductase; trivial name xanthine dehydrogenase; current E.C. 1.1.1.204) was discovered more recently because this enzyme is less stable, especially in mammalian tissues.

NAD$^+$-dependent xanthine oxidoreductase is the form found in vivo. Rat liver xanthine: acceptor oxidoreductase equilibrates between two forms: D, D/O, and O, which differ in their electron acceptor specificity. The D form is primary and uses NAD$^+$ exclusively as the electron acceptor. The D/O form transfers electrons to oxygen in the absence of NAD$^+$ but prefers NAD$^+$ as an electron acceptor. Form O does not occur in fresh liver enzyme preparations but appears during storage. Form O uses molecular oxygen as the preferred electron acceptor. Form O can transfer to other electron acceptors such as methylene blue, but oxygen is preferred in physiological conditions. During storage, form D spontaneously converts to form D/O and then to form O. The reverse conversion occurs when the enzyme is heated with dithiothreitol or cysteine, reagents which reduce disulfide bridges.

Little is known about the properties of nonliver xanthine oxidoreductase. Heart tissue is especially important because cardiac purine and purine nucleotide metabolism are important in cardiac ischemia. This paper characterizes xanthine: acceptor oxidoreductase from rat heart.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (250–300 g), housed in groups of six in a ventilated rodent facility at 20–25°C, were on a 0700–1900 light and 1900–0700 dark cycle. Standard rat chow 5012 (Ralston Purina) and water were given ad libitum. Rats were decapitated between 0900 and 1100 and the hearts removed immediately.

Reagents

Hypoxanthine (6-hydroxypurine), xanthine (2,6-dihydroxyxypurine), phenyl-methylsulfonyl fluoride (PMSF), dithiothreitol (DTT), 4-(2-hydroxyethyl)piperazine-N,N,N',N'-tetraacetic acid (HEPPS), [2-(hydroxy-1,1-bis(hydroxymethyl)aminomethyl]-propanesulfonic acid (TAPS), diethylaminoethyl-N,N,N',N'-tetraacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), beef liver L-lactate dehydrogenase (LDH; 1000 U/ml), β-nicotinamide adenine dinucleotide (NAD$^+$) grade V and Sephadex G-25 fine were purchased from the Sigma Chemical Co., St. Louis, Mo. Special enzyme grade ammonium sulfate and density gradient grade sucrose were purchased from Schwartz/
Mann, Orangeburg, N.Y. Disulfiram (tetraethylthio- 
peroxycarboxylic diamide) was prepared and purified 
as described previously.14

**Enzyme Preparation**

Each rat heart was immediately homogenized in a 
chilled razor blade homogenizer for three alternative 
periods of 15 seconds on and 15 seconds off. The 
homogenization buffer contained 50 mM HEPPS/ 
KOH (pH 8.0), 200 mM sucrose, and 10 mM DTT. In 
some experiments, 0.1 mM PMSF was added to the 
homogenization buffer, or the tissue was immediately 
frozen in liquid nitrogen. In some experiments, DTT 
was excluded. The homogenate was centrifuged at 
30,000 g for 30 minutes and the supernatant removed. 
The supernatant was ultracentrifuged at 160,000 
g for 60 minutes. The resulting supernatant was fractioned 
with 3.8 M ammonium sulfate according to Wood.15 
The fraction precipitating in the range of 1.4–2.4 M 
ammonium sulfate contained all of the oxidoreductase 
activity. This fraction was resuspended in 250 mM 
sodium pyruvate, to provide instant NADH reoxi-
dation (3–11 mg of total protein determined by the Goa 
16 modification biuret method) with or without 500 
M NAD+. Some samples contained 40 nktd LDH and 0.5 
mM EDTA (0.25 mM). This did not increase or decrease 
the activity. The enzyme was characterized before and after incuba-
tion with 20 mM DTT at 45°C for 25 minutes. After 
incubation, the enzyme was cooled on ice for 5 min-
utes and passed through a Sephadex G-25 column 
equilibrated with 20 mM TAPS/KOH buffer, pH 8.0. 
The enzyme solution was shown to be DTT free by a 
negative test with Ellman’s reagent (after protein 
precipitation).

**Michaelis Constant Determination for Xanthine**

The $K_m$ of xanthine was determined from a reaction 
curve using 16 $\mu$M initial xanthine concentration and a 
saturating NAD$^+$ concentration (500 $\mu$M). The 
absorption at 302 nm was monitored to complete xanth-
ine exhaustion. The reaction was carried out under 
aerobic conditions with LDH and pyruvate. The partial 
velocities and substrate concentrations were calculated 
from the integrated Michaelis-Menten equation ac-
cording to Waley.17 The $K_m$ was estimated using the 
computer algorithm of Cornish-Bowden et al.18

**Studies on the Time-course of Hypoxanthine 
Hydroxylation**

Hypoxanthine hydroxylation was studied in the 
presence of accumulating NADH or with instantaneous 
NADH reoxidation by LDH and pyruvate. Abs-
sorption at 279, 302, and 340 nm was continuously 
recorded.19,20 TAPS/KOH buffer (950 mM), pH 8.0, 
replaced HEPPS to avoid interference at 279 nm. 

**Results**

The total activity of xanthine: acceptor oxidoreduc-
tase in rat heart (as determined with a saturating xan-
thine concentration of 150 $\mu$M and a NADH reoxidiz-
ing system) is 460 pkat/g of fresh tissue, or 77 ± 3 
nkat/g of total protein in a partially purified prepara-
tion. The total activity of the crude preparation (super-
натant after 30,000 g) was 13 ± 2 nkat/g total protein. 
The Michaelis constant for xanthine was 2.42 $\mu$M, 
with confidence limits of 1.95 to 2.87,18 the same 
value as for rat liver oxidoreductase.21 Uric acid is a 
weak xanthine oxidoreductase inhibitor with a 
$K_i = 1.6$ mM.22 In this experiment, the maximum con-
centration of uric acid during the xanthine $K_m$ determi-
nation was 16 $\mu$M. Even at high concentrations (40 
$\mu$M), the plots of log concentration of hypoxanthine 
disappearance vs. time fit a straight line with a correla-
tion coefficient of 0.996. Since these lines do not curve 
with increasing uric acid concentration, we assume 
that the amount of uric acid formed during the experi-
ment did not inhibit the enzyme.

In contrast to the liver enzyme,11 all three forms of 
xanthine: acceptor oxidoreductase are found in fresh 
heart preparations. Cardiac oxidoreductase activity 
typically contained 85% form D, 7% form D/O, and 
8% form O. Several attempts were made to block form 
O appearance. To this end, the homogenization buffer 
was modified by the addition of PMSF (0.1 mM) or 
EDTA (0.25 mM). This did not increase or decrease 
the appearance of form O.

Form D was completely converted to form O by
treatment with 60 μM Cd²⁺ or 200 μM Cu²⁺. Disulfiram, which modifies sulfhydryl groups in a highly specific manner, also increased oxygen-dependent activity. Table 1 summarizes these results. Table 2 summarizes the results of the experiment in which DTT was absent in the preparation buffer. Preparations were assayed for total activity and the relative abundance of each form was calculated. The preparations were then incubated with 20 mM DTT at 45°C for 25 minutes and assayed again. There was a marked decrease in the relative abundance in form O while the amount of form D/O changed little. The total activity remained unchanged.

The dehydrogenase activity of xanthine: acceptor oxidoreductase (mainly form D) was inhibited by NADH accumulation. Typical data for hypoxanthine, xanthine, and uric acid with and without the regeneration of NAD⁺ from NADH are shown in Figure 1. The hypoxanthine data were fit to a multiexponential model for kinetic analysis using the Biomedical Data Processing Program BMDP-P3R (BMDP Statistical Software, Inc., Los Angeles, Calif.), available to the public on the Michigan Terminal System. The best fit for the hypoxanthine data with and without NAD⁺ regeneration was a two parameter model of the form \( HX(t) = Ae^{-at} \). The best fit data are given in Table 3. The percent inhibition with NADH present was calculated as percent differences in rates (\( \alpha \)). The disappearance of hypoxanthine, most significant to our discussion, was inhibited 48% in the presence of NADH. The percent inhibition did not increase with increasing NADH concentrations. It has previously been shown that enzyme forms D and D/O are inhibited by NADH in rat liver preparations.  

**Discussion**

Heart xanthine: acceptor oxidoreductase is important both as a free radical source and in purine nucleotide catabolism. For many years, biochemists believed xanthine oxidase had only one form and transferred electrons only to oxygen. This incorrect point of view is still deeply rooted in the current literature. Stirpe and Della Corte discovered the enzyme actually to be xanthine: NAD⁺ oxidoreductase. During storage or preparation without sulfhydryl group protecting agents, the enzyme lost NAD⁺-dependent activity and gained oxygen dependence. Stirpe and Della Corte distinguished three forms of xanthine: acceptor oxidoreductase: Type O uses oxygen as an electron acceptor; type D uses only NAD⁺ and is the in vivo form; an "intermediate" form uses both acceptors. The crude enzyme preparation used by Stirpe and Della Corte did not allow the D/O form to be found. Further purification was needed to quantify the activities of the different forms. Crude enzyme preparations contain excess protein which interferes with the spectrophotometric assays, especially at 279 nm. Additionally, a correction for NAD⁺/NADH absorption during uric acid determination at 292 nm was not used in the original Stirpe and Della Corte procedure (see spectra in Bergmeyer13). This correction was included in this study, according to Kaminski and Jezewska.  

The accuracy of the method used has been shown on grass snake liver, which has only NAD⁺-dependent activity.  

The enzyme preparation used had a specific activity of 77 nkat/g protein, 6 times greater than the crude supernatant. This allowed absorption at 302, 340, and 279 nm to be monitored. In rat heart, all three forms of the enzyme are interconvertible. Cardiac enzyme preparations with 10 mM DTT contained 7% form O, 8% form D/O, and 85% form D. These proportions did not change in the presence of a protease inhibitor (PMSF) or a reagent chelating heavy metal ions (EDTA). Moreover, the presence or absence of DTT played a significant role in the relative abundance of form O without changing the total enzyme activity.  

Chambers et al14 suggested that proteolysis causes the appearance of oxidase activity. Our results show the NAD⁺-dependent activity in heart oxidoreductase preparations disappeared during treatment with the sulfhydryl modifying reagents Cd²⁺, Cu²⁺, and disulfiram. Proteolysis may be a further conversion step, especially during storage where the enzyme is still highly active. The results obtained in these studies support the conclusions of Chambers et al.  

**Table 1. Relative Amounts of Three Different Forms of Xanthine: Acceptor Oxidoreductase in Rat Heart**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Form D</th>
<th>Form D/O</th>
<th>Form O</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT in the isolation buffer</td>
<td>85.8% ± 1.9</td>
<td>5.9% ± 1.1</td>
<td>8.3% ± 1.2</td>
<td>6</td>
</tr>
<tr>
<td>PMSF (+ DTT) in the isolation buffer</td>
<td>85.2% ± 3.4</td>
<td>7.2% ± 1.3</td>
<td>7.5% ± 2.1</td>
<td>6</td>
</tr>
<tr>
<td>EDTA (+ DTT) in the isolation buffer</td>
<td>84.3% ± 2.7</td>
<td>7.6% ± 1.6</td>
<td>8.1% ± 1.1</td>
<td>6</td>
</tr>
<tr>
<td>Cd²⁺ (60 μM) or Cu²⁺ (450 μM) incubated in the reaction mixture</td>
<td>2.2% ± 1.0</td>
<td>1.0% ± 0.8</td>
<td>96.8% ± 4.6</td>
<td>6</td>
</tr>
<tr>
<td>Disulfiram (450 μM) in the reaction mixture</td>
<td>5.8% ± 2.2</td>
<td>8.3% ± 1.7</td>
<td>85.9% ± 3.9</td>
<td>4</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE.

**Table 2. Relative Abundance of the Different Forms of Xanthine: Acceptor Oxidoreductase in Rat Heart**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Form D</th>
<th>Form D/O</th>
<th>Form O</th>
<th>Total activity</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT absent in prep. buffer</td>
<td>72.8% ± 5.3</td>
<td>11.4% ± 3.1</td>
<td>15.8% ± 3.6</td>
<td>75 ± 4</td>
<td>4</td>
</tr>
<tr>
<td>Sample incubated with DTT (20 mM, 45°C, 25 min)</td>
<td>90.2% ± 2.3</td>
<td>8.7% ± 1.3</td>
<td>1.1% ± 0.8</td>
<td>80 ± 3</td>
<td>4</td>
</tr>
</tbody>
</table>

The isolation procedure was first performed without DTT. Later the preparation was incubated with DTT.
but the first alteration seems to involve sulphydryl group modifications. This has been well documented in vitro for the rat liver enzyme.\textsuperscript{12,14,24,25} Form O rich preparations (15\%) incubated with 20 mM DTT decreased strikingly in form O activity. Again, the total enzymatic activity remained unchanged. This supports the hypothesis that the first conversion step from D to O form is nonproteolytic. It does not exclude the possibility that form O may be a better substrate for subsequent proteolysis. Of special significance to cardiac pathology and pharmacology, form O can generate superoxide radical ions. Experiments using allopurinol (4-hydroxy-pyrazolo-[3,4d]-pyrimidine) show a marked decrease in myocardial reperfusion injury.\textsuperscript{26-28} The extreme inhibitory potency\textsuperscript{29} and specificity of allopurinol for mammalian xanthine: acceptor oxidoreductase suggests that the inhibition of all forms, especially form O of xanthine oxidase (E.C. 1.1.3.22) may play an important role in the reperfusion injury process.\textsuperscript{3}

Table 3. Best Fit of Data for Hypoxanthine

<table>
<thead>
<tr>
<th>Hypoxanthine</th>
<th>−NADH</th>
<th>+NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>49.0 ± 1.0</td>
<td>44.8 ± 0.4</td>
</tr>
<tr>
<td>α</td>
<td>−0.116 ± 0.004</td>
<td>−0.0556 ± 0.0007</td>
</tr>
<tr>
<td>Residual mean square</td>
<td>2.42</td>
<td>0.43</td>
</tr>
<tr>
<td>% Difference between αs</td>
<td>48%</td>
<td></td>
</tr>
</tbody>
</table>

Best fit of data for hypoxanthine using BMDP to the model HX = Ae\textsuperscript{α}. Data are given with standard errors (SE).
Hypoxanthine hydroxylation followed the same pattern for NADH dependence as the liver enzyme.\(^{10}\) This suggests another consequence of D to O conversion, that is, the increased depletion of the cardiac adenylate nucleotide pool (see Mauser et al\(^{10}\) for an extensive discussion). Hypoxanthine is the last substrate in purine catabolism salvageable for purine nucleotide synthesis. It should be noted that myocardium has poor de novo purine nucleotide synthesis.\(^{30}\) Consequently, hypoxanthine loss depletes AMP levels; ATP production falls even with elevated phosphocreatine. This may decrease the Atkinson energetic potential and contribute to myocardial ischemic damage. We found most of the enzyme in the D and D/O forms, both strongly inhibited by NADH, unlike form O. NADH accumulates during ischemia, but the oxidase activity increases.\(^3\) Form O escapes inhibition due to an increasing NADH/NAD\(^+\) ratio and may deplete the adenylate nucleotide pool. The physiological and clinical significance of this is as yet unknown. The ratio between D + D/O (total dehydrogenase activity at physiological conditions) and O after myocardial ischemia needs to be determined.

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