Conversion of Xanthine Dehydrogenase to Oxidase in Ischemic Rat Tissues

Todd D. Engerson, T. Greg McKelvey, Darryl B. Rhyne, Elizabeth B. Boggio, Stephanie J. Snyder, and Harold P. Jones
Departments of Biomedical Sciences and Biochemistry, University of South Alabama, Mobile, Alabama 36688

Abstract

In response to global ischemia, tissue xanthine dehydrogenase was converted to xanthine oxidase in all tissues with half-times of conversion at 37°C of ~ 3.6, 6, 7, and 14 h for the liver, kidney, heart, and lung, respectively. The time course of enzyme conversion at 4°C was greatly extended with half-conversion times of 6, 5, 5, and 6 d for the respective tissues. Increases in xanthine oxidase activity were accompanied by the appearance of a distinct new protein species with greater electrophoretic mobility. The oxidase from ischemic rat liver was purified 781-fold and found to migrate with a higher mobility on native gels than the purified native dehydrogenase. Sodium dodecyl sulfate profiles revealed the presence of a single major band of 137 kD for the native dehydrogenase, whereas the oxidase had been partially cleaved generating polypeptides of 127, 91, and 57 kD. Polypeptide patterns for the oxidase resemble those seen following limited in vitro proteolysis of the native dehydrogenase supporting a proteolytic mechanism for the conversion of xanthine dehydrogenase to oxidase in ischemic rat liver.

Introduction

Superoxide has been implicated as playing a major role in postischemic or reperfusion injury in a wide variety of tissues including intestine, heart, kidney, liver, and brain. Superoxide dismutase, an enzymatic scavenger of superoxide, provides extensive protection against reperfusion injury in both in vivo and in vitro heart, kidney, intestine, and liver models (1–7).

One major source of superoxide in these reperfused tissues is the enzyme xanthine oxidase. Allopurinol and pterinaldehyde, two structurally dissimilar inhibitors of xanthine oxidase, are both highly protective against certain types of reperfusion injury. Allopurinol blocks reperfusion injury in the heart, kidney, intestine, and liver (3, 4, 7, 8), improves posttransplant renal function and graft survival (9, 10), and increases survivability in response to circulatory shock (11), whereas pterinaldehyde potently decreases postischemic injury in the intestine (12). In the heart, allopurinol also provides protection against reperfusion-induced arrhythmias (13).

The mechanism of xanthine oxidase–mediated reperfusion injury was first proposed by Granger et al. (5). According to their hypothesis, two important events were required during the ischemic period to poise the tissue for injury once oxygen returned. One of these events, the breakdown of ATP to AMP to hypoxanthine, would provide substrate for xanthine oxidase. The second event, the intracellular conversion of xanthine dehydrogenase to xanthine oxidase, would cause the enzyme to use oxygen, rather than NAD, as an oxidant. As a result of the conversion, the active enzyme would produce superoxide, rather than NADH, as hypoxanthine was oxidized. Thus when oxygen was reintroduced into the tissue the action of the oxidase would provide a burst of superoxide and subsequently derived active oxgen species that would inflict damage on the organ.

In support of this model, Roy and McCord (14) demonstrated that a dramatic conversion of xanthine dehydrogenase to xanthine oxidase activity occurred in rat intestinal segments that had been subjected to global ischemia with almost total conversion of dehydrogenase to oxidase occurring within 1 min. However similar studies with rat liver, lung, and kidney showed little conversion (only 10%) during a 1-h period of global ischemia.

In their original paper, Granger et al. (5) proposed that this conversion might be due to proteolytic processing of the native dehydrogenase. This was suggested by the irreversibility of the conversion with dithiothreitol and by previous reports by other investigators showing that conversion of xanthine dehydrogenase to oxidase can be accomplished in vitro by limited proteolysis (15–17). However, dehydrogenase-to-oxidase (D-to-O)[1] conversion can be promoted in vitro by a variety of other treatments including heating at 37°, anaerobiosis, perturbation with organic solvents, and sulfhydryl oxidation (17).

If the model as proposed by Granger et al. (5) is to be generalized to explain reperfusion injury in tissues other than the intestine, it is important to demonstrate that D-to-O conversion occurs in these tissues in a time frame consistent with the ischemia-reperfusion injury process. Also, because the model calls for enzyme modification via proteolysis, it is important to document that physical changes in the xanthine dehydrogenase/xanthine oxidase enzyme do accompany changes in dehydrogenase and oxidase activities within the tissue and that those changes are due to proteolysis.

In this paper, we report the kinetic parameters of the conversion of xanthine dehydrogenase to xanthine oxidase in ischemic rat liver, lung, heart, and kidney, the temperature dependence of this conversion, and the appearance of an ischemia-induced xanthine oxidase that is structurally distinct from the xanthine dehydrogenase found in normal tissue. In addition, we report the purification of this converted oxidase form from ischemic rat livers and present evidence that the conversion of xanthine dehydrogenase to oxidase in ischemic tissues is mediated by proteolysis.

Methods

Materials. DEAE Sephacel and G-25 Sephadex were purchased from Pharmacia Inc., Piscataway, NJ, and HA-Ultrogel from LKB Instruments,

Address correspondence to Dr. Harold P. Jones, Chairman, Department of Biomedical Sciences, University of South Alabama, Mobile, AL 36688.

Received for publication 25 September 1986 and in revised form 22 January 1987.

J. Clin. Invest.

0021-9738/87/06/1564/07 $1.00
Volume 79, June 1987, 1564–1570

1. *Abbreviation used in this paper:* D-to-O, dehydrogenase-to-oxidase.

Inc., Gaithersburg, MD. Acrylamide and ammonium persulfate were obtained from Boerhinger Mannheim Biochemicals, Indianapolis, IN, and bisacrylamide, sodium dodecyl sulfate, and molecular weight standards from Bio-Rad Laboratories, Richmond, CA. α-chymotrypsin was obtained from Worthington Diagnostic Systems, Inc., Biochemical Products Division, Freehold, NJ, and bovine pancreatic trypsin from Sigma Chemical Co., St. Louis, MO. Protein was assayed with the Bio-Rad Laboratories protein assay kit using bovine serum albumin purchased from Sigma Chemical Co. as a standard. Leupeptin was obtained from Boerhinger Mannheim Biochemicals. All other protease inhibitors, antibiotics, and chemicals were purchased from Sigma Chemical Co.

Tissue preparation and homogenation for measurement of conversion. Tissues were surgically excised from male Sprague-Dawley rats, 250–275 g, that had been euthanized by decapitation. Tissues were rinsed in a phosphate-buffered saline (PBS) solution (0.15 M potassium phosphate, 0.9% NaCl, pH 7.4) to remove residual blood elements and then incubated for various time periods in the same buffer. Incubations were conducted at either 37, 25, or 4°C. After the prescribed period of incubation, individual organs were frozen in liquid nitrogen and then homogenized with a Polytron Tissue Homogenizer, Brinkmann Instruments Co., Westbury, NY, (30 s at high speed) in an ice-cold buffered solution containing 0.05 M potassium phosphate, 0.1 M EDTA, 0.5 mM dithiothreitol (to prevent reversible D-to-O conversion), 0.5 mg/liter leupeptin, and 0.2 mM phenylmethylsulfonylfluoride, pH 7.8. The homogenate was centrifuged at 27,000 *g* at 4°C for 30 min and the pellet discarded. The supernatant fraction was chromatographed on Sephadex G-25 in the same buffer to remove endogenous substrates. The resultant eluate was utilized for measurement of xanthine dehydrogenase and oxidase activity, protein measurements, and electrophoresis samples.

Enzyme and protein assays. Xanthine dehydrogenase and oxidase activities were assayed by measurement of uric acid formation at 295 nm in the presence and absence of NAD at 37°C as described by Waud and Rajagopalan (16). 1 U of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of xanthine to uric acid per minute at 37°C, and specific activity as micromoles of xanthine converted to uric acid per minute per milligram protein. Protein was assayed according to Bradford (18) using bovine serum albumin (BSA) as a standard.

Data display. Conversion data were plotted as scattergrams fitted to fourth order curves generated by the curve-fitting function of IBM-PC Statistician (Danbury, CT). Each curve fits the prescribed data with a significance value of $P < 0.005$.

Purification of xanthine dehydrogenase and oxidase. Livers were excised from male Sprague-Dawley rats, 200–250 g, and washed in cold (4°C) buffer A (10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/liter leupeptin, 0.7 mg/liter pepstatin, and 0.2 mM phenylmethylsulfonylfluoride, pH 7.8). The washed livers were then homogenized in 4 vol (wt/vol) of cold (4°C) buffer A with 0.25 M sucrose added. Livers were homogenized with a Polytron disruptor and then further homogenized with a motor-driven Teflon pestle in a glass-grinding vessel (three strokes, high speed). The homogenate was centrifuged at 27,000 *g* for 30 min at 4°C and the resultant supernatant fraction was used as the starting material.

The supernatant fraction was applied to 300 ml of DEAE-Sephacel preequilibrated with buffer A in a Buchner funnel (diameter, 9 cm). After application, the gel was washed with three 200-ml aliquots of buffer A and the enzyme was then eluted by washing the column with successive 100-ml aliquots of buffer A with 0.1 M NaCl. Each 100-ml fraction was collected and assayed for xanthine dehydrogenase plus xanthine oxidase activity and active fractions (fractions 2–6) pooled.

The pooled fractions were then fractionated with cold acetone (−20°C). Cold acetone was added with vigorous stirring to the enzyme fraction until an acetone/homogenate ratio of 43:57 was achieved. The preparation was immediately centrifuged at 5,000 *g* for 10 min at 4°C. The resultant supernate was discarded and the pellet resuspended in 50 ml of buffer A. Material that would not redissolve was then removed by centrifugation at 27,000 *g* for 10 min at 4°C.

The redissolved acetone precipitate was applied to a 45 × 2.5–cm G-25 Sephadex column preequilibrated with buffer A. The void volume, light brown in coloration, was applied to a 35 × 2.5–cm DEAE-Sephacel column preequilibrated with buffer A enzyme activity and eluted with a 500 ml, 0.05–0.25 M NaCl gradient in buffer A. 5-ml fractions were collected and assayed for activity.

Pooled fractions from DEAE-Sephacel were desalted by chromatography on G-25–equilibrated with 50 mM potassium phosphate, 1 mM dithiothreitol, 1 mM EDTA, and 0.2 mM phenylmethylsulfonylfluoride, pH 7.0. The enzyme preparation was then applied to an hydroxylapatite column (HA-Ultrogel) (48 × 1.5 cm). The column was washed with the same buffer until no detectable protein eluted from the column as judged by absorbance measurements at 280 mM. Enzyme activity was then eluted from HA-Ultragel with a 500-ml, 0.05–0.40 M potassium phosphate gradient, pH 7.0, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonylfluoride. 5-ml fractions were collected and assayed for xanthine dehydrogenase plus xanthine oxidase activity and protein estimated by spectral absorbance at 280 nM.

Samples of each pooled fraction were assayed for xanthine dehydrogenase activity, xanthine oxidase activity, and protein, and samples were frozen for future electrophoretic examination.

Purification procedures for the xanthine oxidase from ischemic livers were identical with two exceptions. First, the excised livers were subjected to warm ischemia (37°C) in PBS, pH 7.4, (0.15 M potassium phosphate, 0.9% NaCl) for 6 h before homogenization, and secondly, 0.175 M NaCl was used to elute the enzyme from the initial DEAE batch step.

Gel electrophoresis. Protein samples were electrophoresed on 6.0% polyacrylamide slab gels according to the method of Gabriel (19) and then stained for either protein with Coomassie Brilliant Blue or for xanthine dehydrogenase plus xanthine oxidase activity as described by Waud and Rajagopalan (16). Sodium dodecyl sulfate (SDS) gel electrophoresis was performed in the presence of 5% β-mercaptoethanol on 7.5% polyacrylamide gels according to Laemmli (20). Molecular weight standards included myosin (200,000), B-galactosidase (116,000), phosphorylase *b* (88,000), BSA (66,000), and ovalbumin (44,000).

Preparation of chymotrypsin and trypsin: converted oxidase for electrophoresis. Conversion of rat liver xanthine dehydrogenase to oxidase by limited chymotryptic and tryptic digestion was accomplished as described by Waud and Rajagopalan (16). Xanthine dehydrogenase was incubated with each protease at 37°C for 1 h (1 part protease:100 parts xanthine dehydrogenase [wt/wt]) in a buffer containing 0.05 M potassium phosphate, pH 7.8, and 10 mM dithiothreitol. Digested samples were assayed and found to be converted to the oxidase form. Digested samples were immediately prepared for electrophoresis by denaturation in SDS.

Results

Dehydrogenase to oxidase conversion at 37°C. Incubation of ischemic livers, kidneys, hearts, and lungs at 37°C resulted in a four- to six-fold increase in xanthine oxidase activity in those tissues (Fig. 1). In each case, at time 0, xanthine oxidase represented only 10–20% of the total xanthine dehydrogenase plus xanthine oxidase activity. However xanthine oxidase activity rose to 80–100% of the total activity given a sufficient period of ischemia at 37°C. The increase in oxidase activity was judged to have resulted from conversion of xanthine dehydrogenase to oxidase because xanthine dehydrogenase activity fell and the total activity of xanthine oxidase plus xanthine dehydrogenase remained relatively constant throughout the ischemic period. Specific activities for liver, kidney, heart, and lung were 5.8, 1.9, 1.1, and 1.8 mU/mg protein, respectively. Similar studies conducted in the presence of antibiotics (10,000 U penicillin G, 10 mg streptomycin sulfate, and 25 μg amphotericin B per ml diluted 1:100 in PBS) showed no appreciable difference in either the rate or extent of conversion.

Of the four tissues studied, the conversion process at 37°C proceeded most rapidly in the liver with ~ 50% of the enzyme existing in the oxidase form after 3.6 h of warm (37°C) ischemia.

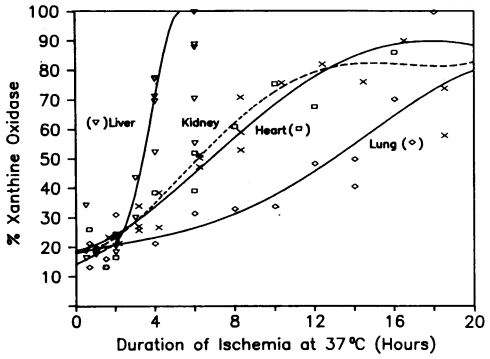

Figure 1. Tissue comparisons of rates of conversion of xanthine dehydrogenase to oxidase. Rat tissues subjected to various lengths of ischemia at 37° were assayed for xanthine oxidase activity and xanthine dehydrogenase plus xanthine oxidase activity. Data is plotted as that percentage of xanthine dehydrogenase plus xanthine oxidase in each tissue present in the oxidase form as a function of length of ischemia at 37°.

Conversion in the kidney and heart proceeded at a somewhat slower rate with half conversion occurring after 6 h in the kidney and after 7 h in the heart. Xanthine dehydrogenase was most slowly converted in the lung where ~ 14 h of ischemia were required for 50% conversion of the enzyme.

Conversion at 4 and 25°C. A similar conversion process occurred in each of the tissues when they were kept ischemic at 4°C. However, as one might expect, the length of ischemia required for conversion was greatly extended. Liver, kidney, heart, and lung were 50% in the oxidase form following ischemic periods of 6, 5, 5, and 6 d at 4°C (Fig. 2, *A* and *B*). At 25°C, the conversion process in liver was 50% complete after 20 h (data not shown). In each case the total concentration of xanthine dehydrogenase plus xanthine oxidase did not appreciably change over the time course.

Electrophoretic examination of xanthine dehydrogenase and oxidase in control and ischemic tissues. Samples from control livers, kidneys, hearts, and lungs and from livers, kidneys, hearts, and lungs subjected to warm ischemia (8 h for liver, 24 h for all other tissues) were electrophoresed on 6% discontinuous polyacrylamide gels and stained for xanthine dehydrogenase and oxidase activity. As shown in Fig. 3, xanthine-oxidizing activity migrated with a higher mobility in each of the ischemic tissues than in their matched controls, with the change in mobility least pronounced in the ischemic heart.

Purification of rat liver xanthine dehydrogenase. Results of the purification of xanthine dehydrogenase from control rat livers are shown in Table I. The enzyme was purified 1,060-fold relative to the original 27,000 *g* supernatant fraction and has a specific activity for dehydrogenase activity of 4,980 mU per mg protein. 47% of the initial activity was recovered using this purification protocol with ~ 4 mg of pure enzyme recovered from the 100 g of fresh liver.

The inclusion of protease inhibitor and the sulfhydryl reagent dithiothreitol throughout the procedure successfully prevented the conversion of xanthine dehydrogenase to xanthine oxidase during purification. The percentage of activity present in the dehydrogenase form remained relatively constant at ~ 88% throughout the entirety of the process.

The xanthine dehydrogenase preparation was judged to be pure based upon its elution from hydroxylapatite as a single peak of protein coincident with activity (Fig. 4) and upon electrophoresis of samples collected at various stages of protein purification that showed the progressive purification of a single protein species (Fig. 5 *A*) comigrating with xanthine dehydrogenase activity (Fig. 5 *B*). Fig. 5 *B* also indicates that no structural changes that can be detected by electrophoresis occur within the dehydrogenase during the purification procedure.

Purification of rat liver xanthine oxidase from ischemic livers. Results of the purification of xanthine oxidase from ischemic rat liver are shown in Table II. The ischemia-induced oxidase was purified 781-fold with a specific activity for oxidase of 3,300 mU/mg protein. The yield of xanthine oxidase from the starting materials was 19 percent or 3 mg of protein from 140 g of ischemic rat liver. As with the purification of the dehydrogenase, under the condition utilized for purification, there was little interconversion between oxidase and dehydrogenase forms, with the final product being 88% oxidase.

The oxidase from ischemic livers was judged pure, based upon its elution as a single, coincident peak of activity and protein from hydroxylapatite (Fig. 6) and upon its appearance as a single major band of protein on native polyacrylamide gels (Fig. 7 *A*) comigrating with xanthine oxidase activity (Fig. 7 *B*). As judged by activity staining, no change in electrophoretic mobility occurred during the course of enzyme purification. This suggests that the final product is indeed the "native" form of the ischemia-induced xanthine oxidase. A small minor band of both protein and coincident activity (< 5%) appeared at a slightly lower mobility than the major activity.

Electrophoretic comparisons of xanthine dehydrogenase and ischemia-induced xanthine oxidase. Fig. 8 shows an electrophoretic comparison on native gels of xanthine dehydrogenase from control rat livers and xanthine oxidase from ischemic rat liver. Also shown are rat liver xanthine oxidase forms generated by the limited proteolysis of rat liver xanthine dehydrogenase by chymotrypsin and trypsin. Under nondenaturing conditions, xanthine oxidase from ischemic liver migrates with greater mo-

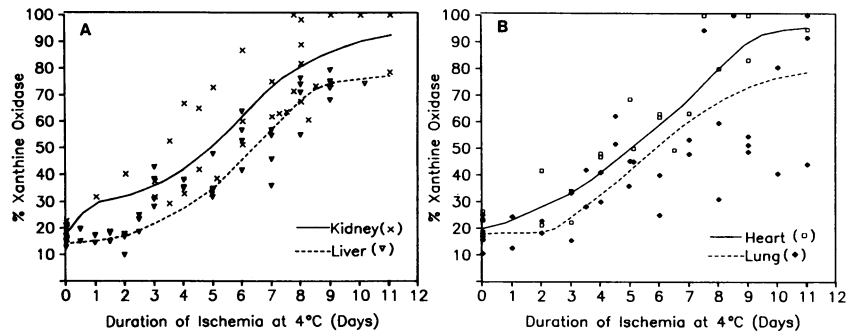

Figure 2. Conversion of xanthine dehydrogenase to xanthine oxidase at 4°. Rat tissues subjected to various lengths of ischemia at 4° were assayed for xanthine oxidase activity and xanthine dehydrogenase plus xanthine oxidase activity. Data is plotted as the percentage of xanthine dehydrogenase plus xanthine oxidase present in the oxidase form as a function of duration of ischemia at 4°C. (*A*) kidney and liver; (*B*) heart and lung.

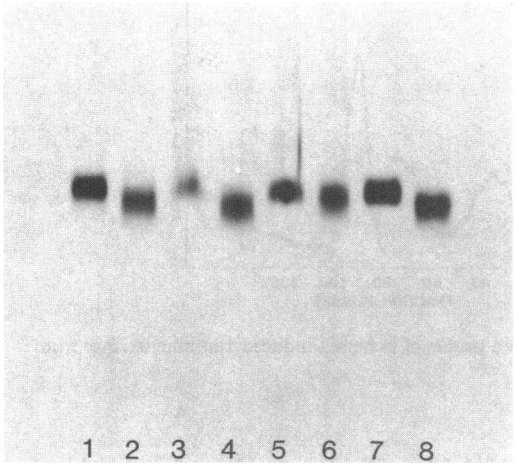

Figure 3. Electrophoretic patterns of xanthine dehydrogenase and xanthine oxidase obtained from control and ischemic tissues. High-speed supernatant fractions prepared from samples of control liver, kidney, heart, and lung and from corresponding samples subjected to ischemia at 37° (liver, 8 h; kidney, heart, and lung, 24 h), electrophoresed on 6% polyacrylamide gels and then stained for xanthine dehydrogenase and oxidase activity. Each sample contained 18.0 mU of activity. Lane *1*, control liver; lane *2*, ischemic liver; lane *3*, control kidney; lane *4*, ischemic kidney; lane *5*, control heart; lane *6*, ischemic heart; lane *7*, control lung; lane *8*, ischemic lung.

bility on 6% polyacrylamide gels than does rat liver xanthine dehydrogenase. This shift in mobility is similar to that observed when xanthine dehydrogenase is subjected to limited hydrolysis by either chymotrypsin or trypsin, suggesting that the ischemia-induced oxidase is a proteolytic product of the native xanthine dehydrogenase.

This is further supported by SDS gel electrophoretic comparisons of the rat liver dehydrogenase and oxidase species presented in Fig. 9. Native rat liver xanthine dehydrogenase, isolated from control rat liver, migrates primarily as a single polypeptide species with an apparent molecuar weight of 137 kD. However, when xanthine oxidase from ischemic rat liver is electrophoresed under denaturing conditions, a much more complex pattern is observed. The single-protein species on native gels is disassociated into three major peptide species with molecular weights of 127, 91, and 57 kD. A small amount of residual 137-kD molecular weight material, consistent with the residual 12% dehydrogenase in the sample, remains. The smaller peptides seen when the ischemia-induced oxidase is electrophoresed in the presence of SDS are similar to those generated by the limited digestion of native xanthine dehydrogenase with chymotrypsin and trypsin. With chymotrypsin, major peptides are found at molecular weights of 127, 91, and 37 kD, and with trypsin, major peptides have molecular weights of 91, 57, and 33 kD. The similarities between the peptide patterns of these protease-derived oxidase forms and that of the ischemia-induced rat liver xanthine oxidine are consistent with in vivo proteolytic conversion of xanthine dehydrogenase to xanthine oxidase during ischemia.

Discussion

In agreement with the model proposed by Granger et al. (5), xanthine dehydrogenase was converted to xanthine oxidase in each of the four rat tissues examined (liver, kidney, lung, and heart) when those tissues were subjected to global ischemia at 37°C. However, the time of ischemia required for maximal conversion was significantly longer than the period monitored by Roy and McCord (14). At the final time point in their study (60 min) only 20% of the total enzyme is present in its oxidase form. Our studies revealed that significantly longer periods of time are required before substantial conversion began to occur with half times for conversion ranging from 3 h for the liver to 14 h for the lung. However, in each case conversion continued until 80–100% of the total xanthine dehydrogenase was converted to the oxidase form. Whereas oxidase activity increases, the concentration of total xanthine dehydrogenase plus oxidase did not significantly change during the ischemic period indicating that interconversion of the two forms occurred. Measured activities of xanthine dehydrogenase plus oxidase in the various tissues were consistent with those previously reported by others (21).

The observation that ischemia induces D-to-O conversion in a wide variety of tissues suggests that this conversion may be an important concern in organ preservation. Hypothermic preservation of organs greatly decreased the rate of D-to-O conversion, suggesting that hypothermia will decrease the generation of xanthine oxidase–derived oxidants in reperfused tissues. This could be of special importance because glutathione concentrations fall rapidly during ischemia leaving the tissue vulnerable to oxidative attack.

When initially proposing the ischemia-induced conversion of xanthine dehydrogenase to xanthine oxidase, Granger et al. (5) predicted that the conversion was mediated through the limited proteolysis of xanthine dehydrogenase. This was suggested by the work of several investigators who had demonstrated that xanthine dehydrogenase could be converted to xanthine oxidase in vitro through either sulfhydryl oxidation or limited proteolysis. The in vitro conversion of the oxidase by sulfhydryl oxidation is readily reversed by dithiothreitol, but proteolytic conversion

Table I. Purification of Rat Liver Xanthine Dehydrogenase (XDH)

Purification step	Total protein	XDH + XO activity	% XDH	XDH specific activity	XDH % yield	XDH fold purification
	mg	*total mU*		*mU/mg protein*		
27,000 *g* Supernate	9,522	49,500	89	4.7	100	—
0.1 M DEAE batch	2,012	47,200	83	19.5	89	4
43% Acetone precipitate	204	36,900	84	152	70	32
DEAE Sephacel pool	32	25,100	84	650	47	138
HA Ultrogel	4	23,700	88	4,980	47	1,060

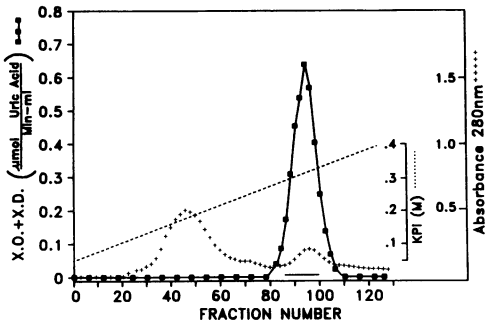

Figure 4. Elution profile of xanthine dehydrogenase from HA-Ultrogel.

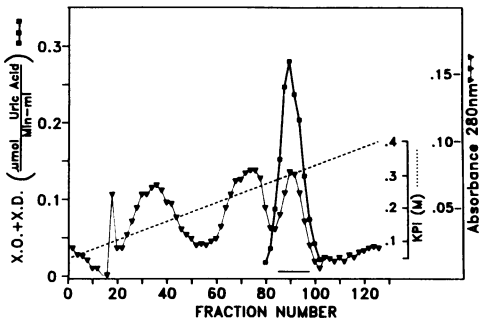

Figure 6. Elution profile of ischemia-induced xanthine oxidase from HA-Ultrogel.

is irreversible (16, 22). Because the newly formed oxidase activity in ischemic intestine can not be reconverted to the dehydrogenase form with dithiothreitol (5), it was proposed that the activation event is proteolytic in nature.

The discovery of a structurally distinct species of the enzyme that arises concurrently with conversion of the enzyme from its dehydrogenase to oxidase form is consistent with that proposal. This distinct form, isolated from the various ischemic tissues, migrates with a higher electrophoretic mobility, as does xanthine oxidase generated from in vitro proteolysis of xanthine dehydrogenase (15, 20).

In an attempt to study the nature of the alteration resulting in this distinct form, the dehydrogenase form of the enzyme from control rat liver and the oxidase form from ischemic rat liver were purified. The utilized protocols produced highly purified enzyme species with specific activities and fold purifications greater than those previously reported (16, 23). More importantly though, through the use of protease inhibitors and dithiothreitol, it was possible to prevent any interconversion or alterations of the enzyme forms during the course of purification. The enzyme from control liver was maintained as 88% dehydrogenase throughout the procedure and represents the highest percentage of dehydrogenase activity in any preparation to date. The purified enzymes were stable for > 8 wk when stored at −70°C in the presence of dithiothreitol. Xanthine oxidase from the ischemic tissue remained in the oxidase form as well during the purifi-

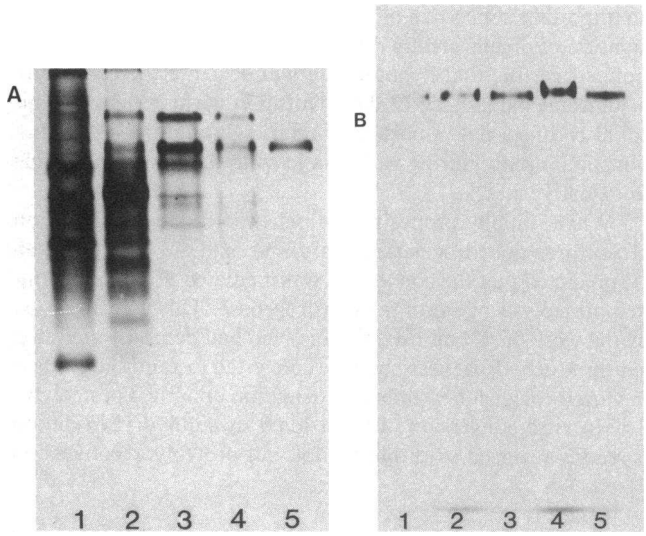

Figure 5. Electrophoretic examination of the purification protocol for xanthine dehydrogenase. Samples at various stages of purification of xanthine dehydrogenase were electrophoresed on 6% native polyacrylamide gels and then stained for either protein with Coomassie Brilliant Blue or for activity according to Waud and Rajagopalan (5). (*A*) is the protein stain, with lane *1* containing 150μg of the 27,000 *g* supernate; lane *2*, 100 μg of the pooled DEAE-batched step; lane *3*, 50 μg of the resuspended acetone pellet; lane *4*, 20 μg of the DEAE-column pool; and lane *5*, 5 μg of the HA-Ultrogel pool. (*B*) is activity-stained fractions. Each fraction contains 1.25 mU of total activity. Lane *1*, 27,000 *g* supernate; lane *2*, DEAE-batch step; lane *3*, acetone precipitate; lane *4*, DEAE-column pool; and lane *5*, HA-Ultrogel pool.

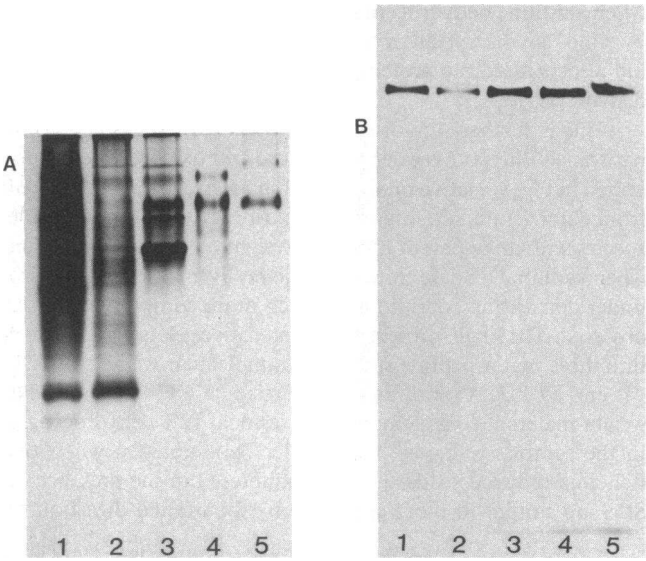

Figure 7. Electrophoretic examination of the protocol used for purification of xanthine oxidase from ischemic rat livers. Samples at various stages of purification of ischemia-induced xanthine oxidase were electrophoresed on 6% native polyacrylamide gels and then stained for either protein with Coomassie Brilliant Blue or for activity according to Waud and Rajagopalan (5). (*A*) is the protein stain, with lane *1* containing 150 μg of the 27,000 *g* supernate; lane *2*, 100 μg of the pooled DEAE-batched step; lane *3*, 50 μg of the resuspended acetone pellet; lane *4*, 20 μg of the DEAE-column pool; and lane *5*, 5 μg of the HA-Ultrogel pool. (*B*) is activity-stained fractions. Each fraction contains 1.25 mU of total activity. Lane *1*, 27,000 *g* supernate; lane *2*, DEAE-batch step; lane *3*, acetone precipitate; lane *4*, DEAE-column pool; and lane *5*, HA-Ultrogel pool.

Table II. Purification of Rat Liver Xanthine Oxidase (XO)

Purification step	Total protein	XDH + XO activity	% XO	XO specific activity	XO % yield	XO fold purification
	mg	*total mU*		*mU/mg protein*		
27,000 *g* Supernate	12,130	52,000	100	4.3	100	—
0.175 M DEAE batch	1,357	51,800	100	38.1	99	9
43% Acetone precipitate	87	28,600	84	277	46	64
DEAE Sephacel pool	20	15,400	75	579	22	135
HA Ultrogel	3	12,000	84	3,360	19	781

cation process. Gel patterns of samples electrophoresed and stained for activity showed that no physical changes resulting in changes in electrophoretic mobility occurred during the courses of purification. This integrity of the nature of the starting material in the final preparation was critical if comparisons were to be made between the two forms because in vitro interconversion of the dehydrogenase to the oxidase could have occurred if proper precautions were not taken (15–17, 23). We believe that these data demonstrate that the utilized purification protocols maintained the general integrity of both the dehydrogenase and the ischemia-induced oxidase.

A comparison of rat liver xanthine dehydrogenase and its ischemic-induced oxidase form indicated that the oxidase is a proteolytic product of the dehydrogenase. Whereas both forms of the enzyme (dehydrogenase and oxidase) migrated as single species on nondenaturing polyacrylamide gels, denaturation revealed that the oxidase form had been cleaved. Xanthine dehydrogenase has one major subunit of molecular weight 137 kD, whereas the ischemia-induced oxidase slowed multiple polypeptides of 127, 91, and 57 kD along with some residual 137 kD polypeptide. Cleavage of the enzyme during ischemia produced an SDS gel profile similar to that observed when xanthine dehydrogenase was converted to the oxidase form by limited digestion with trypsin or chymotrypsin and resulted in a shift in mobility for the enzyme on native gels similar to that observed when the dehydrogenase was converted to the oxidase by limited proteolysis.

The molecular weight for the rat liver dehydrogenase and its proteolytic products are comparable to those previously reported by Waud and Rajagopalan (16). The native dehydrogenase in those studies was reported to have a subunit molecular weight on SDS-urea gels of 150 kD compared with our measurement of 137 kD. Differences in molecular weight estimates between the studies may reflect differences in the electrophoretic systems (the presence or absence of urea) or differences in standards because in the study of Waud and Rajagopalan (16) bovine milk xanthine oxidase was used as the upper limit standard and was ascribed a molecular weight value of 150 kD. Nevertheless, the molecular weights of the degradation products were similar. In each case a major protein species is present at a molecular weight 10–15 kD smaller than the native dehydrogenase. Similar bands are also present at molecular weights of ~ 90 and 60 kD.

There are at least two potential types of proteases that might mediate the conversion process in ischemic tissues. Lysosomal proteases, physically separated from the dehydrogenase in normal tissues, could be released from lysosomes damaged during ischemia. The released proteases could then mediate the con-

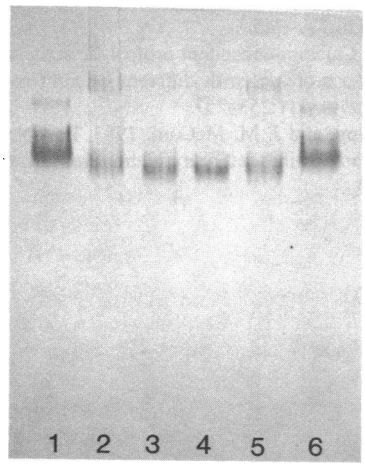

Figure 8. Electrophoretic comparisons of native rat liver xanthine dehydrogenase and ischemia-induced xanthine oxidase. 10-μg samples of the native xanthine dehydrogenase (lanes *1* and *6*), ischemia-induced xanthine oxidase (lanes *2* and *5*), trypsin-treated xanthine dehydrogenase (lane *3*), and chymotrypsin-treated xanthine dehydrogenase (lane *4*) were electrophoresed as 6% native polyacrylamide gels and stained for protein with Coomassie Brilliant Blue.

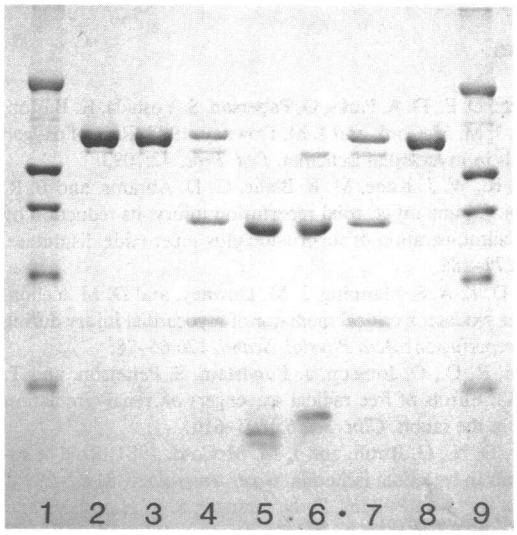

Figure 9. SDS gel profiles of native rat liver xanthine dehydrogenase and ischemia-induced xanthine oxidase. Samples of native rat liver xanthine dehydrogenase (5 μg), ischemia-induced xanthine oxidase (15 μg), trypsin-treated xanthine dehydrogenase (20 μg), and chymotrypsin-treated xanthine dehydrogenase were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS and B-mercaptoethanol and then stained with Coomassie Brilliant Blue. Standards in lanes *1* and *9* include myosin (200,000), B-galactosidase (116,000), phosphorylase *b* (88,000), BSA (66,000), and ovalbumin (44,000). Lanes *2*, *3*, and *8* contain the native dehydrogenase; lanes *4* and *7*, the ischemia-induced oxidase; lane *5*, trypsin-treated dehydrogenase; and lane *6*, chymotrypsin-treated dehydrogenase.

version process. Alternatively, conversion could be mediated by a cytosolic protease whose activity is stimulated during ischemia. One potential candidate would be the cytosolic calcium-dependent proteases.

Cytosolic proteases activated by micromolar concentrations of calcium are present in the liver (24), and it is well documented that intracellular calcium concentrations rise during ischemia to levels that would trigger the action of these proteases. Schaffer et al. have shown that the dehydrogenase can be converted to the oxidase by increases in intracellular calcium even in the absence of ischemia (25), and recently, we have found in preliminary studies that calcium-dependent proteases cause the conversion of rat liver xanthine dehydrogenase to xanthine oxidase in vitro (data not shown). Nevertheless, convincing evidence for the involvement of the calcium-dependent proteases in the conversion process must await careful analysis of the cleavage products of the ischemia-induced oxidase and comparison of those products to those obtained by the in vitro conversion of the enzyme by the calcium-dependent proteases.

In conclusion, proteolytic conversion of xanthine dehydrogenase to xanthine oxidase occurs during ischemia in the liver. This conversion may be an important factor in reperfusion injury in both the liver and other organs. Therefore, understanding the conversion process and its regulation may prove important in designing new agents and devising new strategies for organ preservation and for limiting reperfusion injury in a variety of other pathological situations leading to reperfusion injury.

Acknowledgments

The authors wish to thank Dr. Matthew Grisham and Dr. D. N. Granger for their help in preparation of the manuscript.

References

1. Chambers, D. E., D. A. Parks, G. Patterson, S. Yoshida, K. Burton, L. F. Parmley, J. M. McCord, and J. M. Downey. 1983. Role of oxygen derived radicals in myocardial ischemia. *Fed. Proc.* 42:1093.

2. Jolly, S. R., W. J. Kane, M. B. Bailie, G. D. Abrams, and B. R. Lucchesi. 1984. Canine myocardial reperfusion injury: its reduction by the combined administration of superoxide plus superoxide dismutase. *Circ. Res.* 54:277–286.

3. Hearse, D. J., A. S. Manning, J. M. Downey, and D. M. Yellon. 1986. Xanthine oxidase: a critical mediator of myocardial injury during ischemia and reperfusion? *Acta Physiol. Scand.* 126:65–78.

4. Hansson, R. O., O. Jonsson, J. Lundstam, S. Petterson, and T. Schersten. 1983. Effects of free radical scavengers on renal circulation after ischemia in the rabbit. *Clin. Sci.* 65:605–610.

5. Granger, D. N., G. Rutili, and J. M. McCord. 1981. Role of superoxide radicals in intestinal ischemia. *Gastroenterology.* 81:22–29.

6. Parks, D. A., G. B. Bulkley, D. N. Granger, S. R. Hamilton, and J. M. McCord. 1982. Ischemic injury in the cat small intestine: role of superoxide radicals. *Gastroenterology.* 82:9–15.

7. Adkison, D., M. E. Hollwarth, J. N. Benoit, D. A. Parks, J. M. McCord, and D. N. Granger. 1986. Role of free radicals in ischemia-reperfusion injury to the liver. *Acta Physiol. Scand.* 126:101–107.

8. Parks, D. A., and D. N. Granger. 1983. Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. *Am. J. Physiol.* 245:G285–289.

9. Owens, M. L., H. Lazarus, M. W. Wolcott, J. G. Maxwell, and B. Taylor. 1974. Allopurinol and hypoxanthine pretreatment of canine kidney donors. Transplantation 17:424–427 (1974).

10. Koyama, I., G. B. Bulkley, G. M. Williams, and M. J. Im. 1985. The role of oxygen free radicals in mediating the reperfusion injury of cold-preserved ischemic kidneys. *Transplantation (Baltimore).* 40:590–595.

11. Crowell, J. W., C. E. Jones, and E. E. Smith. 1969. Effect of allopurinol of hemorrhagic shock. *Am. J. Physiol.* 216:744–748.

12. Granger, D. N., M. E. Hollwarth, and D. A. Parks. 1986. Ischemia-reperfusion injury: role of oxygen-derived free radicals. *Acta Physiol. Scand.* 126:47–63.

13. Manning, A. S., D. J. Coltart, and D. J. Hearse. 1984. Ischemia and reperfusion-induced arrhythmias in the rat. Effects of xanthine oxidase inhibition with allopurinol. *Circ. Res.* 55:545–548.

14. Roy, R. S., and J. M. McCord. 1983. Superoxide and ischemia: conversion of xanthine dehydrogenase to xanthine oxidase. *In* Proceedings of the Third International Conference on Superoxide and Superoxide Dismutases. R. Greenwald and G. Cohen, editors. Elsevier/North Holland Biomedical Press, New York. 145–153.

15. Batelli, M. G., E. Lorenzoni, and F. Stirpe. 1973. Milk oxidase type D (Dehydrogenase) and type O (Oxidase). Purification, interconversion, and some properties. *Biochem. J.* 131:191–198.

16. Waud, W. R., and K. V. Rajagopalan. 1976. Purification and properties of the NAD^+-dependent (Type D) and O_2-dependent (Type O) form of rat liver xanthine dehydrogenase. *Arch Biochem. Biophys.* 172:354–364.

17. Della Corte, E., and F. Stirpe. 1972. The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme. *Biochem. J.* 126:739–745.

18. Bradford, M. 1970. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:240–254.

19. Gabriel, O. 1971. Analytical disc gel electrophoresis. *Methods Enzymol.* 22:565–577.

20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

21. Krenitsky, T. A., J. V. Tuttle, E. L. Catlau, and P. Wang. 1974. A comparison of the distribution and electron acceptor specificities of xanthine oxidase and aldehyde oxidase. *Comp. Biochem. Physiol.* 49B: 687–703.

22. Batelli, M. G., E. Lorenzoni, and F. Stirpe. 1973. Milk oxidase type D (Dehydrogenase) and type O (Oxidase). Purification, interconversion, and some properties. *Biochem. J.* 131:191–198.

23. Della Corte, E., and F. Stirpe. 1972. The regulation of rat liver xanthine oxidase. *Biochem. J.* 126:739–745.

24. DeMartino, G. N. 1981. Calcium-dependent proteolytic activity in rat liver: identification of two proteases with different calcium requirements. *Arch Biochem. Biophys.* 211:253–257.

25. Schaffer, S. W., R. S. Roy, and J. M. McCord. 1983. Possible role for calmodulin in calcium paradox-induced heart failure. *Eur. Heart J.* 4:81–87.