Bovine Milk Xanthine Oxidase: Effect of Limited Proteolysis on Kinetics and Structure

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Bovine milk xanthine oxidase was purified by an improved nonproteolytic method. This enzyme and a proteolytically purified preparation were kinetically characterized and compared. Even though there were some similarities between the two preparations in Q_{10} and energy of activation, proteolytically derived xanthine oxidase (PDXO) migrated faster in electrophoresis and its kinetic behavior was significantly different than that of nonproteolytically derived xanthine oxidase (NPDXO). At subsaturating substrate concentrations, the activity of PDXO was much less in 0.1 M Tris-HCl buffer than in 0.1 M pyrophosphate buffer but the activity of NPDXO was the same in both buffers. With xanthine at pH 8.3, NPDXO had a 93% higher specific activity and a 147% higher V_{max} than PDXO. With xanthine (pH 8.7) and hypoxanthine (pH 8.3), NPDXO showed 52% and 68%, respectively, higher V_{max} than PDXO. At pHs 8.3 and 8.7, NPDXO had a 51% and 59%, respectively, greater affinity for xanthine than PDXO. At pH 8.3, NPDXO demonstrated a 83% greater affinity for hypoxanthine than PDXO. Furthermore, NPDXO showed greater affinity for competitive inhibitors folic acid, allopurinol, and pterin-6-carboxylic acid than PDXO. These data suggest that proteolysis, by modifying the primary structure, has reduced the enzyme's conformational stability, catalytic efficiency, and affinity for both substrates and competitive inhibitors.

Bovine milk xanthine oxidase (EC 1.2.3.2) is a dimer of two identical subunits having molecular weights estimated between 140 000 and 160 000. Each monomeric subunit contains a molybdenum (Mo) center, a flavin adenine dinucleotide (FAD) center, and two distinct iron sulfur centers, designated Fe/SI and Fe/SII. The proximal locations of these constituents within each independent subunit is presumed to compose an intramolecular electron transport mechanism that facilitates the rapid transfer of a pair of electrons from the substrate to molecular oxygen. The precise arrangement of these constituents within each monomer remains undetermined. However, there is general agreement that the Mo and FAD are present as independent noninteracting moieties while the Fe/SI and Fe/SII redox centers interact directly with both Mo and FAD (Barber and Siegel, 1982). The oxidation of substrate by O_2 is proposed to occur via a covalently bound intermediate from which two electrons are removed, thus reducing the Mo center from the VI to the IV oxidation state (Olson et al., 1974). These electrons then pass singly within the Fe/S reducible centers and exit the enzyme via the FAD moiety (Barber and Siegel, 1982).

In nearly all methods published since 1939, bovine milk xanthine oxidase has been purified by using proteolytic and lipolytic enzymes and denaturing organic reagents (Ball, 1939; Avis et al., 1955; Gilbert and Bergel, 1964; Hart et al., 1970; Massey et al., 1972; Briley and Eisenthal, 1974). Use of these agents increases the enzyme yield. Lipolytic enzymes break up the milk fat globule membrane and release membrane-bound xanthine oxidase. Pancreatin has been used to degrade casein micelles to lower molecular weight components so that they may be eluted behind xanthine oxidase in chromatographic separations. However, pancreatin (which is a mixture of proteases; Hartlett and Gjessing, 1962; Uriel and Avrameas, 1965) exerts its effect on all proteins. In fact, it was recently shown that xanthine oxidase isolated by using pancreatin copurified with proteases from pancreatin thereby making it difficult to purify the milk enzyme (Nathans and Hade, 1975). More recently, these difficulties have been circumvented by employing much milder chemical procedures for the purification of the milk enzyme (Zikakis and Biasotto, 1976; Nathans and Hade, 1975; Waud et al., 1975; Mangino and Brunner, 1977; Zikakis, 1979). Although several investigations have indicated that the use of pancreatin in the preparation of milk xanthine oxidase does not adversely affect enzymatic activity and/or electron transport mechanism (Avis et al., 1955; Hart et al., 1970), it has been demonstrated that this type of procedure alters the primary structure of the protein (Nagler and Vartanyan, 1973; Waud et al., 1975; Nagler and Vartanyan, 1976). In the present investigation a sample of milk xanthine oxidase prepared by a mild nonproteolytic method was kinetically characterized and compared to a sample of xanthine oxidase purified by the conventional proteolytic procedure.

MATERIALS AND METHODS

Materials. Xanthine was purchased from Eastman Organic Co., Rochester, NY. Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Hypoxanthine was purchased from ICN Pharmaceuticals, Inc., Cleveland, OH. Folic acid and pterin-6-carboxylic acid were purchased from Sigma Fine Chemicals Co., St. Louis, MO. Disodium ethylenediaminetetraacetate (EDTA) was purchased from Fisher Scientific, King of Prussia, PA. Other reagents and solvents were of reagent grade. Glass distilled–deionized water was used throughout. Fresh raw milk was obtained from the University of Delaware dairy Guernsey and Holstein herds.

Enzyme Preparations. The NPDXO prepared in this laboratory was purified by the method of Zikakis and Biasotto (1976) and Zikakis (1979). The PDXO was purified by the method of Gilbert and Bergel (1964) and is available from ICN Pharmaceuticals, Inc., Cleveland, OH.

Polyacrylamide Disc Gel Electrophoresis (PAGE). PAGE was performed with various amounts of each sample ranging from 0.025 to 0.50 mg of protein using a Buchler

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18 tube polyanalyst. Pore sizes in the gels were based on 3.5% acrylamide in the stacking gel and 10% acrylamide in the separating gel (Ornstein, 1964).

Gels designated for protein stain first were fixed in 12.5% trichloroacetic acid; then gels were stained in a mixture containing 1% Coomassie Brilliant Blue and 12.5% trichloroacetic acid in a ratio of 1:20 Coomassie Brilliant Blue to trichloroacetic acid and destained in 7.5% acetic acid. Localization of active enzyme in PAGE gels was accomplished by staining the unfixed duplicate gels with neotetrazolium chloride according to the method of Zikakis (1981). Total protein concentration in samples was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Enzyme Activity Determinations. All activity assays were performed by using a Gilford Model 250 spectrophotometer. The constant temperature of the cuvette chamber was maintained within ±0.5 °C by using a Forma Scientific circulating bath. Xanthine oxidase activity was determined in a manner similar to that described by Avis et al. (1955, 1956a,b). The conversion of xanthine to uric acid was monitored at 295 nm in a 2.5-mL reaction volume of 0.1 M sodium pyrophosphate buffer (pH 8.3), containing 0.1 mM xanthine, 1.0 mM EDTA, and 75% oxygen saturation at 23 °C. Changes in absorbance at 295 nm were converted into international units per milliliter (IU/mL) of activity (micromoles of substrate oxidized per minute) by using the extinction coefficient difference of 9.5×10^3 cm^{-1} M^{-1} between xanthine and uric acid. The extinction coefficient difference between hypoxanthine and uric acid was negligible at 295 nm and thus 1.2×10^4 cm^{-1} M^{-1} (extinction coefficient for uric acid) was substituted in the calculation when hypoxanthine was used as the substrate.

Effect of pH on Xanthine Oxidase Activity. The optimum pH value for milk xanthine oxidase has been reported to range between pH 8.2 and pH 8.4 (Bray and Malmstron, 1964; Bray, 1975; DeRenzo, 1956). This pH range has been correlated to a pK_a value that would represent the ionization of a catalytically significant sulfur group. In the present investigation the effect of changing pH was studied at subsaturating concentrations of substrate. Assay conditions were similar to those described for activity determinations. The two sample preparations were examined by using 0.1 M sodium pyrophosphate buffer from pH 5.0 to pH 11.0 and 0.1 M Tris-HCl buffer from pH 7.0 to pH 10.0.

Determination of Maximum Velocities and Michaelis Constants. Michaelis constants and maximum velocities were determined from Hanes plots generated from assay data obtained over a range of final concentrations of xanthine from 5 to 20 μM at pH 8.3 and 5 to 35 μM at pH 8.7 and 23 °C, in 2.5 mL of 0.1 M sodium pyrophosphate buffer containing 1.0 mM EDTA. Reactions were initiated by the introduction of 5.0 μL (3 μg) of either enzyme preparation.

In a similar set of experiments, hypoxanthine was employed as the substrate at pH 8.3. The final concentration of substrate in these experiments was reduced to the 1 μM range due to a substrate inhibition phenomenon observed at higher concentrations. Protein concentration for all PDXO and NPDXO samples was adjusted to equality with either 0.1 M sodium pyrophosphate or Tris-HCl buffer.

Determination of Apparent Inhibitor Affinity. The approximate affinity between the enzyme and inhibitor, designated K_I, was determined by using three competitive inhibitors: folic acid, allopurinol, and pterin-6-carboxylic acid. The three K_I values were determined from Dixon plots generated by varying the concentrations of each inhibitor over a range of fixed xanthine concentrations at pH 8.3 and 23 °C, in 2.5 mL of 0.1 M sodium pyrophosphate buffer containing 1.0 mM EDTA.

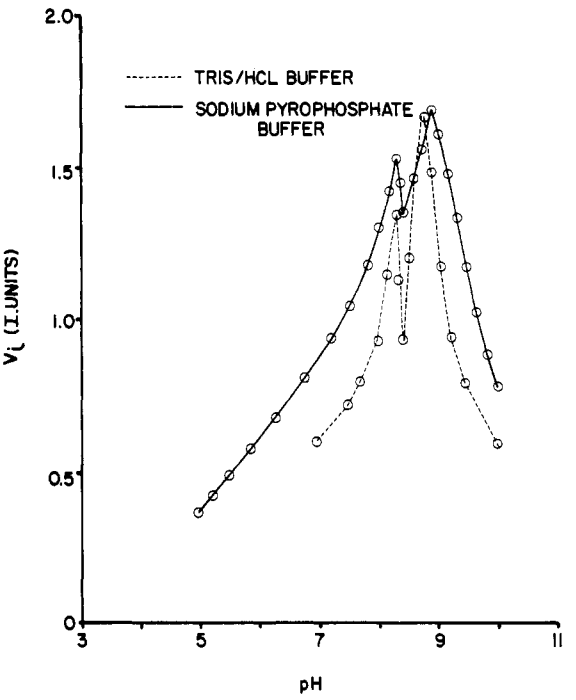

Figure 1. pH profile of nonproteolytically derived bovine milk xanthine oxidase in two buffer systems: 0.1 M sodium pyrophosphate and 0.1 M Tris-HCl. The substrate was xanthine and was used at subsaturating concentrations.

RESULTS

PAGE of PDXO and NPDXO samples indicated that PDXO migrated faster than NPDXO. The aparent migrational differences between the two preparations are most likely a direct result of alterations in the primary structure of the PDXO preparation.

Figures 1 and 2 demonstrate the effect of changing pH at subsaturating concentrations of xanthine on the NPDXO and PDXO preparations, respectively. Examination of these profiles shows that the NPDXO preparation exhibits quite dissimilar behavior in comparison to the PDXO preparation. Although both preparations show a tendency toward enhanced activity around pH 8.7, only the NPDXO samples demonstrated elevated activity between pH 8.1 and pH 8.3 and diminished activity between pH 8.4 and pH 8.5. Furthermore, the activity of PDXO was much less in 0.1 M Tris-HCl buffer than in 0.1 M pyrophosphate buffer (Figure 1). Even though these profiles show a tendency for higher enzymatic activity at subsaturating concentrations and pH 8.7, maximum velocities obtained at saturating concentrations of xanthine defined the pH optima of both preparations at pH 8.3 (Table I).

Table I summarizes and compares the various characteristics of PDXO and NPDXO preparations. As stated under Materials and Methods, the protein concentration for both PDXO and NPDXO was equalized to 5.55 mg/mL by using the appropriate buffer. Several important differences were found between the two preparations. With xanthine, at pH 8.3, the NPDXO preparation had a 93% higher specific activity and a 147% higher maximum velocity than the PDXO preparation. Furthermore, with xanthine (pH 8.7) and hypoxanthine (pH 8.3) the NPDXO had 52% and 68%, respectively, higher maximum velocity than the PDXO. It should be noted here

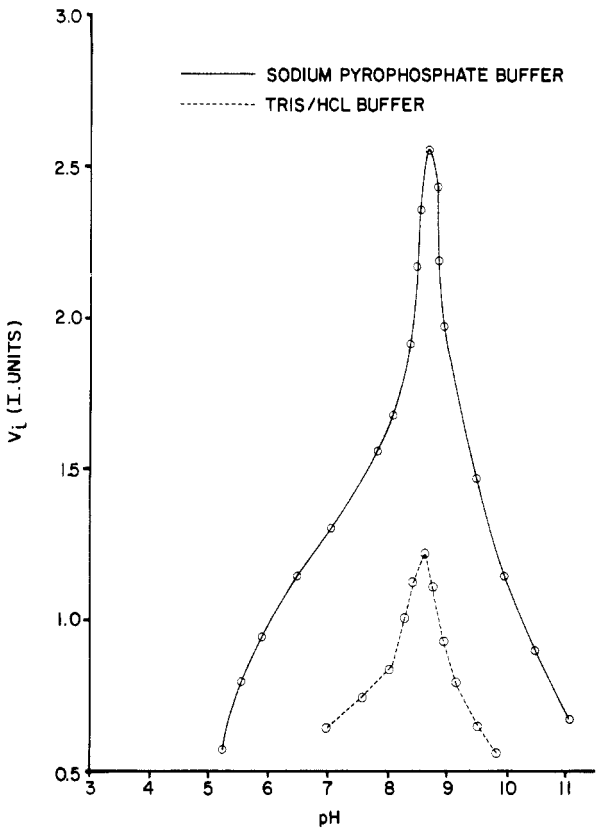

Figure 2. pH profile of proteolytically derived bovine milk xanthine oxidase in two buffer systems: 0.1 M sodium pyrophosphate and 0.1 M Tris-HCl. Xanthine was used in subsaturating concentrations.

Table I. Comparison of Characteristic Data for Proteolytically and Nonproteolytically Derived Xanthine Oxidase Preparations

characteristic	PDXO[a]	NPDXO[b]
total protein, mg/mL[d]	5.55	5.55
specific activity, IU/mg	3.64	7.04
protein/flavin ratio	4.55	3.90
E_a, kcal/mol	13.7	14.4
Q_{10}	2.17	2.26
V_{max} (pH 8.3, xanthine), IU/mL	1.88	4.65
K_m (pH 8.3, xanthine), μM	62.0	41.0
V_{max} (pH 8.3, hypoxanthine), IU/mL	1.16	1.95
K_m (pH 8.3, hypoxanthine), μM	12.8	7.0
V_{max} (pH 8.7, xanthine), IU/mL	2.06	3.13
K_m (pH 8.7, xanthine), μM	31.7	20.0
K_I (folic acid), μM	1.79	1.15
K_I (allopurinol), μM	0.38	0.31
K_I (P6C), μM[c]	1.5	1.0

[a] PDXO = proteolytically derived xanthine oxidase. [b] NPDXO = nonproteolytically derived xanthine oxidase. [c] P6C = pterin-6-carboxylic acid. [d] Protein concentration adjusted with 0.1 M pyrophosphate or Tris-HCl buffer.

that when hypoxanthine was tested in the same concentration range as with xanthine, substrate inhibition was observed. This inhibition was overcome by reducing hypoxanthine concentration to the 1 μM range.

At pHs 8.3 and 8.7, NPDXO showed 51% and 59%, respectively, higher affinity for xanthine than PDXO. Also, at pH 8.3, NPDXO demonstrated an 83% higher affinity for hypoxanthine than PDXO. Furthermore, the NPDXO preparation showed higher affinity (lower K_I) for three competitive inhibitors folic acid, allopurinol, and pterin-6-carboxylic acid than PDXO. The protein to flavin ratio for NPDXO was 17% lower than PDXO, indicating the former preparation was of higher purity. The activation energies and Q_{10} values for the two preparations were fairly close.

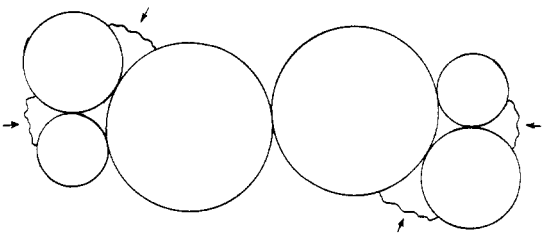

Figure 3. A two-dimensional molecular model for native bovine milk xanthine oxidase proposed by Nagler and Vartanyan (1976). Arrows indicate the four protein segments removed by proteolysis.

DISCUSSION

In previous investigations it has been established that purified bovine milk xanthine oxidase differs according to the purification method employed (Hart et al., 1970; Nelson and Handler, 1968; Waud et al., 1975; Nagler and Vartanyan, 1973; Nagler and Vartanyan, 1976). In 1976, Nagler and Vartanyan proposed a structural model (Figure 3) of a native molecule of bovine milk xanthine oxidase based on a comparison study of a sample prepared by the proteolytic procedure of Gilbert and Bergel (1964) and a sample prepared nonproteolytically. These investigators demonstrated that the use of pancreatin results in the cleavage of at least four segments of the primary structure of the molecule with a total molecular weight of about 12 000 (Nagler and Vartanyan, 1976). Although this modification does not appear to significantly change the catalytic activity of the enzyme, it does reduce the total molecular weight and alters significantly the migration patterns during electrophoresis. Furthermore, Nagler and Vartanyan (1976) found that during SDS electrophoresis, the nonproteolytic preparation resolved as a single band corresponding to 150 000 molecular weight whereas the proteolytic preparation resolved as three bands corresponding to subunits of 92 000, 42 000, and 20 000. Moreover, these investigators showed that the Mo and FAD moieties were located in the 92 000 and 42 000 molecular weight subunits, respectively.

In the present investigation, PDXO migrated faster in PAGE than did the NPDXO preparation. It is concluded that proteolysis altered the primary structure of the enzyme, an observation in agreement with previous reports (Waud et al., 1975; Nagler and Vartanyan, 1976).

In kinetic comparisons, it was found that the two preparations demonstrated dramatic differences in behavior. At subsaturating concentrations of substrate in 0.1 M Tris-HCl buffer, the PDXO preparation showed significantly less activity than in 0.1 M sodium pyrophosphate buffer. In contrast, the activity of NPDXO was the same in both of these buffers. This suggests that proteolysis, by modifying the primary structure, has reduced the enzyme's conformational stability. Furthermore, substantial differences were observed between the two preparations in K_m and V_{max}. With xanthine at pH 8.3, the K_m and V_{max} values for the NPDXO and PDXO preparations were 41 μM and 4.65 IU/mL and 62 μM and 1.88 IU/mL, respectively. This indicates that the NPDXO preparation had a 51% greater affinity for the substrate and a 147% higher maximum velocity than the PDXO preparation. Similarly, at pH 8.3 NPDXO showed higher affinity and maximum velocity for hypoxanthine than PDXO. Moreover, the trend toward higher affinity (lower K_I) with the NPDXO preparation was also observed with the three competitive inhibitors folic acid, allopurinol, and

pterin-6-carboxylic acid (Table I).

These differences in kinetic behavior may be directly related to the arrangement of the subunit globules described in Nagler and Vartanyan's molecular model (Nagler and Vartanyan, 1976) (Figure 3). For optimum catalytic efficiency, the electron transport chain at the active site of the enzyme requires that the molybdenum- and FAD-containing globules must be within a favorable proximity to each other. This arrangement is in agreement with the recent magnetic interaction model for xanthine oxidase proposed by Barber et al. (1982). If the protein segments described in Nagler and Vartanyan's model are represented as segments of the enzyme's primary structure that closely interact with the amino acid side chains of the globules, then it is possible that these segments might have a more significant structure-to-function relationship to the intact enzyme molecule than realized heretofore. The greater catalytic efficiency and higher affinity for both substrates and inhibitors observed in this study with the NPDXO preparation support this conclusion.

Registry No. Xanthine oxidase, 9002-17-9; P6C, 948-60-7; allopurinol, 315-30-0; folic acid, 59-30-3; xanthine, 69-89-6; hypoxanthine, 68-94-0.

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Organic Nitrates and Nitriles in the Volatiles of Cooked Cured Pork

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Headspace volatiles from slices of cooked cured pork loin containing residual sodium nitrite concentrations between 63 and 1057 μg/g were collected on Tenax GC and analyzed by gas chromatography–mass spectrometry. Benzonitrile, phenylacetonitrile, and four alkyl nitrates were found in the headspace volatiles of all the bacon samples. Several alkanenitriles were also found in samples with high residual nitrite levels. All these nitrogen compounds were also found in a volatile extract of high residual nitrite bacon prepared by steam distillation–solvent extraction. Possible mechanisms for the formation of these compounds from reactions between lipid and sodium nitrite and the importance in cured flavor are discussed.

A number of studies using sensory panels have clearly demonstrated that sodium nitrite is essential for the formation of the characteristic flavor of cured meats (MacDougall et al., 1975; Gray et al., 1981). However, there are few reports on flavor voltiles from cured meats, a marked contrast to the wealth of literature relating to cooked beef flavor (MacLeod and Seyyedain-Ardebili, 1981). Recently, benzonitrile, phenylacetonitrile, and several alkyl nitrates were tentatively identified in the headspace volatiles of cured pork (Mottram et al., 1984). None of the compounds were found in control samples of uncured pork or in nitrite-free salt pork.

This paper reports further work to establish the extent of formation of nitriles and nitrates in cooked cured pork and to determine if the amount of sodium nitrite used in the cure affects the concentration of these compounds in the headspace volatiles.

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MATERIALS AND METHODS

Preparation of Cured Pork. Slices (3 mm thick) of fresh pork loin were randomly distributed between treatment groups. Curing was carried out using a slice–cure based on the method described by Holmes (1960) using brines containing 200 g/L sodium chloride and so-

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