

Purification and Properties of a Novel Ferricyanide-Linked Xanthine Dehydrogenase from *Pseudomonas putida* 40

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The isolation of a xanthine dehydrogenase from *Pseudomonas putida* 40 which utilizes ferricyanide as an electron acceptor at high efficiency is presented. The new activity is separate from the NAD⁺ and oxygen-utilizing activities of the same organism but displays a broad pattern for reducing substrates typical of those of previously studied xanthine-oxidizing enzymes. Unlike the previously studied enzymes, the new enzyme appears to lack flavin but possess heme and is resistant to cyanide treatment. However, sensitivity of the purified enzyme to methanol and the selective elimination of the activity when tungstate is added to certain growth media suggest a role for molybdenum. The enzyme is subject to a selective proteolytic action during processing which is not accompanied by denaturation or loss of activity and which is minimized by the continuous exposure of the activity to EDTA and phenylmethylsulfonyl fluoride. Electrophoresis of the denatured enzyme in the presence of sodium dodecyl sulfate suggests that the enzyme is constructed of subunits with a molecular weight of approximately 72,000. Electrophoresis under native conditions of a purified enzyme previously exposed to magnesium ion reveals a series of major and minor activity bands which display some selectivity toward both electron donors and acceptors. An analysis of the effect of gel concentration on this pattern suggests that the enzyme forms a series of charge and size isomers with a pair of trimeric forms predominating. Comparison of the rate of sedimentation of the enzyme in sucrose gradients with its elution profile from standardized Sepharose 6B columns suggests a molecular weight of 255,000 for the major form of the native enzyme.

Pseudomonas putida strain 40 was originally isolated in our laboratory on caffeine as a sole source of carbon and nitrogen (40) and was included in our studies of the distribution of xanthine dehydrogenase specificity types among bacteria. Since that survey, a number of xanthine-oxidizing flavoproteins have been isolated from several genera of bacteria (24, 30, 38, 43), and the electron acceptor properties of these purified enzymes can account for the patterns we found to be representative of the extracts of these different groups of bacteria (42).

We have shown that members of the genus *Pseudomonas* characteristically induce high levels of xanthine-oxidizing activities towards molecular oxygen, ferricyanide, and NAD⁺ when grown on purine substrates (41). Such a behavior would be consistent with the lack of specificity that many xanthine-oxidizing enzymes display towards electron acceptors (3, 8). However, in attempting to purify the xanthine-oxidizing activity from the extracts of *P. putida* 40, it readily became apparent that the activity associated with each of these acceptors is primarily due to a different and separable enzyme form. This also would not be without precedent since the mammalian xanthine dehydrogenase is converted to a xanthine oxidase through actions involving the removal or oxidation of certain sulfhydryl groups (11, 39).

This paper is concerned with the isolation and characterization of the ferricyanide-linked activity from *P. putida* 40. It does not seem likely that the new enzyme is derived from the other xanthine-oxidizing activities since the properties of the prosthetic center of the ferricyanide-utilizing enzyme differ radically from those of any previously studied xanthine-oxidizing enzyme, including the NAD-linked xanthine dehydrogenase from strain 40 and several strains of *Pseudomonas synxantha* (34) and *Pseudomonas acidovorans* (36).

MATERIALS AND METHODS

Standard purification of the enzyme. Step 1. Preparation of the extract. *P. putida* 40 was grown as previously described (40) in 18-liter carboys with forced aeration except that 0.1 M phosphate and 0.35% caffeine were employed. Stationary cells were collected with the Sharples super centrifuge at 62,000 × *g* and flushed with 0.1 M phosphate buffer (pH 7.0). The cells were suspended in the buffer and centrifuged at 8,200 × *g* at 4°C for 10 min and deliberately frozen at -75°C until needed. Approximately 300 g (wet weight) of cells was thawed and suspended in purification buffer (PB) which consisted of 0.1 M potassium phosphate, 10⁻⁴ M sodium EDTA, and 10⁻⁵ M phenylmethylsulfonyl fluoride (pH 7.0) and centrifuged as before. The supernatant, containing significant protein as a result of the freezing process but little activity, was discarded, and the pellet was suspended in 260 ml of PB to which 27% (wt/vol) sucrose had been added. After equilibration at 22°C for 10 min the mixture was pelleted as before, and the sucrose-containing solution was drained from the cells, which were then vigorously suspended in 360 ml of 4°C water, equilibrated for 10 min, and recentrifuged. The supernatant shock fluid was discarded, and the cells were suspended in PB to give a thick paste and subjected to sonic vibrations, followed by centrifugation to give extracts as previously described (41). The pelleted material was resuspended in about half the volume of PB used originally, and the extraction process was repeated. This process was performed two more times, and the four supernatants were combined to give the starting crude extract from which the enzyme was purified (Table 1).

Step 2. Phase separation with polyethylene glycol. Glycerol (102 ml) was added to the crude extract as described in Table 1, followed by the addition of 102 ml of a 10% solution of

TABLE 1. Purification of the ferricyanide-linked xanthine dehydrogenase from *P. putida* 40

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)	Fold purification	Total units	Yield (%)
1. Crude extract	395	10.2	1.4	1	5,640	100
2. Supernatant from phase separation	125	2.0	9.0	6.4	2,250	39
3. Fraction from DEAE-cellulose column	226	0.08	35	25	633	11
4. Concentrate after blue agarose chromatography	9.5	0.16	154	110	234	4
5. Ammonium sulfate precipitate	1.0	0.8	163	116	130	2.3

dextran T-100 (Sigma Chemical Co.) in PB and then 188 ml of a 60% (wt/vol) solution of polyethylene glycol in PB and equilibrated for at least 30 min at 4°C. The mixture was dispensed in 50-ml tubes and centrifuged at $8,200 \times g$ for 10 min. As much as possible (about 700 ml) of the dark clear upper phase, containing most of the NAD⁺-linked xanthine dehydrogenase activity and most of the protein of the extract, was poured off. The lower phase (clear) together with extensive insoluble matter which had accumulated at or near the upper surface was washed by mixing with 130 ml of a 6% solution of polyethylene glycol in PB and centrifuged. The upper wash was discarded, the lower phase was washed with 130 ml of the polyethylene solution to which a 20% (vol/vol) saturated solution of ammonium sulfate (4°C) had been added, and the resulting suspension was stirred at 4°C for 10 min and centrifuged at $28,700 \times g$ for 30 min. The expanded clear lower phase was then carefully drained from the floating insoluble material. The ferricyanide-linked enzyme could be extracted from this insoluble material by stirring in PB at 4°C for 30 min, followed by centrifugation at the higher speed used before. The pelleted material was extracted twice again with decreasing volumes of PB, and the eluates were combined to give fraction 2 found in Table 1.

Step 3. Elution from DEAE-cellulose. Fraction 2 was dialyzed against PB in which the phosphate concentration had been reduced to 1 mM (1 mM PB). Virtually the entire dialyzed volume was applied to a column of DEAE-cellulose (2.5 by 38 cm; Whatman DE 11) equilibrated with 1 mM PB and eluted with the same buffer until the UV-absorbing material not adsorbing to the column under these conditions was collected. The elution of the column was then continued with a stepwise increase in the phosphate concentration of the PB to 5 mM (5 mM PB). The ferricyanide-linked activity was found in the first peak of protein eluting with this buffer (Fig. 1A). The main portion of the protein can then be stripped from the column with several washes of higher salt concentration. NAD-linked xanthine dehydrogenase elutes towards the front of the main protein profile after the addition of higher salt concentration, but the activity is absent at this point of the purification.

Step 4. Elution from Affi-Gel Blue agarose. The fractions containing appreciable activity from the DEAE-cellulose column were combined and added to a column (diameter, 2.5 cm) containing a bed volume of 100 ml of 100-to-200 mesh Affi-Gel Blue agarose (Bio-Rad Laboratories) equilibrated with PB. The bulk of the protein eluted shortly from this column was stripped of its xanthine dehydrogenase activity (Fig. 1B). NAD⁺-linked xanthine dehydrogenase eluted towards the end of the main protein profile, but the activity was no longer present. The ferricyanide-utilizing enzyme

then made its appearance and eluted over a large volume. The enzyme-containing eluate (850 ml) was concentrated by ultrafiltration through an Amicon Diaflo XM-50 filter to nearly the point of dryness. During the concentration, the surface of the filter became bright orange. The filter was washed by several applications of 5 mM PB and each time brought nearly to the point of dryness by ultrafiltration. The filter was removed, and the surface was repeatedly rinsed with a small volume of PB by the aid of a capillary pipette equipped with a rubber bulb until all of the colored material had been resuspended to give fraction 4 (Table 1).

Step 5. Precipitation with ammonium sulfate. Fraction 4 was treated with an equal volume of a 100% saturated ammonium sulfate solution (4°C) and centrifuged at $12,800 \times g$ for 10 min. This was just sufficient to remove most of the colored material from the supernatant with which the activity coprecipitated. The precipitate was suspended in PB and recentrifuged, and the supernatant was poured off to give fraction 5 (Table 1).

Alternative procedure for the purification of the enzyme. Relatively early in this investigation, the enzyme was purified by a variation of the procedure finally adopted and compared in Results. The sonicate was obtained from cells which had been frozen and washed once in PB. The extract was treated with 2% streptomycin sulfate, and the protein from the supernatant precipitating between 33 and 50% saturation with ammonium sulfate was collected, suspended, and dialyzed against 1 mM PB. The ammonium sulfate fraction was first applied to the blue agarose column used in the standard procedure which had been equilibrated with 1 mM PB and eluted with the same buffer. The volume containing the enzyme was concentrated by ultrafiltration as before, washed, and suspended with 1 mM PB. This fraction was applied to the DEAE-cellulose column described above equilibrated with 1 mM potassium phosphate buffer plus 1 mM MgCl₂ (pH 7.0). This Mg²⁺ concentration was maintained as the column was eluted with an increasing phosphate gradient. The ferricyanide-linked xanthine dehydrogenase coincided with the first UV-absorbing peak to elute from the column at a phosphate concentration of about 0.025 M and centered around an elution volume of 170 ml. The volume containing this activity peak was collected and concentrated by ultrafiltration as previously described except the concentration was performed with plain 0.1 M potassium phosphate buffer (pH 7.0).

Enzyme activities. In general, the equipment and methods used in the assay of the xanthine-oxidizing activities with various reducing and oxidizing substrates have been previously described (41, 44). Sulfite oxidation was tested by direct spectrophotometric observation with molecular oxygen as the acceptor (5) or by the use of ferricyanide as in the

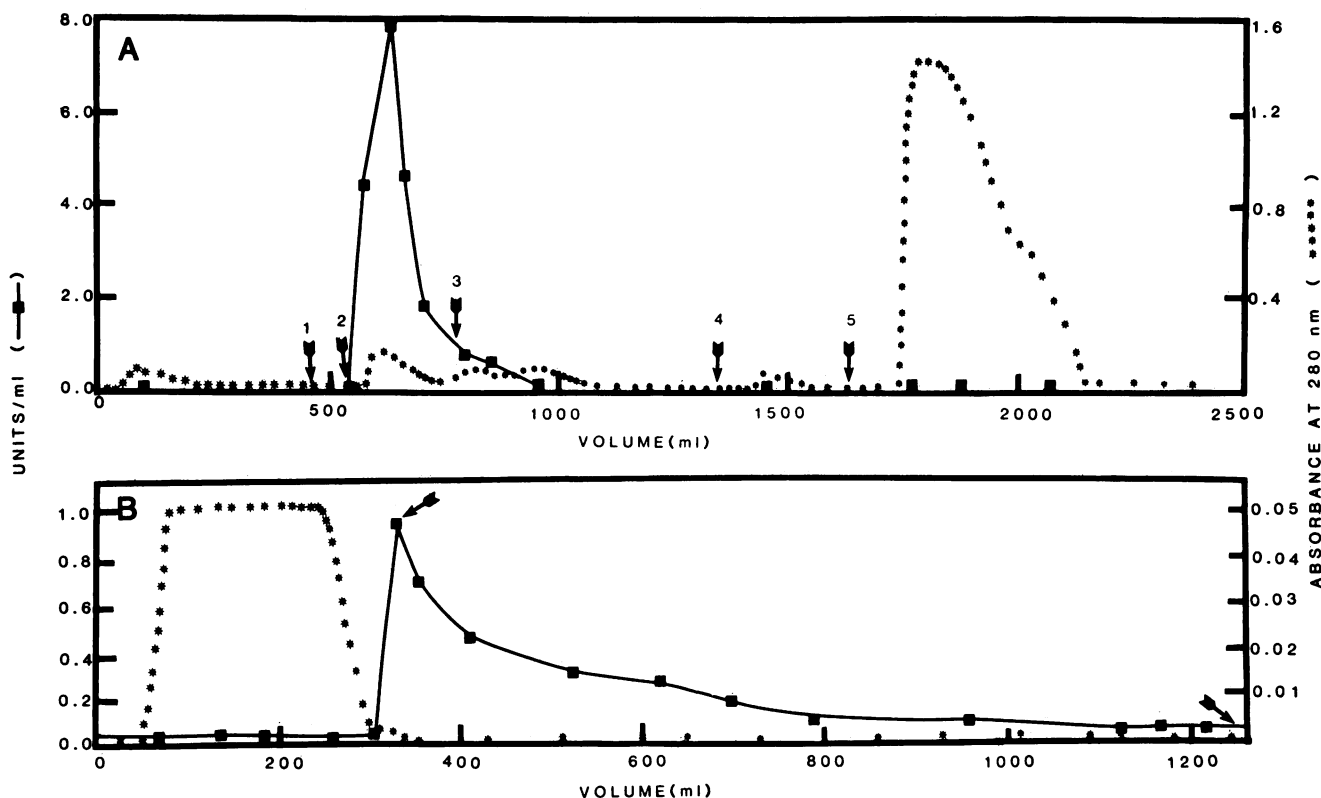


FIG. 1. Activity profiles of the ferricyanide-linked xanthine dehydrogenase during purification (Table 1). (A) Chromatography on DEAE-cellulose. Arrows: 1, point at which 1-mM PB elution buffer was increased to 5 mM PB; 2 and 3, the volume taken for further purification; 4, point at which the 10-mM elution PB was started; 5, point at which elution with 2 M NaCl was initiated. (B) Chromatography on Affi-Gel Blue agarose. The volume between the two arrows was taken for further purification.

xanthine dehydrogenase assay. The ferricyanide-linked xanthine dehydrogenase was routinely measured in a total volume of 480 μ l with 0.6-ml-capacity microcuvettes containing 400 μ l of PB and a final concentration of xanthine and ferricyanide of 1 mM each by following the disappearance of ferricyanide at 410 nm (44). One unit of enzyme is that amount that will reduce 1 μ mol of ferricyanide per min. Unless otherwise stated, the same buffering conditions were employed with the preincubations and assays involving the effect of various substrates, inhibitors, and effectors on activity.

Other assays. Protein was estimated by the method of Lowry according to Layne (22). Heme content of the purified enzyme was determined after extraction of the enzyme with alkaline pyridine (31) by comparison of the reduced and oxidized spectra by the method of Porra and Jones (32) employing the extinction coefficient for pyridine protochromochrome IX. The purified enzyme was examined for the presence of flavins (16) as previously employed (44).

Electrophoretic techniques. Native enzyme preparations were examined for purity by the analytical disc gel technique of Davis (10) and by this same method with the buffering system of Hedrick and Smith (15). The method of the latter workers for analyzing the charge and size of isomer composition of purified proteins was very useful for application to certain of our preparations which exhibit complex behavior. A low-power microscope was useful in determining very low relative mobility values. We have found some irreproducibility in relative mobility values with given enzyme standards from one gel preparation to another, but the Hedrick and Smith plots (15) always gave the same slope values and

were identical to those of the previous workers. Accordingly, we utilized a standard curve obtained from 17 proteins by the latter workers in estimating the molecular weights of the components in certain preparations of the xanthine dehydrogenase.

Enzymatic activity was exhibited on gels by a previously described method (43, 44) except that the Nitro Blue Tetrazolium was substituted by an equal weight of 3-(*p*-iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl 2H-tetrazolium chloride, and 0.1 mg of NAD⁺ per ml was routinely included in the reagent mixture along with the phenazine methosulfate. However, these adjustments were not critical in demonstrating the activity bands reported, all of which were xanthine dependent. Gels were stained for heme proteins by the method of Haut et al. (14) except that, for safety reasons, 100 mg of benzidine was dissolved in 5 ml of methanol instead of using a saturated solution of the benzidine hydrochloride, and the volumes of the other solutions to make the reagent were reduced accordingly. Densitometric tracings of gels exhibiting activity and protein bands were performed as previously described (43, 44).

The sizes of the peptide components of enzyme preparations were estimated exactly as described by Weber and Osborn (40) except that destaining was achieved by diffusive elution with 7% acetic acid. Also, the more rigorous denaturing conditions described by Lane (20) were employed with our enzyme preparations before the electrophoresis in the presence of sodium dodecyl sulfate (SDS). The following proteins were used as standards: bovine serum albumin, catalase, creatine kinase, lactic dehydrogenase, pepsin, trypsin, myoglobin, RNase, lysozyme, and cytochrome *c*.

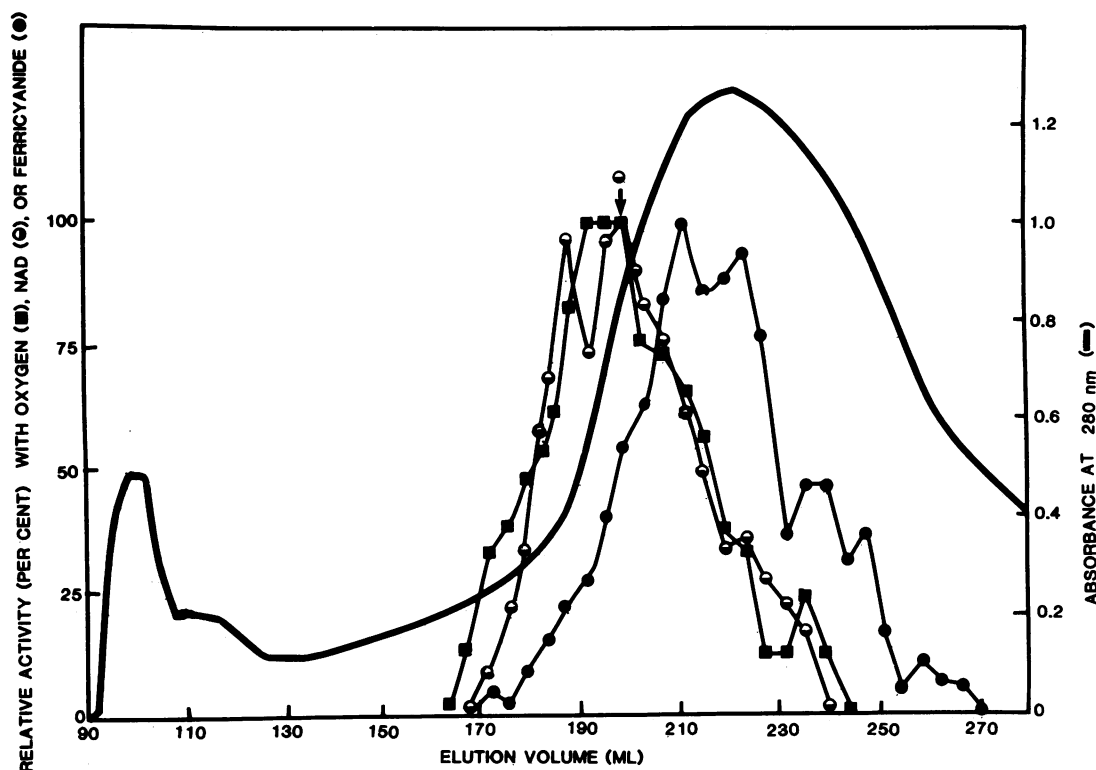


FIG. 2. Gel filtration of the xanthine-oxidizing activities from extracts of *P. putida* 40 on a column (2.5 by 56.5 cm) of Sepharose 6B, with a flow rate of 0.6 ml per min. A fraction (14 ml) which had been carried through the ammonium sulfate precipitation and dialysis step of the alternative method containing 26 mg of protein per ml, 37.5 U of ferricyanide activity per ml, and most of the oxygen- and NAD⁺-linked activity of the crude extracts was applied to the column. Relative activity represents the percent activity of the fraction relative to that given by the peak fraction. Relative activity of 100% represented 10.0 U of ferricyanide, 2.1 U of NAD⁺, and 0.036 U of oxygen activity.

Other physical techniques. The equations provided by Siegel and Monty (35) were used in calculating the molecular weight and f/f_0 values of the xanthine dehydrogenase from a combination of sedimentation and gel filtration data. Sucrose gradient sedimentation was performed by the method of Martin and Ames (25) except that an SW 50.1 rotor was used. In addition to catalase ($s_{20,w}^{0,725} = 11.3$) used by other workers (25, 35), we included serum bovine albumin as a second marker, assuming an $s_{20,w}^{0,725}$ value of 4.31S (13). The Stokes radius of the purified enzyme was estimated by the use of the Sepharose 6B column used in Fig. 2 ($V_0 = 92$) except that a 1.5-ml sample volume containing the subject or standard enzymes was used. Standard proteins with Stokes radii from the literature were as follows: *Escherichia coli* alkaline phosphatase, 2.99 nm (32); catalase, 5.2 nm (37); ferritin, 7.9 nm (35); urease, 6.1 nm (35); thyroglobulin, 8.1 nm (1). A standard curve was obtained from K_{av} values, and the radii were plotted by the method of Laurent and Kilander (21, 35).

RESULTS

Isolation of ferricyanide-linked dehydrogenase and its separation from other xanthine-oxidizing activities. Elution on a Sepharose 6B column of a relatively unpurified fraction from *P. putida* 40 in the presence of EDTA revealed a broad but different pattern of activity peaks with each of the three electron acceptors employed (Fig. 2), suggesting that a separate aggregation series might be responsible for the activity with each acceptor. Furthermore, the oxygen-linked activity was relatively unstable and disappeared after several days of storage at 4°C. When similar fractions prepared

without the use of EDTA but with 6 mM Mg²⁺ were applied to the column equilibrated with this buffer, the major peaks of ferricyanide activity were found in the region corresponding to the leading shoulders around 185 and 195 ml in the profile of this activity in Fig. 2.

Table 1 presents a typical purification of the ferricyanide-dependent activity by the standard procedure, and comparable specific activities are given by the alternate preparation. Both preparations display no activity when molecular oxygen, NAD⁺, NADP⁺, cytochrome *c*, flavin adenine dinucleotide, or flavin mononucleotide are substituted for the ferricyanide under standard assay conditions. We indicated in the description of the standard purification how the NAD⁺-linked activity might also be highly purified by these same methods. The NAD⁺ activity so purified displays no activity toward molecular oxygen or ferricyanide.

Although the ferricyanide-linked activity of crude extracts is stable to freezing and thawing or storage at 4°C for up to a week, the purified enzymes lose from one-half to three-quarters of the activity under these same conditions. Electrophoresis of an aged, purified enzyme shows the same protein intensity profile given originally, although the activity profiles do not develop. The activity of the purified enzyme was not affected by preincubation with flavin adenine dinucleotide or sulfide under conditions approximating those which wholly or partially restore the activity of the deflavo (18) or desulfo (12) forms of milk xanthine oxidase, and these preincubations did not enable the enzyme to utilize NAD⁺ or molecular oxygen.

Spectral and related properties of the purified enzyme. The spectrum of the purified enzyme (Fig. 3) is dominated by an

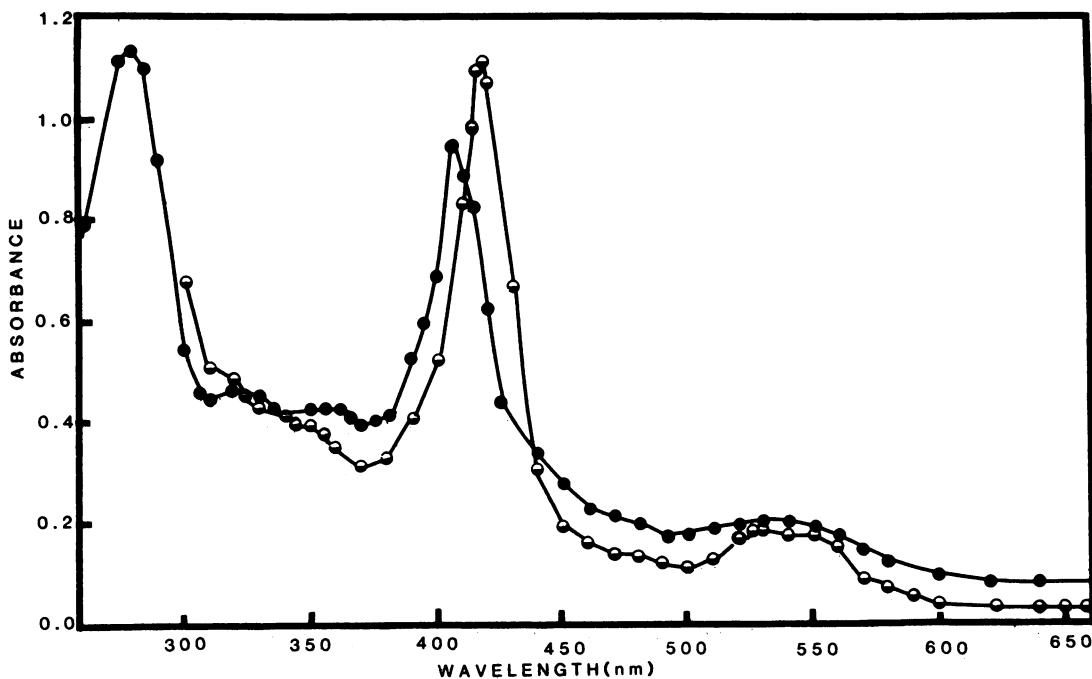


FIG. 3. Spectrum of standard preparation of the enzyme. Symbols: ●, spectrum given by fraction 5, Table 1; ○, spectrum after addition of 6.25×10^{-4} M xanthine. The absorbance was increased by 2.5% to account for the increase in volume due to the addition of xanthine.

intense band around 405 nm and a broad, more weakly absorbing band in the 500-to-580-nm range. It is unlike that of any previously studied xanthine-oxidizing enzyme in which the spectrum is dominated by the flavin component (3, 8). The spectrum more closely resembles that of sulfite oxidase, a molybdenum hydroxylase known to contain heme (7). However, the spectrum is not as clean as that of sulfite oxidase, and there is significantly more absorbance in the trough between the 280 and 405 nm absorption peaks, with shoulders or small peaks at 320 and 360 nm, and more relative absorbance is observed around 450 nm than in sulfite oxidase. The deflavo form of xanthine oxidase exhibits significant absorption in these spectral regions (18). Like sulfite oxidase, there is a shift of the main absorption peak at 405 nm to longer wavelengths and an increased absorbance at the maximum (419 nm), when the enzyme is incubated with its substrate (Fig. 3). This would be consistent with the reduction of a heme group, but we did not observe a net increase in the absorption around 525 and 559 nm that would be expected with the reduction of heme. It is of interest that the deflavo form of xanthine oxidase shows a comparable decrease in the absorption in this region on reduction (18), and it seems possible, depending on the ratios of the various functional groups in the present enzyme, that any increase in this region due to the reduction of the heme may be offset by the reduction of other functional groups. There is, however, some increased definition in the spectrum of the reduced enzyme with more prominent shoulders around 525 and 560 nm. Furthermore, peaks at 525 and 557 nm are observed in the dithionite-reduced alkaline pyridine-extracted enzyme. Calculation from the latter absorption (see Materials and Methods) predicts 2×10^{-6} M heme in a preparation of the enzyme containing 0.34 mg of protein per ml (Lowry). This would amount to about 69% of the value of the enzyme subunit concentration, assuming a molecular weight of 72,000 (see below). The ratio of the absorption of the oxidized enzyme at 405 nm to that at 280 nm is 0.83, a high

value consistent with stoichiometric heme content for a subunit of this size. Supernatants of the denatured enzyme sufficient to contain 7.5 μ g of flavin adenine dinucleotide, on the assumption that each subunit of the protein contains this group, failed to give an indication of flavin components when examined by a method capable of detecting as little as 0.01 μ g of flavin.

Comparison of standard and alternative purified xanthine dehydrogenases by electrophoresis under native and denaturing conditions. When either the alternative (Fig. 4) or the standard (Fig. 5) enzyme preparation is subjected to electrophoresis on native gels, the enzyme activity profile is dominated by two major and somewhat overlapping bands which become visible within seconds after the addition of the xanthine dehydrogenase activity reagents to the gels (Fig. 4, bands 2a and b; Fig. 5A). The protein stains of companion gels display two dominant and overlapping components which correspond to the position of the two major activity bands (compare Fig. 5A with 5B). In addition, standard gels stained for heme-containing proteins display a dark band coinciding with the area occupied by the two intense protein and activity-staining regions.

The alternative preparation displays five additional minor activity bands which become detectable after about 15 min of incubation in the activity reagents (Fig. 4; bands 1a, 1b, 3a, 3b, and 4). The companion gel stained for protein also shows minor discrete protein-staining bands at the position of these minor activity bands. No other protein-staining bands are observed.

The electrophoresis of the standard enzyme under these conditions differs from that of the alternative preparation in that there are no minor discrete activity or protein bands in addition to the two dominant bands. Rather, there is a diffuse distribution of the activity and protein stain over the mobility range from relative mobility 0 to 0.65 (Fig. 5).

The complex pattern of activity bands given by the alternative enzyme preparation is conducive to analysis by the

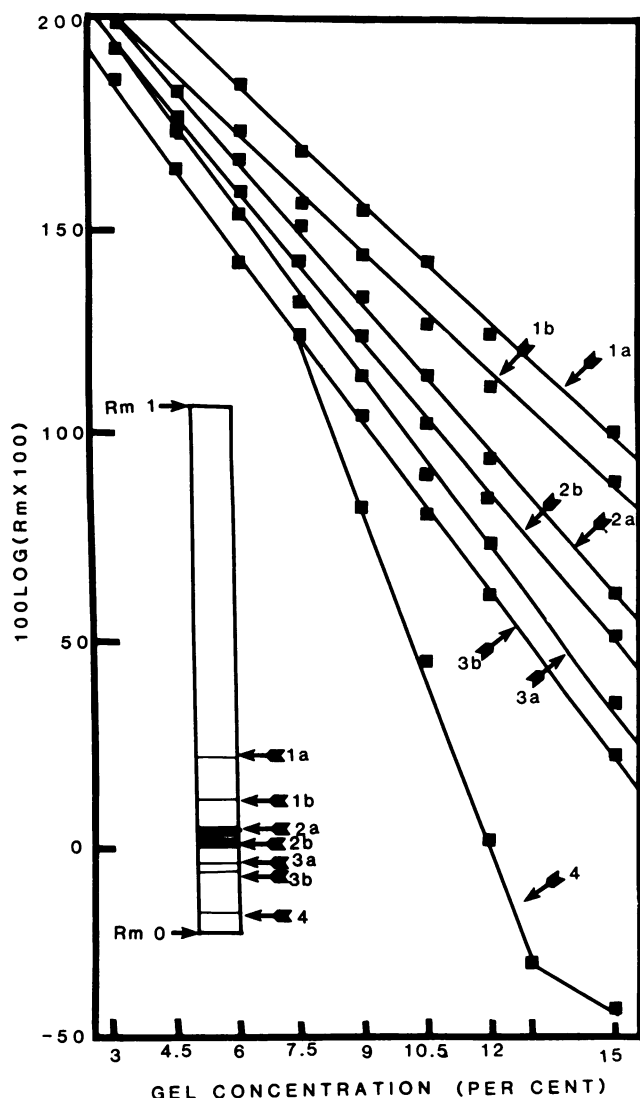


FIG. 4. The Hedrick and Smith (15) plot of activity bands with 60- μ g samples of the alternative enzyme. Inset schematically illustrates the appearance of a 9% gel in which all bands discussed were visible. A similar banding pattern was obtained when these gels were stained for protein. The numbers of the various bands are described in the text.

method of Hedrick and Smith (15) for the estimation of the relative size of the various components (Fig. 4). Hedrick and Smith plots give three pairs of parallel lines (1a and 1b, 2a and 2b, 3a and 3b) with slopes of 9.5, 11.7, and 13.7 plus one additional component of very steep slope (band 4). These slopes correspond to molecular weight values of 147,000 ($2 \times 73,500$), 221,000 ($3 \times 73,600$), and 288,000 ($4 \times 71,900$), respectively. The three leading bands of each parallel pair (Fig. 4; bands 1a, 2a, and 3a) intersect upon extrapolation at around 0% gel concentration, as do the three trailing bands (Fig. 4; bands 1b, 2b, and 3b). The band giving a very steep slope (band 4) is apparently of a still higher level of aggregation but also more negatively charged. The entire pattern could be explained by aggregation of an approximately 70,000-molecular-weight subunit to give a series of size and charge isomers.

Some differences in the catalytic behavior are observed

with the different bands represented in Fig. 4. When 1-methylxanthine is utilized in place of xanthine in the activity stain, the pattern develops as before but more slowly. However, when either 3-methylxanthine or allopurinol is employed as the substrate, the trailing component of each charge isomer set (Fig. 4; bands 1b, 2b, and 3b) and the heavier molecular weight components (Fig. 4; bands 3a, 3b, and 4) are preferentially developed. When the NAD^+ is omitted from the reagent mixture, the components that are more mobile than the main activity band (Fig. 4; bands 1a and b) are reduced in intensity relative to the previously established pattern. When phenazine methosulfate is omitted from the reaction mixture, the trailing component of the main activity set (Fig. 4; band 2b) is not as well developed in respect to the leading component, and the minor components corresponding to higher molecular weight (Fig. 4; bands 3a, 3b, and 4) are relatively poorly developed. When 1 mM Mg^{2+} was added to this enzyme sample for 15 min before performing the electrophoresis with 9% gels, activity bands 2a and b (Fig. 4) retained their dominancy, but there was a relative decrease in the intensity of bands of higher mobility and an increase in the intensity of lower-mobility bands. In addition, a considerable accumulation of activity was observed at the interface of the separating gel. This shift in activity distribution was accompanied by a similar shift in protein stain. Since the standard enzyme preparation (Fig. 5) contains some protein of higher mobility than that observed with the alternative preparation at 9% gel concentration (Fig. 4), it seems possible that this is due to the release of some monomeric subunits in the preparation continuously exposed to EDTA during processing.

When the standard enzyme was subjected to electrophoresis on SDS gels after denaturation by the method of Lane (20), over 90% of the protein stain detected by densitometry was associated with a component corresponding to a molecular weight of 72,000 (Fig. 6A and B). In addition, there were some minor components, each constituting from 2 to 4% of the stain measured by densitometry. Two of these corresponded to molecular weights of 48,000 and 25,000 (Fig. 6B). When the alternative enzyme preparation was examined in the same manner, only about 2% of the protein-staining material corresponded to a component of about 70,000. There was a considerable amount of material associated with 48,000- and 25,000-molecular-weight components and a major band of protein peaking at a position of approximately a molecular weight of 10,000 (Fig. 6C). When the original method of denaturation described by Weber and Osborn was employed with the enzyme prepared by the alternative method, the protein distribution pattern was even more diffuse, with the accumulation of much more relatively small-molecular-weight material.

Hydrodynamic properties of xanthine dehydrogenase. When partially purified fractions of the xanthine dehydrogenase were subjected in triplicate to sedimentation on sucrose gradients, only one major activity peak was detected, and the peak tube was found in fraction 24 in each case (Fig. 7). The average movement relative to catalase and serum bovine albumin was calculated (35), and from these $s_{20,w}^{0.725}$ values of 11.4S and 12.3S, respectively, were estimated for the xanthine dehydrogenase (25). The purified enzyme gave a single peak upon gel filtration on the standardized column of Sepharose 6B, which corresponded to a Stokes radius of 5.45 nm. From this value and the sedimentation constant obtained with catalase, a molecular weight of 255,000 and a frictional ratio (f/f_0) of 1.31 were calculated (35).

Specificity of ferricyanide-utilizing enzyme. In a cursory

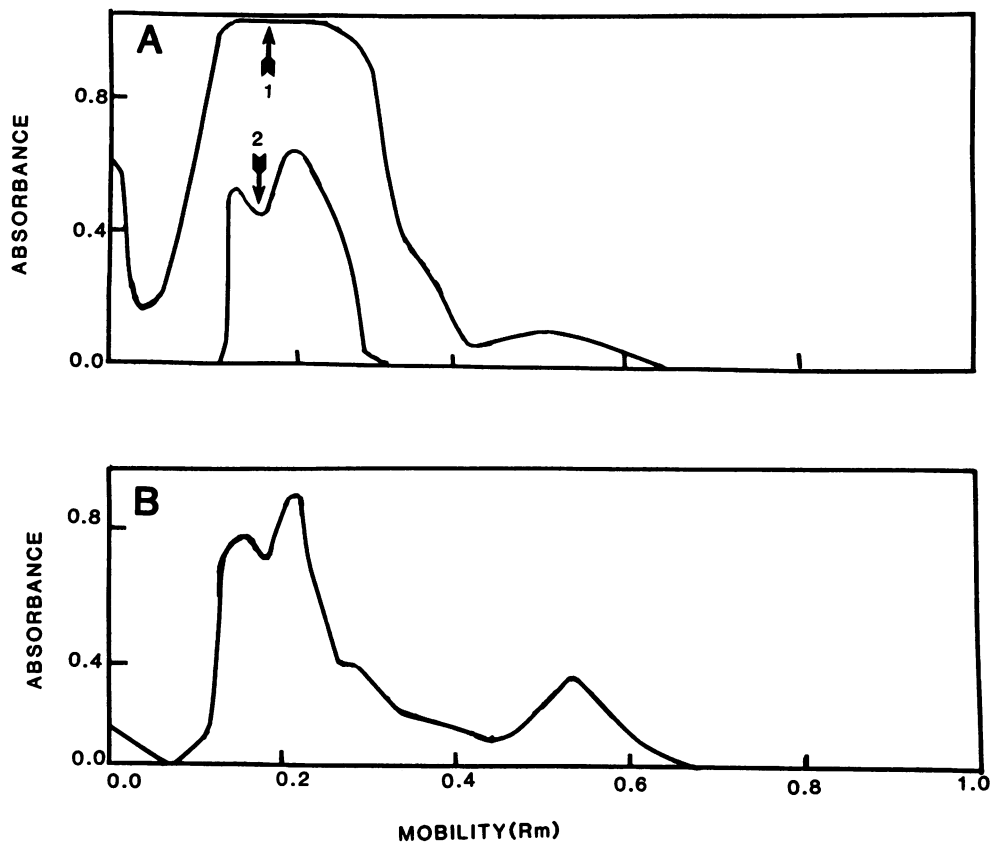


FIG. 5. Densitometric tracings of gels with a 40- μ g standard enzyme sample after electrophoresis on 9% gels by the method of Hedrick and Smith (15). (A) Activity stain after 2 h with phenazine methosulfate omitted. Curve 1, Densitometer was adjusted to read zero with a clear gel. There was considerable damping above 0.8 absorbance. Curve 2, Densitometer was adjusted to read an absorbance of 0.50 at the relatively lightly stained region between the two very intense bands, and the entire gel was scanned at this setting. (B) Densitometric tracing of a companion gel to panel A stained for protein.

examination of the specificity for reducing substrates, it was found that the following compounds were utilized, with the percentages indicating the rate relative to that given by xanthine (K_m , 2.2×10^{-4} M) under standard assay conditions: 6,8-dioxypurine, 77%; purine, 52%; hypoxanthine, 50%; 3-methylpurine, 29%; 1-methylxanthine, 28% (K_m , 3.1

$\times 10^{-4}$ M); 3-methylxanthine, 28% (K_m , 5×10^{-4} M); 2-oxypurine, 15%; allopurinol, 10%. The enzyme supports the NADH-dependent reduction of ferricyanide under the same condition at 35% the rate of the xanthine-dependent reaction.

The enzyme does not catalyze the oxygen- or ferricyanide-

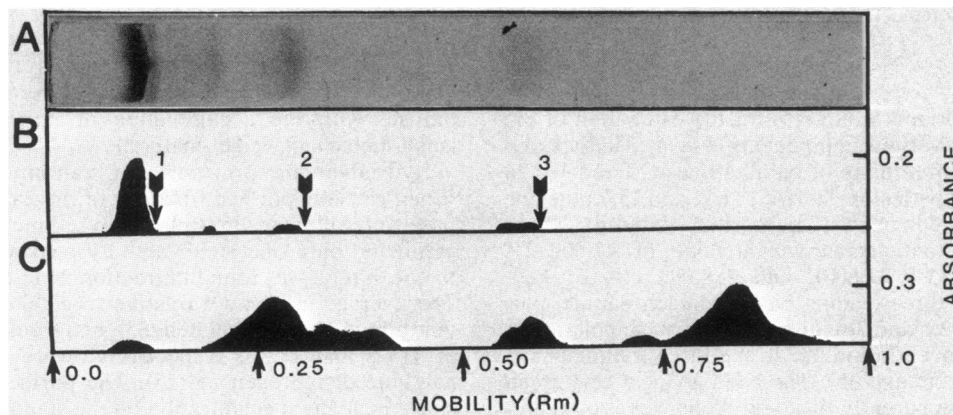


FIG. 6. Electrophoresis of standard and alternative preparations after denaturation with SDS (20) followed by electrophoresis in the presence of SDS (40). (A) Photograph of gel to which 40 μ g of standard enzyme was applied. (B) Densitometric tracing of gel in panel A. Arrows: 1, position of front of a band corresponding to a molecular weight of 72,000 by reference to a standard curve; 2, position of front of a band corresponding to a molecular weight of 48,000; 3, position of a band corresponding to a molecular weight of 25,000. (C) Tracing of gel similar to that shown in panel B with 60 μ g of the alternative enzyme preparation.

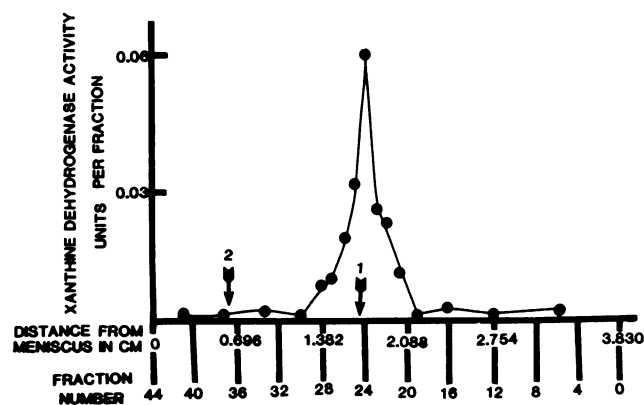


FIG. 7. In the gradient shown, 0.1 ml of a fraction containing 0.19 U of ferricyanide activity (specific activity, 4.1) which had been carried through step 2 of the standard purification, followed by precipitation with 50% saturation with ammonium sulfate and dialysis against 0.1 M PB was layered on the gradient described by Martin and Ames (25) and spun at 36,500 rpm for 356 min. The arrows indicate the relative position assumed by catalase (arrow 1) and bovine serum albumin (arrow 2) on companion gradients.

linked oxidation of sulfite in the presence or absence of AMP (23) nor does it possess the methanol-, formaldehyde-, or formate-dependent ferricyanide reductase activities previously detected in crude extracts of the caffeine-grown cells (42). We are unable to detect an oxygen- or nitrate-linked oxidation of NADH by the enzyme. As reported above, we were unable to detect the utilization of molecular oxygen and other physiological electron acceptors under standard assay conditions. However, it is possible that the enzyme will support xanthine oxidation with molecular oxygen very slowly. During the determination of the spectrum of the enzyme in the presence or xanthine (Fig. 3), we detected a progressive increase in absorbance at 300 nm which could be due to the oxidation of xanthine to uric acid at 2.4×10^{-5} times the rate at which xanthine oxidation is supported by ferricyanide. The purified enzyme will catalyze the xanthine-dependent reduction of 0.1 mM 2,6-dichlorophenolindophenol at 11.5% the rate observed with 1 mM ferricyanide under standard assay conditions.

Inhibition patterns of xanthine-oxidizing enzymes of *P. putida*. Crude extracts were examined for the effects of some reagents commonly employed with molybdenum hydroxylases on the oxygen-, NAD⁺-, and ferricyanide-linked xanthine-oxidizing activities. The activities were stable when incubated in ordinary PB at 21°C during the time periods employed with the inhibitors. When 0.01 M KCN was added to the extracts before assay, it was found that the oxygen-linked activity was completely inactivated in 15 min and that the NAD⁺-linked activity declined according to a 75-min half-life over the 4-h period examined. Under these same conditions, the ferricyanide-linked xanthine dehydrogenase was completely stable. The activities of the purified enzyme with both ferricyanide and 2,6-dichlorophenolindophenol were also stable under these conditions and when preincubated in the presence of cyanide plus 2 mM xanthine.

However, 90% of the ferricyanide activity was lost when preincubated under these same conditions in the presence of 1.78×10^{-3} M arsenite for 1 h 45 min. The purified enzyme activity possessed a 3-min half-life when preincubated with 1.5 M methanol. The sensitivity to methanol was apparently

not enhanced by substrate reduction (8, 33). When the enzyme was assayed in the presence of methanol, the rate of xanthine oxidation declined according to a similar 3-min half-life, whereas the control activity was linear over the 10-min period under the conditions examined.

Effect of tungsten on growth and production of xanthine-oxidizing enzymes of *P. putida*. Laboratory strains of *P. putida* 40 previously grown on caffeine displayed reproducible levels of the three xanthine-oxidizing activities when grown on ordinary nutrient broth (5 g of Difco Bacto-Peptone plus 3 g of Difco beef extract per liter). The specific activities of the ferricyanide-, oxygen-, and NAD⁺-linked xanthine-oxidizing activities in crude sonicates (31) of the broth-grown cells were found to be 0.047, 0.003, and 0.004 U/mg of protein, respectively. These values were 23, 100, and 9%, respectively, of the values previously reported for similar preparations from caffeine-grown cells (41). It was found that addition of from 2.25 to 126 μg of tungsten (as sodium tungstate) per ml to the nutrient broth (27) had no appreciable effect on the growth rate or the amount of growth achieved, but the 2.25-μg/ml level of tungsten was sufficient to completely suppress growth on the caffeine medium to which 0.03 μg of molybdenum per ml is routinely added. With the nutrient broth, the 2.25 μg of tungsten per ml was sufficient to reduce the level of the ferricyanide-linked enzyme to only 3% of the level observed on the plain nutrient broth, and the oxygen- and NAD⁺-linked activities were not detected. No ferricyanide-linked activity could be detected in extracts prepared from cells grown on broth containing 4.5 μg or more tungsten per ml.

DISCUSSION

The elution of relatively unpurified fractions from Sepharose columns suggests that there are at least three different soluble xanthine-oxidizing activities in *P. putida* 40, each specific for one of the three electron acceptors investigated. We have previously described the preparation of a particulate xanthine oxidase from this organism and have argued that it is not related to soluble activities (41, 42). It is further suggested that all three soluble activities may exist at several levels of subunit aggregation and that the ferricyanide activity present in the crude extracts and presumably *in vivo* is of a higher level of aggregation than that obtained after purification in the presence of EDTA. The latter preparations are all dominated by a catalytic component of a molecular weight of approximately 250,000. Comparison with the molecular weight indicated for the subunit (77,000) does not permit a distinction as to whether the major activity is a tetramer or trimer, but Hedrick and Smith plots (Fig. 4) suggest that it is trimeric.

Since a treatment of the purified enzyme with Mg²⁺ before electrophoresis results in a significant enhancement of the minor activity bands, we believe that Mg²⁺ activates the protomer association-dissociation reactions of the protein. One of the more intriguing aspects of the multiplicity of bands is the observation that different size and charge isomers display a selectivity towards oxidizing and reducing substrates. This observation can account for the variability that we have previously reported in the ability of the different preparations of the ferricyanide activity to utilize 1-methylxanthine and 3-methylxanthine (42).

The standard purification procedure has been designed to minimize the effect of proteolysis during purification, which is evidenced by a comparison with the alternate enzyme upon electrophoresis in the presence of SDS (Fig. 6). The alternate enzyme displays, under a variety of gel concentra-

tion and preincubation conditions, a pattern of protein bands on native gels which mimic the activity bands. Therefore, one would expect no major protein component after denaturation that was not derived from the active enzyme. Major bands are observed which correspond to molecular weights of 48,000 and 25,000 (Fig. 6C). This suggests that the corresponding minor components obtained with the standard enzyme preparation (Fig. 6B) may be due to a more limited proteolytic action on a few percent of the subunits during processing and not to contamination with foreign proteins. The molecular weights of these two components total 73,000, suggesting a rather specific and conservative cleavage of the original subunit. These findings are consistent with a general sensitivity of xanthine oxidase and related enzymes to proteolysis at vulnerable sites of the peptide chain which have been presumed to bridge the polyglobular functional domains of the protomer, resulting in a catalytically active structure in which the fragments remain associated (8, 28).

The most distinctive findings of the present paper relate to the apparent absence of flavin and the presence of heme in the ferricyanide-linked dehydrogenase. In this regard, the enzyme is unlike conventional xanthine-oxidizing enzymes and is similar to the molybdenum cofactor containing sulfite oxidase (6, 7). The present activity also resembles sulfite oxidase in its insensitivity to cyanide (7). All xanthine-oxidizing enzymes that have previously been examined are sensitive to cyanide, which removes an essential persulfide group from the active center of these enzymes (3, 8, 9, 12). The finding that the unpurified enzyme and the 2,6-dichlorophenolindophenol activity associated with the purified enzyme are also insensitive to cyanide treatment suggests not only that a persulfide group has not been lost during purification with the formation of a relatively inactive form of the enzyme but also rules out the type of cyanide inhibition observed with nitrate reductase which can be readily reversed by ferricyanide (17).

Some of the catalytic features of the present enzyme may be consistent with the apparent lack of flavin. The deflavo form of xanthine oxidase has been shown to have enhanced activity with ferricyanide (18), although its ability to utilize oxygen is eliminated. Coughlan (8) has suggested that oxygen, NAD^+ , and cytochrome *c* interact with xanthine-oxidizing enzymes at the flavin site and that ferricyanide may act at an iron center (29). Allopurinol may be a reasonably effective substrate of the present enzyme employing ferricyanide, since the latter compound has been shown to readily reverse the inactivated allopurinol-enzyme complex of other xanthine-oxidizing enzymes (26). Most purine-oxidizing enzymes and other relevant molybdenum-containing enzymes utilize ferricyanide at modest rates or not at all when compared with the present enzyme (4, 18, 19, 24, 30, 33, 36, 37, 44). Enzymes which rival the present enzyme in specific activity with ferricyanide include the xanthine dehydrogenases from the clostridia (2, 38) and xanthine oxidase from *Arthrobacter* sp. (43). A particular geometry at the molybdenum- or iron-containing center may be characteristic of the enzymes which utilize ferricyanide so efficiently (8, 29).

The broad pattern of purine substrate oxidation, the extreme sensitivity to methanol, the arsenite inhibition, and the elimination of activity by the addition of tungstate to certain media all argue for a role for molybdenum in the new enzyme. The finding that a bacterial flavin-containing xanthine dehydrogenase preparation contains little or no molybdenum (30) is probably due to losses of the molybdenum

cofactor during purification, and this also may have happened in our preparations since the purified enzyme is relatively unstable but maintains a native protein structure. The most immediate future work proposed for the present enzyme is the determination and characterization of the various prosthetic components, especially molybdenum, and their contents per subunit. This will require isolation and examination of appreciable quantities of freshly prepared enzyme. Although the subunits are of the same size, they may not be identical and may contain different prosthetic components.

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