Purification and Characterization of a Prokaryotic Xanthine Dehydrogenase from *Comamonas acidovorans*†

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ABSTRACT: Xanthine dehydrogenase (XDH) is induced in *Comamonas acidovorans* cells incubated in a limited medium with hypoxanthine as the only carbon and nitrogen source. The enzyme has been purified to homogeneity using standard techniques and characterized. It contains two subunits with M_r values of 90 and 60 kDa. Gel filtration studies show the enzyme to have an $\alpha_2\beta_2$ native structure. No precursor form of the enzyme is observed on Western blot analysis of cell extracts obtained at various stages of enzyme induction. Metal analysis of the purified enzyme shows 1.1 Mo, 4.0 Fe, and 3.6 phosphorus atoms per $\alpha\beta$ protomer. Cofactor analysis shows the enzyme to contain a single molybdopterin mononucleotide and one FAD per $\alpha\beta$ protomer. Electron spin resonance and circular dichroism spectral studies of the oxidized and reduced forms of the enzyme suggest the Fe centers to be two nonidentical [2Fe-2S] clusters. Electron spin resonance signals due to Mo(V) and neutral FAD radical are also observed in the reduced form of the enzyme. Purified enzyme preparations ranged from 70% to 100% functionality. The enzyme is irreversibly inactivated by CN^- and is inhibited on incubation with allopurinol. With xanthine and NAD^+ as substrates the enzyme has a specific activity of 50 units/mg, a k_{cat} value of 120 s^{-1}, an activity/flavin ratio of 1930, and respective K_m values of 66 and 160 mM. Using 8-D-xanthine as substrate, a ^DV value of 1.8 is found with no change in K_m. Thus, the K_m and K_D values of the enzyme for xanthine are equal. These data show *Comamonas* XDH to exhibit structural properties similar to bovine milk xanthine oxidase/dehydrogenase and to chicken liver xanthine dehydrogenase. Although the bacterial enzyme exhibits a 6−7-fold greater turnover rate than the bovine or avian enzymes, the catalytic efficiencies (as measured by *V*/*K*) are similar for all three enzymes.

Xanthine dehydrogenase (1XDH)[1] (xanthine: NAD^+ oxidoreductase; EC 1.1.1.204) is a complex metalloflavoprotein which catalyzes the hydroxylation of xanthine to form uric acid with solvent oxygen rather than O_2 being the source for the incorporated oxygen. A molybdopterin moiety is at the catalytic site for oxidation with FAD at the site for NAD^+ reduction. In addition to these redox cofactors, the enzyme also contains two [2Fe-2S] centers which undergo oxidation−reduction during catalytic turnover. Most of our molecular information on this class of enzymes has come from the extensive literature on bovine milk xanthine oxidase (XO) and on chicken liver XDH. Recent work has shown the bovine milk oxidase probably occurs physiologically as the dehydrogenase and that the change in flavin reactivity (O_2 versus NAD^+) is a result of thiol oxidation and/or proteolysis that occurs during purification (Hunt & Massey, 1992). In addition to the extensive literature on the properties and reactivities of the bound cofactors, the gene sequences and deduced amino acid sequences of enzymes from at least seven species (all eukaryotes, including the human) have been determined [human liver, Ichida et al. (1993) and Xu et al. (1994); rat liver, Amaya et al. (1990); mouse liver, Terao et al. (1992); chicken liver, Sato et al. (1995); *Aspergillus nidulans*, Glatigny and Scazzocchio (1995); *Drosophila melanogaster*, Lee et al. (1987) and Keith et al. (1987); *Calliphora vicina*, Houde et al. (1989)]. These studies have suggested specific domains for cofactor localization which, in the case of the chicken enzyme, has been further documented by limited proteolysis experiments (Sato et al., 1995). To date, however, expression of active recombinant XDH has not been reported although attempts have been made with the rat liver enzyme in the baculovirus system (Nishino et al., 1993) where protein was expressed but in a largely inactive form. Advances in expression of recombinant protein would clearly provide new approaches to the structure and function of XDH since they would allow site-directed mutagenesis approaches and permit isotopic label incorporation experiments to aid in the spectroscopic characterization of the Mo and Fe/S centers .

An approach to provide new insights into the structure and function of XDH which has not been extensively used in the field is to compare in detail the properties of XDH preparations from prokaryotic sources with those of the well-studied eukaryote enzymes. A large number of bacteria exhibit XDH activity, and, in some cases, enzyme activity can be induced by growth of the microorganism in a limited medium with purines as sole carbon and nitrogen source (Woolfolk & Downard, 1977). From a survey of existing

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[1] Abbreviations: XDH, xanthine dehydrogenase; XO, xanthine oxidase; EDTA, ethylenediaminetetraacetic acid; NBT, nitro blue tetrazolium; DCIP, 2,6-dichlorophenol indophenol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; CD, circular dichroism; ESR, electron spin resonance; mT, millitesla.

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literature on bacterial xanthine dehydrogenases, those isolated from *Pseudomonas* appear to exhibit properties similar to the eukaryotic enzymes. Recent XDH preparations from *Pseudomonas putida* (Hettrich & Lingens, 1991) and *Pseudomonas aeruginosa* (Johnson et al., 1991a) show a cofactor stoichiometry similar to that reported for the eukaryotic enzymes. The molybdopterin cofactor from *P. aeruginosa* XDH has been shown to be identical (a pterin monophosphate) with that of the bovine milk enzyme. In a previous study, Sin (1975) purified XDH from *Pseudomona acidovorans* (ATCC 15667) and partially characterized his preparation with respect to kinetic properties and subunit molecular weight. Since the native molecular weight of *P. acidovorans* XDH appeared to be more similar to that of the eukaryote enzymes than the reported native size of the *P. putida* enzyme, we initiated a program to investigate the structure and properties of *P. acidovorans* XDH which is the subject of this communication. This bacterial strain has recently been reclassified as *Comamonas acidovorans* (American Type Culture Collection Catalogue, 1992) and retains the same ATCC number. An improved purification procedure is described which permits the isolation of essentially 100% functional XDH from this organism. The purified preparation exhibits a cofactor content and kinetic behavior quite similar to those of the milk and/or chicken enzymes. This system may be adventageous for future work involving recombinant enzyme technology which may provide new insights into the structure and mechanism of eukaryote xanthine dehydrogenases.

EXPERIMENTAL SECTION

Bacterial Growth and Purification of Xanthine Dehydrogenase. C. acidovorans (ATCC 15667) was maintained on plates containing Difco nutrient media. Large scale fermentations were done at the University of Georgia with cultures grown aerobically in a 100-L fermenter at 30 °C in a minimal medium described by Sin (1975) containing (per L): 6.8 g of KH_2PO_4; 7.2 g of Na_2HPO_4; 0.2 g of $MgSO_4 \cdot 7H_2O$; 0.1 mg of $MnCl_2 \cdot 4H_2O$; 0.2 mg of ferric ammomium citrate; 2 mg of $CaCl_2$; 10 mg of sodium molybdate; 2.5 g of hypoxanthine; 0.5 g of tryptic soy broth. After 24 h of growth, the cells were collected by centrifugation with a yield of 250 g of wet cell paste which was stored at −20 °C.

All purification steps, unless stated otherwise, were carried out at 4 °C. Frozen cells (250 g, wet weight) were thawed in 750 mL of 50 mM KPO_4, pH 7.8, 1 mM EDTA, and 2 mM mercaptoethanol (buffer A), and disrupted by sonication [1 min intervals with alternating 1-min cooling periods at 80% power (full power of 550 watts) for a total of 5 min sonication time using a half-inch probe]. The suspension was centrifuged at 45 000*g* for 30 min, and the supernatant was incubated at 55 °C for 10 min, and cooled to 4 °C on ice for 30 min. After centrifugation (45 000*g* for 30 min) to remove the precipitated protein, the supernatant was loaded onto a DE-52 column (5 × 25 cm) equilibrated in buffer A. The column was washed with 1 L of buffer A and eluted at 1 mL/min with a linear gradient from 0 to 1 M NaCl (1 L each) in buffer A. Active fractions were pooled and concentrated to less than 10 mL by ultrafiltration with a YM-30 membrane (Amicon, Lexington, MA). The protein solution was loaded onto a Sephacryl S-300 column (2.5 × 78 cm) and eluted with 100 mM KPO_4, pH 7.8, 1 mM EDTA, 2 mM mercaptoethanol, and 100 mM NaCl (buffer B) at a flow rate of 12 mL/h. Active fractions were pooled and dialyzed against buffer A and then applied to a Cibacron Blue agarose column (2.1 × 29 cm) equilibrated in buffer A. The column was washed with 100 mL of buffer A and eluted at 1 mL/min with a linear gradient from 0 to 0.6 M NaCl in buffer A. Active fractions were pooled and applied to a Phenyl Sepharose column (2.1 × 20 cm) equilibrated in buffer A. The column was washed with 100 mL of buffer A and eluted at 1 mL/min with 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 2 mM mercaptoethanol (buffer C). Fractions exhibiting enzymatic activity were combined and stored at −80 °C. The specific activities of the purified enzyme from several preparations were consistently found to be 50 units/mg.

Protein concentrations were determined using either the Biuret (Gornall et al., 1949) or Bearden methods (Bearden, 1978) with bovine serum albumin as standard.

Steady State Enzyme Assays and Data Analysis. All assays were carried out spectrophotometrically in 100 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, at 25 °C. For the various spectrophotometric assays (1-cm light path), substrate concentrations, wavelengths, and extinction coefficients used were as follows: xanthine:NAD^+ assay, 0.6 mM xanthine (Sigma), 2 mM NAD^+ (Sigma), $\Delta\epsilon_{295} = 9.6\ mM^{-1}\ cm^{-1}$; xanthine:oxygen assay, 0.6 mM xanthine, air-saturated buffer, $\Delta\epsilon_{295} = 9.6\ mM^{-1}\ cm^{-1}$; xanthine: DCIP assay, 0.6 mM xanthine, 50 mM DCIP, $\Delta\epsilon_{600} = 21\ mM^{-1}\ cm^{-1}$; xanthine: ferricyanide assay, 0.6 mM xanthine, 2.5 mM ferricyanide (Baker Analyzed Reagent), $\Delta\epsilon_{420} = 1000\ M^{-1}\ cm^{-1}$; xanthine:NBT assay, 0.6 mM xanthine, 0.4 mM NBT (Sigma), $\Delta\epsilon_{550} = 28.6\ M^{-1}\ cm^{-1}$; NADH:oxygen assay, 0.5 mM NADH (Sigma), 240 mM O_2, $\Delta\epsilon_{340} = 6.2\ mM^{-1}\ cm^{-1}$; NADH:DCIP assay, 0.5 mM NADH, 50 mM DCIP, $\Delta\epsilon_{600} = 21\ mM^{-1}\ cm^{-1}$; NADH:ferricyanide assay, 1 mM NADH, 2.5 mM ferricyanide, $\Delta\epsilon_{420} = 1000\ M^{-1}\ cm^{-1}$; NADH:NBT assay, 1 mM NADH, 0.4 mM NBT, $\Delta\epsilon_{550} = 28.6\ M^{-1}\ cm^{-1}$.

[8-^{2}H]Xanthine was prepared as described previously by D'Ardenne and Edmondson (1990). Steady state kinetic data were fitted to the Michaelis−Menten equation using a nonlinear regression program (ENZFIT, Elsevier). Enzyme functionality was determined by comparison of the level of reduction of anaerobic enzyme solution at 450 nm after the addition of excess xanthine with the level of reduction after the addition of excess sodium dithionite. AFR (activity to flavin ratio) was calculated by determining the change in absorbance at 295 nm for the conversion of xanthine to uric acid by XDH and division by the A_{450} of the enzyme used in the assay. The AFR is directly proportional to the functionality of the enzyme preparation (Edmondson et al. 1972).

Molecular Mass Determination. The Stokes radius of the purified enzyme was determined by gel-filtration chromatography (1.6 × 80 cm) on Sepharose 6B in 50 mM Tris-HCl, pH 7.8, containing 100 mM KCl. The column was calibrated with apoferritin (443 kDa), amylase (200 KDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) (Sigma). SDS−PAGE (7.5% gels) were performed according to Laemmli (1970). Proteins were detected by staining with Coomassie Brilliant Blue.

Western Blot Analysis of XDH Synthesis. A culture of *C. acidovorans* was grown in bacto nutrient broth (Difco) to late-log phase. The cells were separated by centrifugation

and resuspended in the minimal medium (see Bacterial Growth in Results). At various time intervals, aliquots of culture were withdrawn, centrifuged and the cells resuspended in buffer A, broken by sonication in the presence of 2 mM PMSF, 2 mM benzamidine-HCl, and 2 trypsin inhibitor units (TIU) of aprotinin/mL. The specific activities of the sample crude extracts were determined, and the crude extracts were subjected to immunoelectrophoresis and SDS−PAGE. Electrophoretically separated proteins were blotted onto a nitrocellulose membrane (0.45 mm, Bio-Rad) for 1 h at 100 V (Towbin et al., 1979). The two subunits of XDH were visualized by immunostaining, using goat anti-rabbit IgG peroxidase conjugate (Sigma) as the secondary antibody, according to Bio-Rad's procedures. Rabbit polyclonal antiserum raised against purified *C. acidovorans* XDH was supplied by Pel-Freez Biologicals, Rogers, AR. Rocket immunoelectrophoresis was performed in 1% agarose gels prepared in 15 mM barbital buffer, pH 8.6, and run at 3.5 mA for 15 h at 4 °C in the same buffer (Garvey et al., 1977).

Cofactor, Metal, and Phosphate Analysis. Bovine milk xanthine oxidase [purified according to Massey et al. (1969)] was used as a reference material in all analyses. Purified XDH from *C. acidovorans* was passed through a small Chelex-100 column to remove any adventitious metals prior to metal analysis. Iron and flavin concentrations were determined as described by Vanoni et al. (1992). The flavin coenzyme identity and content on liberation from the denatured enzyme by heat treatment was determined by thin-layer chromatography (*n*-butanol:H_2O:acetic acid, 12/5/3, v/v/v) and by reconstitution of apoglucose oxidase activity (Swoboda, 1969; Morris et al., 1983). Quantitation of the molybdenum content was carried out by the dithiol method used for milk XO (Massey et al., 1969) using a molybdenum atomic absorption standard solution (Sigma).

The molecular weight of the molybdopterin cofactor (released from XDH by denaturation in 2 M guanidine-HCl) was determined as described previously (Krüger & Meyer, 1987; Krüger et al., 1987). The pterin cofactor was oxidized and isolated by method of Aguilar et al. (1992). The phosphorus content of purified XDH was determined using the method of Bartlett (1959). XDH (5−10 nmol) was passed through a small Sephadex G-25 column equilibrated with 50 mM Tris-HCl, pH 7.8 (phosphate-free) buffer. The enzyme was denatured by 5% (w/v) trichloroacetic acid to release all the cofactors. Total phosphorus compositions of the supernatant and pellet from the acid precipitation as well as the native enzyme were determined. A phosphate solution (Phosphate Standard, LabChem Inc., Pittsburgh, PA) was used as standard.

Spectroscopic Methods. Absorption spectra were recorded at ambient temperature on a Perkin Elmer (λ2) spectrometer interfaced to an IBM XT personal computer. Circular dichroism spectra were measured at 26.6 °C on an AVIV 60DS instrument interfaced to an AT&T model 6300 computer.

Low-temperature ESR spectra (70 and 10 K) were recorded on a Bruker ER 200D-SRC spectrometer equipped with an Oxford 3120 Instrument continuous flow cryostat. ESR spectra (140 K) were recorded on a Varian E-4 EPR spectrometer using a Varian nitrogen flow-through temperature controller. Quantitative analysis of the spectral data were performed by double integrations using a Cu EDTA standard and software written in Dr. B. H. Huynh's laboratory in the Department of Physics.

Table 1: Purification of Xanthine Dehydrogenase from *C. acidovorans*

purification step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purification (*n*-fold)
crude extract	20151	9353	0.464	100	1.0
heat treatment	6229	9068	1.456	97	3.1
DE-52	983	5628	5.725	60	12.3
Sephacryl S-300	664	4319	6.507	46	14.0
Cibacron Blue agarose	65	3036	46.500	33	100.0
Phenyl Sepharose	36	1796	50.000	19	108.0

RESULTS

Growth of Bacteria and Purification of XDH. *C. acidovorans* was cultured in a mineral salts medium of same composition used by Sin (1975) with hypoxanthine as the only carbon and nitrogen source. Cell growth reached late-log phase after 24 h with an A_{600} value of 1.0. The yield of wet cell paste from 100 L of culture was typically 250−300 g.

In preliminary experiments in which cells were initially grown on nutrient media and then transferred to a limited medium containing hypoxanthine, XDH activity was induced as judged by an increase in specific activity from 0.02 to 0.46 units/mg in the crude extract. Sin (1975) reported the existence of four bands exhibiting xanthine:NBT oxidoreductase activity on native gel electrophoresis of crude cell extracts made from cells grown on hypoxanthine. In our hands, cells grown on nutrient media exhibit three observable activity bands. One is a minor, slowly migrating band that increases substantially on induction with hypoxanthine and migrates in an identical manner as the purified enzyme reported here. The other two observable activity bands include a reasonably strong band and a weakly-staining band that migrate similarly and exhibit a higher migration than the inducible XDH. The relative staining intensities of these two bands decrease on hypoxanthine induction. Thus, as multiple forms of XDH are observed in *P. putida* (Woolfolk, 1985; Woolfolk & Downard, 1977), *C. acidovorans* also contains at least three forms of XDH, only one of which is inducible by hypoxanthine and is the subject of this communication. The other major noninducible form presumably does not function catalytically with NAD^+ as electron acceptor since low activity is found in crude extracts of noninduced cells.

The purification procedure described by Sin (1975) did not work successfully in our hands. The major problem was a difficulty in obtaining clean ammonium sulfate fractionations of the crude extract, apparently due to polysaccharide or nucleic acid materials which gave a "slimy" nature to the cellular extract. The procedure worked out in our laboratory is relatively fast, reproducible, and utilizes both affinity and hydrophobic column chromatography (Table 1). The results in Table 1 summarize a typical purification procedure from 280 g of wet cell paste. The specific activity of enzyme purified by this procedure is 50 units/mg, which is two-and-one-half times greater than that (20 units/mg) reported by Sin (1975). The purification procedure described here (when compared to that reported by Sin) resulted in higher fold purification (108 versus 65) but gave a lower yield (19% versus 35%).

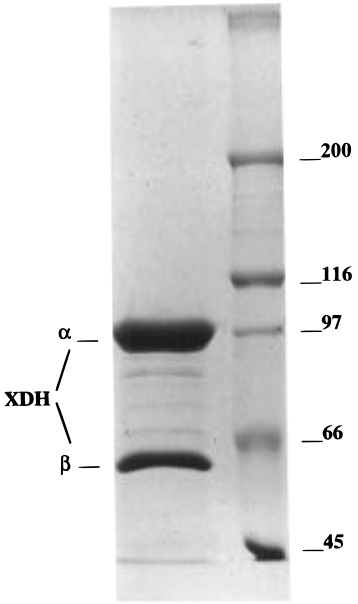

FIGURE 1: Molecular mass determination of *C. acidovorans* XDH. SDS−PAGE of purified XDH (10 μg) and of standard proteins myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), serum albumin (66 kDa), and ovalbumin (45 kDa).

Approximately 35−40 mg of homogeneous XDH is isolated from 300 g of wet cell paste.

The purity of the preparation was assessed by gel electrophoresis under native conditions and exhibits a single band. The enzyme is stable during storage at −80 °C for at least 6 months with no observable proteolytic degradation or loss in activity.

Molecular Mass and Subunit Composition. The Stokes radius of native XDH was determined to be 287 ± 14 kDa by gel filtration. This value is slightly greater than that determined by gel electrophoresis (275 kDa) (Sin, 1975). The size of native *C. acidovorans* XDH therefore appears to be smaller than the native structures of the *P. putida* (550 kDa) (Hettrich & Lingens, 1991) or *Pseudomonas synxantha* A3 (540 kDa) (Sakai & Jun, 1979) enzymes but similar to the mammalian and avian enzymes (α_2 structures of 300 kDa) (Nagler & Vartanyan, 1976; Rajagopalan & Handler, 1967).

SDS−PAGE reveals that the enzyme is comprised of two different subunits (α and β) with respective molecular masses of 90 and 60 kDa (Figure 1), which is similar to the results (81 and 63 kDa) reported by Sin (1975). In agreement with Sin's results, we also find that native *C. acidovorans* XDH exists in an $\alpha_2\beta_2$ structure. Each $\alpha\beta$ protomer (with a molecular weight of 150 kDa) corresponds to the monomeric molecular weight (150 kDa) of the mammalian and avian enzymes. Other bacterial XDH preparations show structural differences either in native enzyme size (composition), or subunit sizes compared to those of mammalian and *C. acidovorans* enzymes. *P. putida* XDH has an $\alpha_4\beta_4$ structure with two subunits in similar size to *C. acidovorans* enzyme (87 and 52 kDa), which results in a native size of 550 kDa (Hettrich & Lingens, 1991). *P. putida* Fu1 XDH also has an $\alpha_4\beta_4$ structure with two smaller subunits (55 and 25 kDa), which results in a native size of about 300 kDa (Koenig & Andreesen, 1990). *P. putida* 40 XDH is reported to have a native molecular mass of 255 kDa with only a single subunit of 72 kDa, which apparently assembles into an α_3 native structure (Woolfolk, 1985). *P. synxantha* A3 XDH exhibits

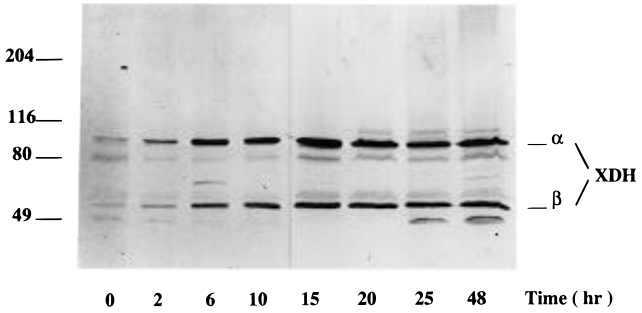

FIGURE 2: Time course of XDH synthesis after induction by hypoxanthine. Samples taken at different time intervals were applied to SDS−PAGE followed by Western blotting (20 μg of total protein in each lane).

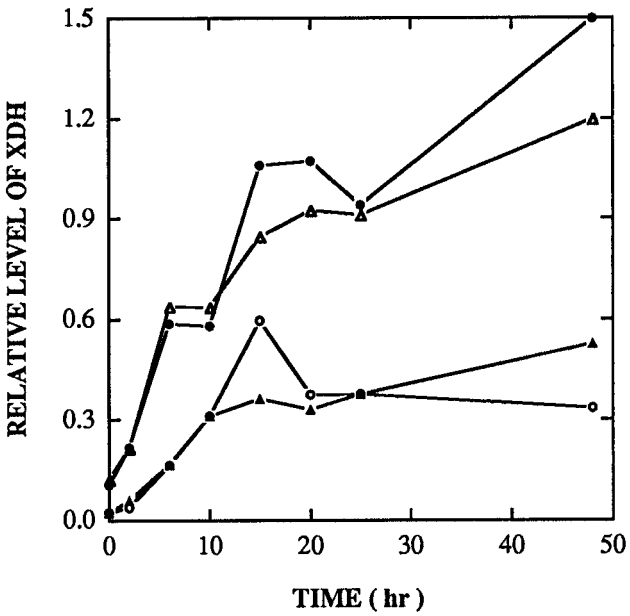

FIGURE 3: Relative levels of xanthine dehydrogenase by Western blot (●, α subunit; ○, β subunit); rocket immunoelectrophoresis assay (▲, height); and activity assay (△, specific activity).

a native molecular weight of 540 kDa, with subunit sizes of 76 and 54 kDa, which suggests an $\alpha_4\beta_4$ structure (Sakai & Jun, 1979).

One possible explanation for the $\alpha\beta$ structure of *C. acidovorans* XDH is that the protomer is initially synthesized as a 150 kDa form which is subsequently processed by proteolysis to the 90 and 60 kDa subunits. To investigate this possibility, the synthesis of XDH was monitored at different time intervals after induction by transferring cells grown on a nutrient medium to a minimal medium containing hypoxanthine. Aliquots of cells were withdrawn from the culture at various times and disrupted by sonication, and the extract was analyzed for XDH activity and for levels of enzyme synthesis from Western blot analysis of the crude extracts. Figure 2 shows a Western blot of cell extracts from samples collected at 0, 2, 6, 10, 15, 20, 25, and 48 h after induction. Immunoreactive bands observable at any time are those corresponding to the α and β subunits of xanthine dehydrogenase. No higher molecular weight precursor form is observed. Densitometry of the intensities of the α and β subunits shows that the level of protein synthesis correlates with the rise in specific activity (Figure 3), which shows cofactor incorporation not to be rate limiting in production of functional enzyme.

Table 2: Cofactor Analysis of *C. acidovorans* Xanthine Dehydrogenase

cofactor	no. of moles/$\alpha\beta$ protomer[a]
Fe	4.0
FAD	1.0
molybdenum	1.1
P_i in trichloroacetic acid supernatant	2.6
P_i in trichloroacetic acid pellet	1.2
P_i in native protein	3.6

[a] Based on an $\epsilon_{450} = 37\,000\ M^{-1}\ cm^{-1}$.

Metal, Cofactor, and Phosphate Content of C. acidovorans XDH. Our current view of mammalian and avian xanthine dehydrogenases is that they contain one molybdopterin cofactor, one FAD coenzyme, and two distinct [2Fe-2S] centers per 150 kDa protomer (Massey et al., 1969; Rajagopalan & Handler, 1967). Studies from this laboratory have shown the presence of a phosphoseryl residue in bovine milk XO (Davis et al., 1984) and in chicken liver XDH (Schieber & Edmondson, 1993), which has been suggested to have a catalytic function (D'Ardenne & Edmondson, 1990b). Thus, it is of interest to examine *C. acidovorans* XDH for cofactor, metal, and covalent phosphorus content to more clearly define its similarities and differences with the eukaryotic enzymes. Studies of *P. putida* XDH show a cofactor ratio of 1 mol of Mo:4 mol of Fe:4 mol of acid-labile S:1 mol of FAD per mol of $\alpha\beta$ protomer (139 kDa), although the enzyme has an $\alpha_4\beta_4$ structure. XDH isolated from the *P. putida* Fu1 strain differs in that it contains a cytochrome *b* instead of FAD and a different ratio of 1.6 mol of Mo:0.9 mol of cytochrome *b*:5.8 mol of Fe:2.4 mol of labile sulfur per mol of native enzyme (Koenig & Andreesen, 1990). The results of *C. acidovorans* XDH cofactor analysis are summarized in Table 2. A ratio of 4 mol of Fe:1 mol of flavin:1 mol of Mo per $\alpha\beta$ protomer is determined by metal and flavin analysis. The flavin cofactor is identified to be FAD on thin-layer chromatography where the flavin extract from the enzyme co-migrated with FAD. The FAD identity was further confirmed by apoglucose oxidase reconstitution experiments showing that purified enzyme contained 0.99 mole of FAD/$\alpha\beta$ protomer.

Gel-filtration analysis of the molybdopterin cofactor released from *C. acidovorans* XDH reveals a molecular weight value of approximately 320, which shows it to be a monophosphate rather than a dinucleotide form. Furthermore, the pterin cofactor isolated from *C. acidovorans* XDH co-elutes in reverse-phase HPLC with the molybdopterin isolated from bovine milk XO. The two isolated pterins exhibit (after oxidation) identical fluorescence spectral properties with emission maxima at 460 nm and excitation maxima (corrected) at 225, 285, and 390 nm, which are characteristic of molybdopterin. Thus, *C. acidovorans* XDH contains molybdopterin mononucleotide which is identical to those of *P. aeruginosa* XDH (Johnson et al., 1991a,b) and bovine milk XO (Kramer et al., 1987) rather than molybdopterin dinucleotide.

The native enzyme, the supernatant, and the pellet obtained from trichloroacetic acid denaturation of enzyme were analyzed for phosphorous content and shown to have 3.6, 2.6, and 1.2 mol of phosphorous/$\alpha\beta$ protomer, respectively. The stoichiometric phosphate content in the pellet from acid-denatured enzyme suggests that it is covalently linked to xanthine dehydrogenase. The presence of a covalent phosphate in *C. acidovorans* XDH has been further confirmed by ^{32}P labeling studies of the enzyme in cell culture (Q. Xiang and D. E. Edmondson, manuscript in preparation). The phosphate in the supernatant of acid-denatured enzyme accounts for the pyrophosphate moiety of the FAD (two phosphates per FAD) and phosphomonoester of molybdopterin (one phosphate per molybdopterin monomer). From consideration of the stoichiometry of 1 mol of Mo:1 mol of FAD:2.6 mol of cofactor phosphate:1 mol of $\alpha\beta$ protomer ratio, we conclude that molybdopterin cofactor exists as a monomeric form with one Mo atom per one molybdopterin moiety rather than a 2:1 pterin:metal ratio as found in the tungsten-dependent *Pyroccocus furiosus* aldehyde−ferredoxin oxidoreductase (Chan et al., 1995).

Table 3: Kinetic Parameters of *C. acidovorans* Xanthine Dehydrogenase

kinetic parameter	value[a]
K_m xanthine	$66 \pm 6\ \mu M$
K_m NAD^+	$160 \pm 12\ \mu M$
k_{cat}	$120 \pm 3\ s^{-1}$
$^Dk_{cat}$	1.8 ± 0.02
$^Dk_{cat}/K_m$	1.8 ± 0.02
K_d xanthine	$66 \pm 6\ \mu M$
activity:flavin ratio	1930
specific activity	50 units/mg
functionality	94%−100% (most preparations) 70% (early preparations)

[a] All kinetic experiments were performed at 25 °C in 50 mM KP_i at pH 7.8.

Kinetic Properties. The kinetic properties of *C. acidovorans* XDH are summarized in Table 3. The K_m values for xanthine (66 μM) and NAD^+ (160 μM) are similar to those values previously published (70 and 120 μM for xanthine and NAD^+, respectively) by Sin (1975). However, the maximum velocity (50 μmol per NAD^+ reduced per min per mg of protein) is two-and-a-half-fold greater than that previously reported. These parameters are quite comparable to those of the *P. putida* enzyme (80 and 70 μM for xanthine and NAD^+ K_m values; 31 units/mg for specific activity) (Hettrich & Lingens, 1991).

A direct comparison of the catalytic constants of *C. acidovorans* XDH with those of the eukaryotic enzyme requires that the level of functionality of the enzyme is known. It is well-known that both functional and nonfunctional forms of eukaryotic XDH exist in purified preparations as a result of the presence or absence, respectively, of a terminal sulfur ligand on the active site Mo center (Edmondson et al., 1972). The level of functionality of *C. acidovorans* XDH preparations was routinely determined by monitoring the level of enzyme reduction on the addition of xanthine relative to that observed on the addition of $Na_2S_2O_4$. The level of functionality of the purified enzyme ranged from 70% (in the initial purification) to 90%−100% in subsequent purifications. These latter values were consistently obtained and are higher than the usual 60%−70% functionality observed in preparations of milk XO or chicken XDH. Correcting for the level of any nonfunctional enzyme, k_{cat} for *C. acidovrans* XDH is found to be 120 s^{-1}, a value approximately 10−20-fold greater than the k_{cat} values reported for milk XO (18 s^{-1}; Olson et al., 1974), chicken XDH (19 s^{-1}; Hille & Massey, 1981), milk XDH (6.3 s^{-1}; Hunt & Massey, 1992), or rat XDH (13.5 s^{-1}; Saito & Nishino, 1989). The K_m value exhibited for xanthine by *C.*

Table 4: Specific Activities of Purified *C. acidovorans* Xanthine Dehydrogenase in Different Assay Systems

	specific activity[a] (units/mg)	
electron acceptor	xanthine → acceptor	NADH → acceptor
NAD^+	50.0	
O_2	1.2	2.9
DCIP	2.2	30.6
ferricyanide	9.5	58.0
NBT	0.5	1.0

[a] Activities are expressed as micromoles of reducing substrate ($2e^-$) oxidized min^{-1}/mg of XDH at 25 ± 0.1 °C and pH 7.8.

acidovrans XDH is also approximately 10-fold higher than those measured for the above eukaryotic XDH preparations. Thus, the catalytic efficiency of *C. acidovorans* XDH (as measured by *V*/*K*) is similar to those of the eukaryotic enzymes.

The action of *C. acidovorans* xanthine dehydrogenase on [8-^{2}H]xanthine has been measured and compared with that of [8-^{1}H]xanthine. A $^Dk_{cat}$ value of 1.8 with no change in K_m for xanthine is found. From these data the K_d for xanthine binding to the catalytic site could be calculated by the method of Klinman and Matthews (1985). Since $^Dk_{cat}$ equals $^D(V/K)$ in *C. acidovorans* XDH, the K_d for xanthine binding equals the K_m for xanthine, which is 66 μM. It is of interest that the K_d for xanthine, binding to the Mo center of *C. acidovorans* XDH is only 2-fold tighter than the K_d estimates for xanthine binding to milk XO or chicken XDH (K_d = 120 μM) (D'Ardenne & Edmondson, 1990a).

Allopurinol (100 μM) rapidly inactivates *C. acidovorans* XDH under anaerobic conditions as found for the mammalian and avian enzymes because of the inhibitory binding of the product (alloxanthine) to Mo(IV) (Massey et al., 1970; Edmondson et al., 1972). Upon exposure to O_2, a partial activation of the allopurinol-inhibited enzyme occurs to 25% of its original level with no additional restoration of activity observable after several hours. Ferricyanide (100 μM) treatment also reactivates the enzyme slowly (to 30% of its original level after 90 min), presumably by oxidation of Mo(IV) to Mo(VI) and the subsequent dissociation of alloxanthine. The failure to observe complete restoration of activity under oxidative conditions as previously shown for bovine milk XO has not been further investigated. CN^- irreversibly inactivates *C. acidovorans* XDH (as it does to the bovine and chicken enzymes) by removal of the terminal sulfur ligand from the Mo center (Massey & Edmondson, 1970). The half-time for inactivation is 2.5 min with 5 mM CN^- at 25 °C and 1.7 min with 10 mM CN^-. The inactivation half-time is shorter than that of *P. putida* XDH (3.0 min with 10 mM CN^-) (Hettrich & Lingens, 1991) and milk XO (5.7 min with 9 mM CN^-) (Massey & Edmondson, 1970).

Electron Acceptor Specificity. The relative efficiencies of several electron acceptors in facilitating the oxidation of xanthine and NADH by *C. acidovorans* XDH are shown in Table 4. It is generally accepted that, in xanthine oxidase/dehydrogenase, xanthine acts at the molybdenum center and NAD^+, NADH, and O_2 act at the flavin site. The remaining electron acceptors used in Table 4 would not necessarily react at a single site only.

C. acidovorans XDH shows both similarities and variations in electron acceptor specificities as compared with those of chicken XDH (Kanda & Rajagopalan, 1972). In the case of the *C. acidovorans* enzyme, DCIP is a better electron acceptor when NADH is the reducing substrate than when xanthine is the reducing substrate (Table 4) which contrasts with the chicken enzyme where the rate of DCIP reduction is independent of whether NADH or xanthine is the reductant. NBT is a sluggish electron acceptor for either chicken or *C. acidovorans* XDH. Although ferricyanide is a poor electron acceptor in chicken XDH assays, it is a better electron acceptor when NADH is the reducing substrate with *C. acidovorans* XDH than when xanthine is used (Table 4). *C. acidovorans* XDH has a very limited xanthine oxidase activity (1.2 units/mg) compared to 50.0 units/mg XDH activity. It catalyzes the dehydrogenase reaction almost exclusively as observed with chicken XDH.

These results show that *C. acidovorans* XDH exhibits a reasonable diaphorase activity; however the xanthine oxidoreductase activity is reasonably specific for NAD^+ as the electron acceptor, relative to other nonspecific electron acceptors commonly used.

Absorption and CD Spectral Properties. The absorption spectrum of the purified *C. acidovorans* XDH, shown in Figure 4A, closely resembles those of *P. putida* XDH and *P. aeruginosa* XDH (Hettrich & Lingens, 1991; Johnson et al., 1991a). It also is essentially identical to those of bovine milk XO and chicken liver XDH (Massey et al, 1969; Rajagopalan & Handler, 1967) and is characteristic of iron−sulfur flavoproteins. In milk XO, the two iron−sulfur centers absorb approximately equally in the 300−700 nm range (Hille et al., 1985) with absorption maxima at 467, 420, and 315 nm. Oxidized FAD also absorbs in the 300−500 nm spectral region with maxima at 450 and 370 nm. The similarity of absorption spectra suggests the presence of iron−sulfur centers in the *C. acidovorans* enzyme. The ratio A_{280}:A_{450} of the purified enzyme is 5.5, similar to the values of purified bovine and chicken enzymes and lower than the value (6.4) reported by Sin (1975) for his preparation.

The addition of xanthine results in the reduction of the enzyme with extensive bleaching in the visible absorption region due to reduction of the iron−sulfur and flavin centers (Figure 4A). The spectrum of the xanthine-reduced form is similar to the dithionite-reduced spectra of *P. putida*, bovine, and chicken enzymes. The addition of dithionite does not result in any additional bleaching of the visible absorption spectrum. Increased absorbance in the region 500−650 nm is seen after reduction with xanthine. This increased absorbance is due to the formation of the neutral flavin semiquinone (also observable by ESR, see below) in several other xanthine dehydrogenases but is not normally observed in bovine milk XO.

It is well-known from studies on milk XO and its deflavo form that the visible CD spectra of the oxidized forms are dominated by contributions from the two oxidized [2Fe-2S] centers (Massey & Palmer, 1969; Komai et el., 1969). In general, [2Fe-2S] centers exhibit considerably more optical activity than [3Fe-4S] or [4Fe-4S] centers (Vanoni et al., 1992).

To obtain information on the structure(s) of the iron−sulfur centers in *C. acidovorans* XDH, the circular dichroism spectra were measured in the visible region in both the oxidized and reduced forms as shown in Figure 4B. The spectrum of the oxidized enzyme in Figure 4B exhibits strong negative dichroic bands at approximately 350−400 and 520−580 nm and intense positive bands between 400 and 500

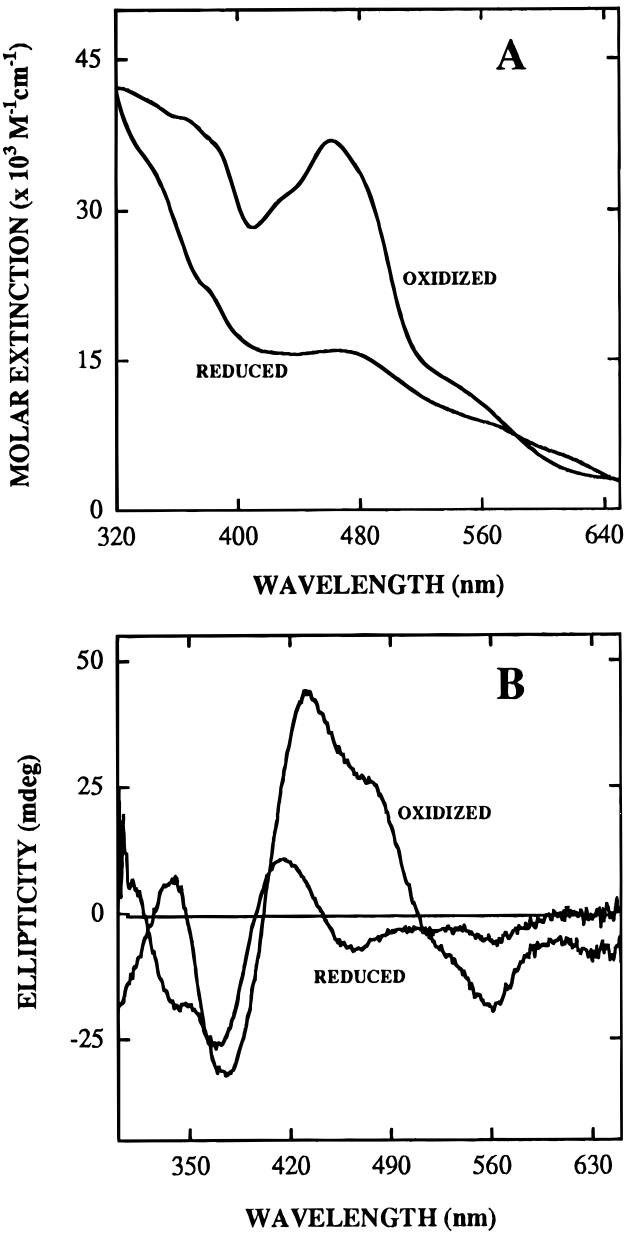

FIGURE 4: Spectral properties of *C. acidovorans* XDH in the visible region. (A) Absorption spectrum in the oxidized state (as isolated) and in the reduced state (reduced with xanthine). (B) CD spectrum in the oxidized state and after reduction with xanthine. No further spectral changes in the xanthine-reduced enzyme sample were observed on adding a few crystals of $Na_2S_2O_4$. The enzyme concentration is 27.6 μM in 50 mM Tris-HCl, pH 7.8.

nm. From the various maxima and inflections of the spectrum, transitions can be identified at 380 (−), 430 (+), 470 (+), and 560 (−) nm. On reduction with xanthine, the spectrum changes markedly with less intense transitions at 370 (−), 420 (+), 460 (+), and 565 (−) nm. The visible CD spectra of oxidized and reduced *C. acidovorans* XDH are very similar in shape and intensity to those of bovine milk XO and deflavo XO (Palmer & Massey, 1969; Komai et al., 1969), which are known to contain two [2Fe-2S] centers.

With consideration of the CD data and the Fe analytical data (see above), these results suggest the presence of two distinct [2Fe-2S] centers in *C. acidovorans* XDH as found with the eukaryotic xanthine dehydrogenases, which is further documented by low-temperature ESR studies of the reduced enzyme.

ESR Studies. ESR spectroscopy has been used to study (a) flavin semiquinone, (b) Mo(V), and (c) iron−sulfur centers of XO/XDH. These studies have shown that eukaryotic enzymes have two non-identical [2Fe-2S] centers with one center (Fe/S II) being observable only at temperatures below 40 K while the other center (Fe/S I) is observable at 77 K (Palmer & Massey, 1969). Thus ESR measurements at various temperatures were carried out to determine whether the [2Fe-2S] centers in *C. acidovorans* XDH suggested by CD spectra (see above) exhibit similar ESR properties with those of eukaryotic XDH or XO.

No ESR signals were observed in the resting form of *C. acidovorans* XDH. Therefore, the enzyme does not contain a [3Fe-4S] center which is known to be paramagnetic and to give a sharp axial signal at low temperature (Emptage et al., 1980; Huynh et al., 1980; Teixeira et al., 1989; Kent et al., 1982). The enzyme (70% functional) was reduced by xanthine under anaerobic conditions, and spectra were taken at 10, 70, and 140 K. After these spectra were obtained, the sample was thawed, further reduced by a few crystals of dithionite, and refrozen, and ESR spectra were measured at the above temperatures. All data were quantified by double integration and compared with a Cu EDTA standard run under identical, nonsaturating conditions.

The ESR spectrum of xanthine-reduced enzyme at 140 K (Figure 5A) shows only signals attributable to the neutral flavin semiquinone ($g = 2.0$) and to Mo(V) signals ($g_{av} = 1.97$) (Bray, 1980). Flavin radical has a g value of 2.00 and a peak to peak line width of approximately 2 mT, characteristic of the neutral form of the semiquinone (Palmer & Massey, 1969). The total integrated spin intensity of the signals is 0.63/$\alpha\beta$ protomer. Dithionite-reduced enzyme (Figure 5A) exhibits a smaller level of flavin semiquinone (about 40% that of the xanthine-reduced sample) due to the further reduction of the flavin semiquinone to the ESR-silent hydroquinone. In contrast, more Mo(V) is observed due to the formation of the "slow" Mo(V) signal (Bray, 1980) which results from reduction of the nonfunctional Mo center (about 30% of total Mo) by dithionite.

When the temperature is lowered to 70 K, an additional ESR signal becomes observable with g values of 1.90 and 1.93 in enzyme samples reduced either by xanthine or by dithionite (Figure 5B). This signal is similar to that of the [2Fe-2S] I center ($S = {}^1/_2$) found in milk XO (Hille et al., 1985). The signal in the $g = 2.0$ region is probably a composite of the [2Fe-2S] I signal and the flavin semiquinone. The total integrated spin intensity of xanthine-reduced enzyme is 1.4−1.5 and per $\alpha\beta$ promoter, and that for the dithionite-reduced enzyme is 1.6 per $\alpha\beta$ protomer. One would expect the spin quantitation of reduced [2Fe-2S] I signal to be 0.7 in the substrate-reduced sample and 1.0 in the dithionite-reduced sample since the sample is 70% functional from previous work on bovine milk XO (Edmondson et al., 1972). Thus, the similarity in total spin quantitation for the two reduced samples (1.4−1.6) is expected since the substrate-reduced sample has a higher level of flavin radical than the dithionite-reduced sample. Comparison of integrated spin intensities at the two temperatures (140 and 70 K) shows an increase of about 1 spin/mol of $\alpha\beta$ protomer due to the appearance of the ESR signal of a single [2Fe-2S] center comparable to Fe/S I in bovine milk XO at 70 K.

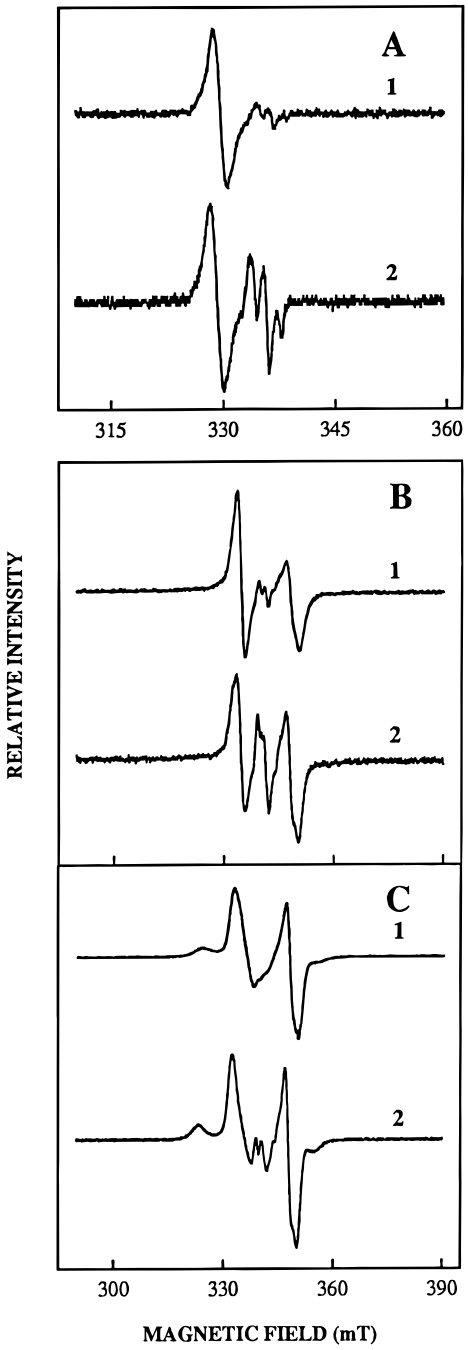

FIGURE 5: ESR spectral properties of reduced *C. acidovorans* XDH at various temperatures. Enzyme concentrations are 93 μM in 50 mM Tris-HCl, pH 7.8. (A) Xanthine-reduced (1) and dithionite-reduced (2) (displayed at a 3-fold higher gain than the spectrum 1) taken at 140 K; microwave frequency, 9.05 GHz; microwave power, 0.258 mW; modulation frequency, 100 KHz; receiver gain, 5×10^4; modulation amplitude, 0.4 mT. (B) Xanthine-reduced (1) and dithionite-reduced (2) taken at 70 K; microwave frequency, 9.43 GHz; microwave power, 0.2 mW; modulation frequency, 100 KHz; receiver gain, 5×10^4 modulation amplitude, 1 mT. (C) Xanthine-reduced (1) and dithionite-reduced (2) taken at 10 K; microwave frequency, 9.43 GHz; microwave power, 0.2 mW; modulation frequency, 100 KHz; receiver gain, 2×10^4; modulation amplitude, 1 mT.

As found with milk XO (Hille et al., 1985) and chicken XDH (Barber et al., 1980), lowering the temperature of either xanthine or dithionite-reduced *C. acidovorans* XDH to 10 K (Figure 5C) results in the appearance of a second ESR signal with properties consistent with [2Fe-2S] II. Although complete analysis of the spectral properties of this second iron−sulfur center is beyond the scope of this paper, it appears to be very similar with the [2Fe-2S] center in eukaryotic XDH preparations with the exception of a slight difference in g_3 (2.07 for *C. acidovorans* XDH and 2.11 for milk XO). Spin quantitation of the xanthine-reduced sample is 1.1 spins/$\alpha\beta$ protomer, which is slightly lower than the expected 1.4 [due to two [2Fe-2S] centers of 70% functional enzyme and little contribution from the flavin radical or Mo-(V) signals since they would be extensively saturated at this temperature]. The integrated spin intensity of the dithionite-reduced sample is 1.5 spins/$\alpha\beta$ protomer. This increase in intensity relative to the substrate-reduced sample is consistent with the estimated functionality of the preparation at 70%.

The ESR data show the presence of two [2Fe-2S] centers with relaxation properties and *g*-values similar to those of eukaryotic XDH. The level of neutral flavin radical observed on substrate reduction is also similar to that observed with chicken XDH (Kanda & Rajagopalan, 1971).

DISCUSSION

The xanthine dehydrogenase/oxidase system has received extensive study with the bovine milk oxidase and chicken and rat dehydrogenase, due to their ready availability in reasonable quantities, and have become the standards of comparison for xanthine oxidizing enzymes isolated from other sources. A more detailed understanding of the relative functional role of the redox cofactor (molybdopterin, two [2Fe-2S] centers, and FAD) found in these enzymes will require the use of site-directed mutants and their characterization as an approach. Initial efforts toward this goal were attempted by Bray's group on a *Drosophila* mutant enzyme (Hughes et al., 1992), although a tremendous amount of work is required to isolate a few milligrams of enzyme (which appears to be extensively proteolyzed) for structural and kinetic studies. Thus, relying on XDH mutations in *Drosophila* as ready sources of mutated XDH appears impractical. Nishino's group attempted to express the rat liver XDH in a baculovirus system (Nishino et al., 1993) and were successful in expressing the protein; which was, however, in a largely inactive form. From these considerations, it appears that the most probable chance for success in expression of XDH mutants would be to investigate bacterial xanthine dehydrogenase from a view of concentrating on one that exhibited structural and catalytic properties similar to those of the eukaryotic enzymes.

The results presented in this paper show that *C. acidovorans* XDH is very similar, if not identical, to the mammalian xanthine dehydrogenases in cofactor identity and content. The molybdenum cofactor is a molybdopterin rather than a molybdopterin dinucleotide, as shown also for the XDH isolated from *P. aeruginosa* (Johnson et al., 1991a). The Mo center of *C. acidovorans* XDH is similar to bovine XO or chicken XDH in possessing a cyanolyzable terminal sulfur ligand since the enzyme is irreversibly inactivated on CN^- treatment, presumably due to formation of SCN^- on cyanolysis of the terminal sulfur ligand (Massey & Edmondson, 1970). This behavior contrasts with other bacterial XDH preparations from *V. alcalescens* (Smith et al., 1967) and *P. putida* 40 (Woolfolk, 1985) which exhibit a resistence to CN^- inhibition. With proper care, XDH preparations of 100% functionality can be routinely isolated from *C. acidovorans* using the procedure described here such that

resolution of functional from nonfunctional enzyme using affinity column techniques (Nishino et al., 1981) does not have to be employed, as is the case with mammalian and avian enzymes.

CD, ESR, and the analytical Fe content of *C. acidovorans* XDH support the conclusion that this bacterial enzyme contains two distinct [2Fe-2S] centers. Both centers are reduced on the anaerobic addition of xanthine to a level dictated by the functionality of the Mo center as found for the mammalian enzymes. The only other bacterial XDH examined in detail has been the enzyme isolated from *Veillonella alcalescens*, which appears to contain only a single Fe/S center (Dalton et al., 1976) which may be a 4Fe-4S center since the Fe:Mo stoichiometry is 4:1 (Smith et al., 1967). The Fe centers of other bacterial XDH preparations have not been characterized, and therefore nothing is known regarding their structures.

The phosphorus content of *C. acidovorans* XDH shows identity to both bovine milk XO and chicken XDH. A total of four phosphorus atoms per $\alpha\beta$ protomer are found, of which three are liberated from the enzyme by acid treatment. Those liberated can be accounted for by the pyrophosphate moiety of FAD (2) and the monophosphate of the molybdopterin cofactor (1). These data show that the Mo center contains only a single pterin cofactor rather than two pterins such as found in the tungsten-containing aldehyde oxidoreductase from *P. furiosus* (Chan et al., 1995). The finding of a protein-bound covalent phophorus residue (1 per $\alpha\beta$ protomer) in *C. acidovorans* XDH has also been shown with bovine milk XO and chicken XDH (Davis et al., 1984; Schieber & Edmondson, 1993). Phosphoserine was demonstrated to be the phosphorylated amino acid residue in the two eukaryotic enzymes. As of this writing, the amino acid phosphorylated in *C. acidovorans* XDH has not been identified. Unpublished work in this laboratory shows the enzyme to be labeled with ^{32}P on the β (60 kDa) subunit (Q. Xiang and D. E. Edmondson, unpublished observations), and work is ongoing in this laboratory to identify the residue phosphorylated and its sequence location. This aspect of XDH/XO has been a controversial area (Johnson et al., 1991b; Howes et al., 1991). The finding of covalent phosphorylation of three distinct sources of XO/XDH (bovine milk, chicken, and *C. acidovorans*) from distinct phyla (mammalian, avian, and bacterial) suggests a basic function which may be an undefined catalytic role (D'Ardenne & Edmondson, 1990b) and merits further investigation.

The turnover number of functional *C. acidovorans* XDH is 6−7-fold faster than bovine milk, chicken, or rat xanthine dehydrogenase. Of interest is the observation that $^{D}(V/K)$ is similar (1.8−1.9) for three of the enzymes tested (this work; D'Ardenne & Edmondson, 1990a). Since $^{D}(V/K)$ measurements are inclusive of all steps up to and including the first irreversible step in catalysis, these data suggest similar commitment factors for the initial steps in catalysis up to the C−H bond cleavage step. Since it is generally thought that product (uric acid) release is rate limiting in catalysis for bovine XO (Olson et al., 1974), the higher turnover number observed for *C. acidovorans* XDH probably is a reflection of a faster rate of product release than found with chicken XDH or milk XO. Supporting this hypothesis is the observation of a larger ^{D}V value (1.8) for *C. acidovorans* XDH than for chicken XDH (1.2) or bovine milk XO (1.1) (D'Ardenne & Edmondson, 1990a). All three enzymes exhibit similar (V/K) values and therefore show similar catalytic efficiencies. Of the electron acceptors tested, NAD^+ is by far the most efficient (Table 4). Typically DCIP and ferricyanide are much better electron acceptors when NADH is the reductant than when xanthine is the reducing substrate. This behavior is quite different from chicken XDH where the rate of DCIP reduction is similar for either xanthine or NADH as reductant and may reflect differences between the bacterial and avian enzymes in flavin and/or Fe/S center oxidation−reduction potentials and/or rates of intramolecular electron transfer.

In contrast to the eukaryotic xanthine dehydrogenases, *C. acidovorans* XDH is composed of two subunits in an $\alpha_2\beta_2$ structure which is the same size as the α_2 structure of the eukaryotic enzymes. No evidence was found for the formation of a larger molecule weight "proform" of either subunit, which suggests the two subunits are separate gene products. The rate of synthesis of each subunit parallels the increase in specific activity which indicates cofactor incorporation does not constitute any rate limitation in the biosynthesis of functional enzyme by the cell. Amino terminal sequence analysis of either subunit shows weak sequence homology with any known eukaryotic XDH sequences. Neither the α nor the β subunits of *C. acidovorans* XDH nor the bovine milk XDH cross-react with antisera raised against chicken XDH, which suggests that there are no similar epitopes on the avian with the bacterial and bovine enzymes.

In conclusion, the results presented here show that *C. acidovorans* XDH exhibits cofactor, structural, and catalytic properties that are quite similar to the well-studied eukaryotic xanthine oxidase/dehydrogenase. This bacterial enzyme thus appears to be a suitable model to address a number of questions currently not technically feasible with the eukaryotic enzymes, which should provide new insights into cofactor functional roles and electron transfer pathways in this multiredox center class of enzymes.

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