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PURIFICATION AND PROPERTIES OF XANTHINE DEHYDROGENASE FROM *PSEUDOMONAS ACIDOVORANS*

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Summary

Xanthine dehydrogenase (EC 1.2.1.37) from *Pseudomonas acidovorans* has been purified to near homogeneity (approx. 65-fold). The enzyme has a molecular weight of about 275 000. Electrophoresis in gels containing sodium dodecyl sulphate showed the presence of two types of subunit with molecular weights of about 81 000 and 63 000. Thus the intact molecule probably contains two of each type of subunit. Xanthine and hypoxanthine are good substrates, and NAD^+ is an effective electron acceptor. With xanthine and NAD^+ as substrates the purified enzyme has a specific activity of about 20 μmol NADH formed/min per mg protein. Michaelis constants for xanthine and NAD^+ are 0.07 and 0.12 mM, respectively, and for hypoxanthine and NAD^+ 0.29 and 0.16 mM, respectively.

Introduction

Pseudomonas species are known to be capable of oxidising xanthine to uric acid [1,2], presumably with a xanthine oxidase, but little is known of the properties and nature of this enzyme. Dikstein et al. [1] and Clarke and Meadow [2] reported that the xanthine oxidase activity of *Pseudomonas aeruginosa* was not stable to sonication and thus could only be studied in intact cells, with consequent limitations on the information obtainable.

This paper describes the purification and some properties of a soluble xanthine dehydrogenase (xanthine: NAD^+ oxidoreductase, EC 1.2.1.37) from *Pseudomonas acidovorans* grown on hypoxanthine as sole carbon, nitrogen and energy source.

Abbreviation: NBT, 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride.

Materials and Methods

Organisms and culture media. Ps. acidovorans (ATCC 15667) was grown aerobically at 30°C in medium containing per l: 6.8 g KH_2PO_4; 8.9 g $Na_2HPO_4 \cdot 2H_2O$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 mg $MnCl_2 \cdot 4H_2O$; 0.2 mg ferric ammonium citrate; 2 mg $CaCl_2$; 10 mg sodium molybdate; 2.5 g hypoxanthine; 0.5 g trypticase soy broth. Cells were washed in 0.1 M phosphate buffer, pH 6.8, and resuspended (1 g cells/15 ml) in 0.05 M Tris, 0.10 M $(NH_4)_2SO_4$, 0.5 mM EDTA, pH 7.9 (Tris/EDTA buffer) containing 2 mM 2-mercaptoethanol, and disrupted in an MSE 100-w ultrasonic disintegrator by sonication for a total of 1.5 min, with intermediate cooling. The sonicated cell suspension was centrifuged at 120 000 × *g* for 20 min.

Enzyme assays. Xanthine dehydrogenase was determined essentially as described by Watt [3]. The total incubation volume was 2.0 ml of 0.10 mM xanthine, 0.6 mM NAD^+ (Boehringer) in Tris/EDTA buffer. Kinetic experiments were performed at 30°C, in a Zeiss PMQII spectrophotometer. Other assays were performed at room temperature (approx. 22°C).

Gel electrophoresis. Disc gel electrophoresis was performed by the method of Davis [4]. Protein was stained with Amido black [4] or Coomassie blue [5]. The xanthine dehydrogenase activity stain contained xanthine, NAD^+, and 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride (NBT) (Sigma) in 50 mM Tris · HCl buffer, pH 7.8.

Molecular weight determination was performed by analytical disc gel electrophoresis using the technique of Hedrick and Smith [6], and the buffer system of Davis [4]. The data was evaluated as described by Rodbard and Chrambach [7] and Junge et al. [8]. Mobilities were measured on photographs of the gels, and the slopes of the plots of log mobility versus acrylamide concentration determined by the method of least squares.

The determination of subunit molecular weight was performed by sodium dodecyl sulphate gel electrophoresis as described by Laemmli [9]. Proteins were dissociated by heating at 80°C for 20 min in 5% 2-mercaptoethanol, 2% sodium dodecyl sulphate, 10% glycerol, 0.0625 M Tris · HCl, pH 6.8.

Absorption spectra were recorded on a Cary 118 spectrophotometer.

Protein was determined by the modified Folin method of Lowry et al. [10] and also by the ratio of absorbances at 205 and 280 nm, as described by Scopes [11].

Enzyme purification. (1) $(NH_4)_2SO_4$ fractionation: Crude extract was brought to 65% saturation by addition of solid $(NH_4)_2SO_4$, and allowed to stand at 4°C for at least 1 h. The resulting suspension was centrifuged at 12 000 × *g* for 10 min and the precipitate resuspended in 1.7 M $(NH_4)_2SO_4$, 50 mM Tris, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, pH 8.0. After 1 h this suspension was centrifuged at 30 000 × *g* for 15 min and the precipitate, which contained the xanthine dehydrogenase activity, was redissolved in 0.1 M potassium phosphate, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.65, in a volume of about half that of the original crude extract.

(2) DEAE-cellulose column chromatography: Enzyme from the previous step, after dialysis against 0.1 M potassium phosphate, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.65, and centrifugation to remove insoluble material,

was applied to a column (3 × 11 cm) of DEAE-cellulose (Serva) equilibrated with the same buffer. The column was washed with 250 ml of starting buffer, and enzyme was eluted with a linear gradient consisting of 200 ml 0.10 M potassium phosphate, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.65, and 200 ml 0.25 M potassium phosphate, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, pH 6.8. Active fractions were pooled and protein precipitated by adding $(NH_4)_2SO_4$ to 65% saturation.

(3) Sephadex G-200 chromatography: The enzyme from the DEAE-cellulose column was dissolved in a small volume of 50 mM potassium phosphate, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.7, and applied to a column (2.3 × 45 cm) of Sephadex G-200 (Pharmacia), equilibrated with the same buffer. 6.5-ml fractions were collected and assayed for xanthine dehydrogenase.

Results

Purity and stability of xanthine dehydrogenase. The peak fractions from Sephadex G-200, as judged from densitometry of polyacrylamide gels stained with Amido black or Coomassie blue, were approx. 90% pure (Fig. 1). Gels containing 8 or 10% acrylamide showed one sharp, major protein band, which corresponded to xanthine dehydrogenase as detected by the activity stain.

The enzyme, both in crude extract and purified form, readily forms insoluble inactive aggregates. It is not stable to freezing and thawing.

Visible absorption spectrum. Fig. 2 shows the visible absorption spectrum of purified xanthine dehydrogenase. A broad absorption peak around 450 nm, characteristic of xanthine oxidases and dehydrogenases, is present. The ratio A_{280nm}/A_{450nm} is 6.4, which is a little higher than that of 5.54 reported for a highly purified xanthine dehydrogenase obtained from chicken livers [12].

Molecular weights of xanthine dehydrogenase species. The molecular weights of the species present in cells grown on hypoxanthine were determined by electrophoresis of crude extracts according to the method of Hedrick and Smith [6].

After gel electrophoresis of a sample of crude extract, prepared in the presence of 2-mercaptoethanol or dithiothreitol, cells grown on hypoxanthine

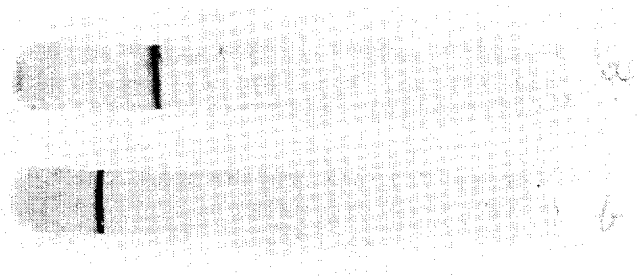

Fig. 1. Gel electrophoresis of purified xanthine dehydrogenase. Conditions of electrophoresis are described in the text. Samples contained approx. 10 μg protein, and were stained with Amido black. Direction of migration is from left to right. a, 8% gel; b, 10% gel.

TABLE I

PURIFICATION OF XANTHINE DEHYDROGENASE FROM *PS. ACIDOVORANS*

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	240	75	0.31	100	1
$(NH_4)_2SO_4$ fraction	100	70	0.70	93	2.3
DEAE-cellulose	12	44	3.7	59	12
Sephadex G-200	3	42	14	56	45
Peak fractions	1.3	26	20	35	65

and xanthine showed, respectively, four and five bands with xanthine dehydrogenase activity. Staining of all these bands was dependent on xanthine, but the band specific to cells grown on xanthine stained equally strongly whether NAD^+ was present or absent. There was very weak staining of the other bands in the absence of NAD^+.

The standard curve for determining molecular radius is shown in Fig. 3. The molecular radius of the major xanthine dehydrogenase species, the one obtained by the purification procedure, is about 4.32. This corresponds, for a spherical protein, to a molecular weight of 275 000. The other minor species have molecular radii of 4.99, 2.83, and 2.35, which correspond to molecular weights of 400 000, 77 000, and 44 000, respectively.

Subunit molecular weight of xanthine dehydrogenase. Purified preparations of xanthine dehydrogenase, which contained almost exclusively the 275 000 molecular weight species, were subjected to electrophoresis in gels containing sodium dodecyl sulphate. Electrophoresis in 8, 10, and 12% gels revealed, in three separate enzyme preparations, the presence of two non-identical subunits, with molecular weights of 81 000 and 63 000 (Fig. 4). The smaller subunit stained poorly with Coomassie blue, and this band steadily faded during destaining and storage of the gels.

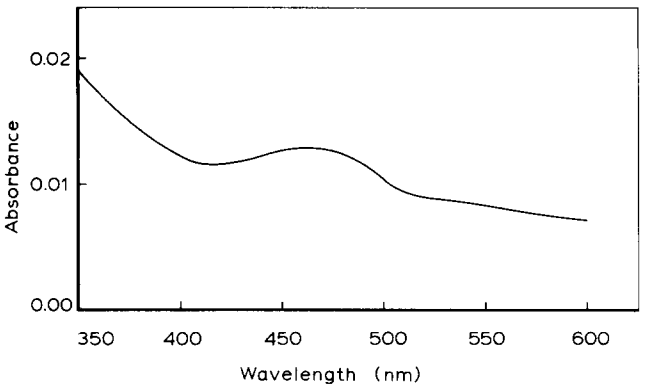

Fig. 2. Visible absorption spectrum of purified xanthine dehydrogenase. The protein concentration was approx. 40 μg/ml in Tris/EDTA buffer.

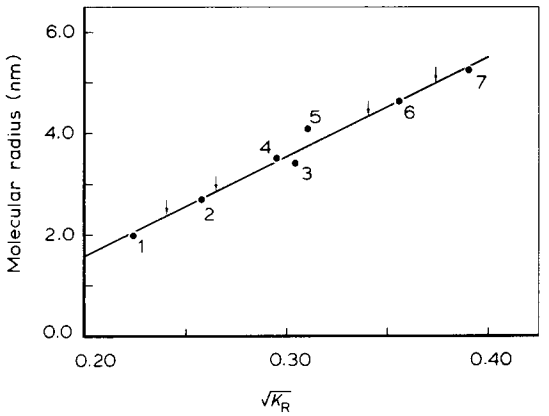

Fig. 3. Standard curve for the determination of molecular weight by electrophoresis in a range of gel concentrations. Measurement of gels and analysis of data was as described in the text. Standard proteins and molecular radii are: 1, α-chymotrypsinogen A (Sigma) 1.96; 2, bovine serum albumin (Boehringer) 2.69; 3, bovine serum albumin dimer 3.40; 4, yeast alcohol dehydrogenase (Boehringer) 3.52; 5, catalase (Boehringer) 4.08; 6, glutamate dehydrogenase (Boehringer) (beef) 4.63; 7, jack-bean urease (Sigma) 5.22. Positions of xanthine dehydrogenase species are indicated by arrows.

Optimum pH for xanthine dehydrogenase activity. The pH optimum in both potassium phosphate and Tris · HCl buffers was about 7.9. Activity was higher in phosphate buffer than in Tris buffer at the same pH value.

Specificity for substrates and inhibitors. The activity of xanthine dehydrogenase with a variety of potential substrates is shown in Table II. Hypoxanthine

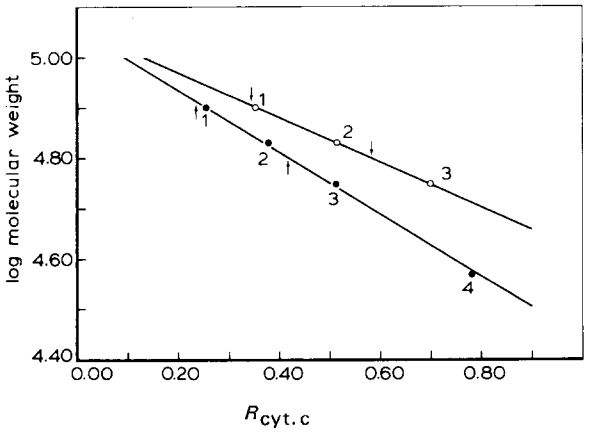

Fig. 4. Mobility of standard proteins in sodium dodecyl sulphate gels of two different acrylamide concentrations. Conditions of electrophoresis and preparation of protein samples are described in the text. Between 10 and 40 μg of each standard protein was applied per gel, with 10 μg of cytochrome *c*. Mobilities were measured relative to the distance migrated by cytochrome *c*. 1, urease; 2, bovine serum albumin; 3, glutamate dehydrogenase; 4, yeast alcohol dehydrogenase. Mobilities of xanthine dehydrogenase subunits are indicated by arrows. ○, 8% gel; ●, 10% gel.

TABLE II

SUBSTRATE SPECIFICITY OF XANTHINE DEHYDROGENASE

Incubations contained 10 μl of xanthine dehydrogenase, from the peak fraction of a DEAE-cellulose column, in a total volume of 2.0 ml 50 mM Tris, 0.10 M $(NH_4)_2SO_4$, 0.5 mM EDTA, pH 7.9. Other additions were as indicated. NADH production was followed at 340 nm.

Substrates	Concentration (mM)	Activity (percent of control)
Xanthine NAD^+	0.10 2.0	100*
Xanthine $NADP^+$	0.10 2.0	11
Hypoxanthine NAD^+	0.10 2.0	65
Purine NAD^+	1.0 2.0	4
Adenine NAD^+	1.0 2.0	0
Guanine NAD^+	saturated soln 2.0	0
Xanthine Air	0.10	3**
NADH Air	0.013	0.3

* 10.9 μmol NADH per min per mg protein.

** Xanthine oxidase activity was measured at 295 nm. E_{295nm} uric acid = $9.62 \cdot 10^3\ M^{-1} \cdot cm^{-1}$; E_{295nm} xanthine = $1.67 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

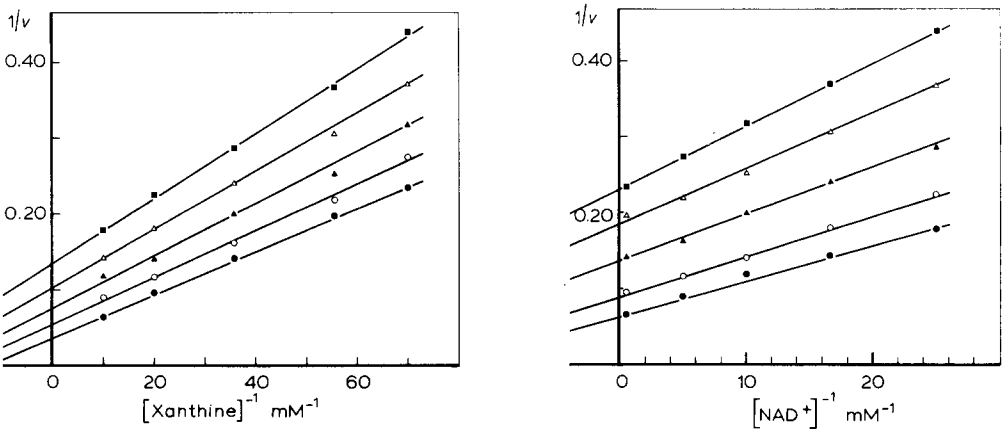

Fig. 5. Kinetics of the reaction between xanthine and NAD^+ catalyzed by xanthine dehydrogenase. Results are presented in double reciprocal form. Reaction was at 30°C in Tris/EDTA buffer. (a) Dependence of initial rate on xanthine concentration at the following concentrations of NAD^+: 2.0 mM (●); 0.20 mM (○); 0.10 mM (▲); 0.06 mM (△); 0.04 mM (■). (b) Dependence of initial rate on NAD^+ concentration at the following concentrations of xanthine: 0.10 mM (●); 0.05 mM (○); 0.028 mM (▲); 0.018 mM (△); 0.0143 mM (■).

and xanthine were good substrates, purine was rather poor and the other compounds tested did not act as substrates. In addition to NAD^+, some dyes, $Fe(CN)_6$, 2,6-dichlorophenolindophenol, but not phenazine methosulphate or NBT, served as electron acceptors. FAD did not affect the rate of reaction. The reoxidation of NADH was extremely slow.

A number of compounds were tested for possible inhibition of the reaction between xanthine and NAD^+. Adenine, guanine, and purine all caused some inhibition, but urate, uracil, and NADP had little or no effect.

Stoichiometry of reaction between xanthine and NAD^+. In a reaction mixture containing initially 1.8 μmol NAD^+ and 0.0387 μmol xanthine, at equilibrium 0.0381 μmol NADH were present.

Kinetics. The kinetics of NADH formation were investigated as a function of xanthine (or hypoxanthine) and NAD^+ concentrations. Results for the reaction of xanthine with NAD^+ are shown in Fig. 5. The Michaelis constant for xanthine was 0.07 mM and for NAD^+ 0.12 mM, while the maximum velocity was 26 μmol per min per mg protein. For the reaction of hypoxanthine with NAD^+ the Michaelis constant for hypoxanthine was 0.29 mM and for NAD^+ 0.16 mM. The maximum velocity was 38 μmol per min per mg protein. Whether xanthine or hypoxanthine was used as substrate, the double reciprocal plots were not strictly parallel.

Inhibition by adenine. The inhibition by adenine with respect to xanthine was found to be non-competitive [13], both slope and intercept being dependent on adenine concentration (Fig. 6). Inhibition constants for slope and intercept effects [13] were 0.04 and approx. 0.15 mM, respectively.

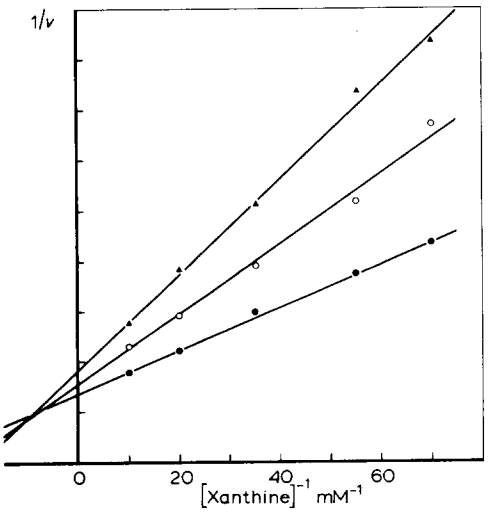

Fig. 6. Inhibition of xanthine dehydrogenase by adenine. Reaction was at 30°C, in Tris/EDTA buffer. Incubations contained 0.04 mM NAD^+. Adenine concentrations were: zero (●); 0.025 mM (○); 0.050 mM (▲). Lines of best fit were drawn by the method of least squares.

Discussion

This study, the first to report the properties of a xanthine dehydrogenase from an aerobic bacterium, shows that xanthine dehydrogenase from *Ps acidovorans* has many properties in common with xanthine dehydrogenases from a variety of mammals [14,15], birds [16], and insects [3,17]. Both hypoxanthine and xanthine are good substrates, and there is a slight reaction with purine. In addition to NAD^+, some dyes can serve as electron acceptors. Adenine is an inhibitor, but urate, which is reported to be a potent inhibitor of xanthine dehydrogenases from *Colias* butterflies [3] and *Micrococcus lactilyticus* [20], has little effect. The oxidation of xanthine with NAD^+ as electron acceptor proceeds at a rate in excess of 20 μmol per min per mg protein, a rate considerably higher than those reported for xanthine dehydrogenase from other sources [3,14,16—18]. The double-reciprocal plots for the reaction between xanthine or hypoxanthine and NAD^+ did not strictly follow the parallel pattern observed for milk xanthine oxidase [19,20], but more detailed kinetic studies are needed to establish whether the reaction mechanism differs from that of milk xanthine oxidase.

Xanthine dehydrogenase from *Ps acidovorans* has a molecular weight of about 275 000, in the same range as those from other organisms [3,14,16—18]. The detection of two non-identical subunits with molecular weights of 81 000 and 63 000 suggests that the intact molecule contains two of each kind of subunit. Xanthine oxidases/dehydrogenases from bovine milk [14], Drosophila [17] and rat liver [15] have been split into two identical subunits of about 150 000, but smaller subunits have not been found.

The relationship between the species which stain for xanthine dehydrogenase in gels run from crude extract, remains unclear. The species with molecular weights of 44 000 and 77 000 are less charged at pH 9 than the major xanthine dehydrogenase species. Thus if the species of molecular weight 77 000 is related to the 81 000 subunit observed on sodium dodecyl sulphate, the other smaller subunit must have a high negative charge to give the intact molecule its observed mobility. The poor staining of the smaller subunit is consistent with its containing many acidic residues [21], but further studies are needed to characterise both the subunits and the minor xanthine dehydrogenase species.

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