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PURIFICATION AND CHARACTERIZATION OF XANTHINE DEHYDROGENASE FROM *CLOSTRIDIUM ACIDIURICI* GROWN IN THE PRESENCE OF SELENIUM

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Xanthine dehydrogenase has been purified from *Clostridium acidiurici* to homogeneity. The purified enzyme exhibited a specific activity of 385 units per mg protein with xanthine as substrate and methyl viologen as most effective electron acceptor. However, the enzyme activity was quite unstable. Michaelis constants for xanthine and methyl viologen were 1.35 and 0.44 mM, respectively. Uric acid, xanthine, hypoxanthine, 6-mercaptopurine, allopurinol and purine could serve as substrates. NAD, NADP and ferredoxin were inactive as acceptors. The molecular mass of the enzyme was estimated to be 224 kDa. After SDS-gel electrophoresis, five different polypeptides were detected. Analysis of the native enzyme gave values for non-haem iron, acid-labile sulphur, FAD, molybdenum, tungsten and selenium in a molar ratio of 7.7:7.5:1.7:1.8:0.12:0.13, respectively. Free ESR radical signals, presumably due to flavin semiquinone, were observed after reduction with xanthine, dithionite, or NADH. Despite the requirement for selenium for the synthesis of the active xanthine dehydrogenase, there was no evidence for involvement of selenium in the iron-sulphur or molybdenum centres. Reduction with xanthine induced a signal analogous to the rapid Mo(V) species of other xanthine dehydrogenases and xanthine oxidase, indicating that active molybdenum centres are present. In addition, a 'slow' desulphomolybdenum signal was observed after prolonged reduction with dithionite, and a 'desulpho-inhibited' signal in the protein reduced with dithionite plus 9 M ethanediol. Spectra of two iron-sulphur centres I + II, corresponding to the [2Fe-2S] clusters of milk xanthine oxidase, were observed after reduction with dithionite.

Introduction

Clostridium acidiurici is specialized in anaerobic purine degradation, like *Clostridium cylindrosporum* and *Clostridium purinolyticum* [1]. The enzyme xanthine dehydrogenase catalyzes the initial transformations of the intact purine ring system [2,3]. The formation of an active xanthine dehydrogenase requires the presence of selenite compounds during growth of these three species [4,5]. In addition to xanthine dehydrogenase, selenium is also needed for formate dehydrogenase formation. The latter enzyme requires the further addition of molybdate or tungstate, depending on the species and strain studied [5–7]. The trace element requirement of the type strain of *C. acidiurici* is of special interest, for tungsten is known to be an antagonist of molybdate [8]. In contrast to formate dehydrogenase [9], xanthine dehydrogenase generally is a molybdenum-con-

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate.

taining iron-sulphur flavoprotein [3]. This has been demonstrated in case of *C. cylindrosporum* [10]. The cofactor composition of the homogeneous xanthine dehydrogenase from *C. acidiurici* in respect to selenium and tungsten and some of its properties were of special interest.

Experimental

Materials

Biochemicals and chemicals were purchased from Boehringer Mannheim, Mannheim (bovine serum albumin, standard protein samples for the determination of molecular weight, enzymes), BDH Chemicals. Poole, U.K. (cytidine, cytosine), Calbiochem Giessen (8-azaguanine, 6-mercaptopurine), Difco Laboratories, Detroit, MI (yeast extract), Ega-Chemie, Steinheim (4-aminoimidazole-5-carboxamide, imidazole). Pharmacia Fine Chemicals, Uppsala (Sepharose 6B, Sephacryl S-300 superfine), Serva Feinbiochemica, Heidelberg (acrylamide, bisacrylamide, Coomassie brilliant blue R-250, *p*-nitroblue tetrazolium chloride, sodium dodecyl sulphate, viologen dyes, xanthosine), Sigma Chemical Co., St. Louis, MO (allopurinol, 8-azaadenine, uric acid, isonicotinic acid, 3-methyl xanthine, nicotic acid amide, orotic acid, picolinic acid, theobromine, theophylline, thymine, uracil, xanthopterine) and Whatman (DE-52). Biochemicals and chemicals not listed were obtained from Merck, Darmstadt. The materials used were of the highest grade commercially available. Desulpho xanthine oxidase from milk was a gift from Dr. R.C. Bray.

Methods

Growth of organism

Clostridium acidiurici (DSM 604, ATCC 7906) was grown anaerobically on uric acid in the presence of sodium selenite, molybdate and tungstate ($1 \cdot 10^{-7}$ M each) as described previously [4,6]. The cell yields (wet wt. cells/20 l medium) averaged about 13 g.

Assays of xanthine dehydrogenase and formate dehydrogenase

The extracts were prepared exactly as previously described [6]. Xanthine dehydrogenase was assayed in crude extracts under the following conditions (mmol/l in a total incubation volume of 1 ml): Tris (pH 8.5), 100; xanthine, 3.5; methyl viologen (neutralized), 10. Assays for purified xanthine dehydrogenase were run under identical conditions except for the methyl viologen concentration (3 mmol/l). Formate dehydrogenase assays contained in a total volume of 1 ml (in mmol/l); potassium phosphate buffer (pH 7.9), 100; sodium formate, 20; methyl viologen (neutralized), 20; dithioerythritol, 1. The assays were started routinely by the addition of enzyme. Blanks were run without xanthine (formate), but an appreciable reaction was never found using methyl viologen as acceptor. For calculations, a molar extinction coefficient of $\epsilon = 1.3 \cdot 10^4$ ($l \cdot mol^{-1} \cdot cm^{-1}$), at 600 nm was used for methyl viologen. XDH activity was determined with various electron acceptors in mixtures containing 100 mmol/l Tris-HCl buffer (pH 8.5), 3.5 mmol/l xanthine and one of the following electron acceptors (mmol/l; millimolar extinction coefficient; wavelength): ferredoxin (1.5 mg protein per test; 30.0; 390 nm); Benzyl viologen (20; 15.6; 555 nm); NAD(P) (1.0; 6.18; 334 nm); FAD (1.0; 11.3; 450 nm); FMN (1.0; 12.5; 445 nm); Methylene blue (0.04; 13.1; 570 nm); DCIP/PMS (0.2 : 0.2; 16.1; 600 nm); $K_3Fe(CN)_6$ (1.0: 1.0; 420 nm); nitroblue tetrazolium (0.5; 12.0; 535 nm); oxygen (saturated; 12.2; 290 nm). Ferredoxin from *Clostridium pasteurianum* was prepared according to the method of Schönheit et al. [11].

Substrate specificity of xanthine dehydrogenase was examined under standard assay conditions by varying the substrate concentration in a range of 0.35–17.5 mmol/l. The results listed in Table II were obtained at a substrate concentration of 3.5 mmol/l. Effects of pH were investigated with xanthine (3.5 mmol/l) and methyl viologen (3 mmol/l) using the following buffer systems (each 100 mmol/l): triethanolamine-HCl (pH 7.0–8.0), potassium phosphate (pH 7.0–7.9), Tris-HCl (pH 7.5–8.8) and glycine-NaOH (pH 8.8–10.0). To demonstrate the effects of temperature in a range of 10–70°C, xanthine dehydrogenase was tested

under standard assay conditions. Enzyme activity was calculated from the initial reaction velocity. The activation energy expressed in $kJ \cdot mol^{-1}$ was read from the Arrhenius plot (rate versus reciprocal temperature in K). Uric acid reduction was measured using sodium dithionite-reduced methyl viologen as electron donor and different buffer systems (triethanolamine-HCl, Tris-HCl, potassium phosphate, 100 mmol/l) in a pH range of 7.0–8.5. The following assay conditions were found to be optimal (mmol/l): uric acid (1.0), reduced methyl viologen (0.2), sodium dithionite (0.2) and triethanolamine-HCl, pH 7.0 (100). A unit of enzyme is defined as that amount which converts 1 μmol of substrate (2 electrons) per min. Activities are expressed in units per mg of protein. The enzyme activity was routinely assayed at 37°C under anaerobic conditions. The protein content of crude extracts was determined by the biuret method [12]. Protein concentration of purified enzyme preparations was determined by the method of Bradford [13].

Enzyme purification

All preparation steps were carried out under anaerobic conditions [14] at a temperature of 4°C.

$(NH_4)_2SO_4$ fractionation. The crude extract was brought to 48% saturation by addition of solid $(NH_4)_2SO_4$, and stirred for at least 15 min. The precipitate formed was removed by centrifugation at $10000 \times g$ for 30 min, and discarded. Solid ammonium sulphate was added to the supernatant to give 65% saturation. After stirring for 15 min, the resulting precipitate was collected by centrifugation at $10000 \times g$ for 30 min and dissolved in anaerobic potassium phosphate buffer (50 mmol/l; pH 7.5). The volume of the protein solution which contained 99% of the xanthine dehydrogenase and about 50% of the formate dehydrogenase activity was about 4–5 ml.

Gel filtration. Gel filtration was performed with a Sepharose 6B column (2.5 × 100 cm, bed volume 450 ml) equilibrated with potassium phosphate buffer (pH 7.5, 50 mmol/l) containing ammonium sulphate (50 mmol/l) and 2-mercaptoethanol (3 mmol/l). Protein solutions were eluted with the same buffer system at a flow-rate of 18 ml/h. Eluates were collected under a nitrogen atmosphere (glove box) in fractions of 3.3 ml.

For molecular mass determination, 2 mg xanthine dehydrogenase were loaded on a column containing Sepharose 6B and in addition on a column of Sephacryl S-300 superfine (2.5 × 100 cm, bed volume 350 ml). Urease (molecular weight 483000, 12 mg), catalase (240000, 2 mg), aldolase (158000, 4 mg), bovine albumin (136000/68000, 4 mg), and cytochrome *c* (12500, 3 mg) were used as standards. Elution was performed in a buffer system described above, but without ammonium sulphate. The fraction volume was 1.0 ml.

DE-52 chromatography. Concentrated xanthine dehydrogenase solution was loaded onto a column of DE-52 cellulose (1.0 × 15 cm, bed volume 12 ml), equilibrated with potassium phosphate buffer (50 mol/l, pH 7.5). Protein was eluted using a linear gradient of ammonium sulphate (100–200 mmol/l, in potassium phosphate buffer, 50 mmol/l, pH 7.5). Peak fractions were combined and concentrated.

Preparative and analytical gel electrophoresis. Preparative gel electrophoresis was performed on 3% polyacrylamide gels including 0.5% agarose [15]. Up to 100 μg xanthine dehydrogenase per gel column were subjected to electrophoresis which was conducted at a constant current of 5 mA per column for 50 min at 15°C. The xanthine dehydrogenase activity band was made visible by incubating the gel columns and slab gels in anaerobic standard assay mixture at 37°C. Xanthine dehydrogenase-containing protein bands were cut out, homogenized in potassium phosphate buffer (50 mmol/l, pH 7.5) and stored for 24 h at 4°C in the dark under an atmosphere of nitrogen. Gel fragments were sedimented by centrifugation at $100000 \times g$ for 45 min. The supernatant was concentrated and contained the homogenous xanthine dehydrogenase as could be shown by immediate re-electrophoresis. Analytical SDS-gel electrophoresis was carried out on 7.5% polyacrylamide gels containing 0.1% SDS at a constant current of 6 mA per column for 4 h [16]. The molecular mass of the xanthine dehydrogenase subunits was estimated by comparison with parallel runs of the standard marker proteins: RNA polymerase α (molecular weight, 39000), β (165000), bovine serum albumin (68000), catalase (58000), aldolase (40000), chymotrypsinogen (25700), trypsin inhibitor (21500) and cytochrome *c* (12500). Pro-

teins were dissociated by heating 2 min at 95°C in potassium phosphate buffer (10 mmol/l, pH 7.0) in the presence of 2% (w/v) sodium dodecyl sulphate and 0.9% (v/v) 2-mercaptoethanol. The gels were stained for protein with Coomassie brilliant blue R-250 [17].

Concentration of protein solution. Protein concentration steps were carried out in ultrafiltration cells (Amicon, Oosterhout, Netherlands) using PM-10 filters with an exclusion limit of 10000 molecular mass. The filtration procedure was done under nitrogen pressure of 3 kPa/cm^2 at 4°C. Protein samples smaller than 2 ml were concentrated in Minicon A 25 cells (exclusion limit of 25000 molecular mass).

Molecular components

Flavin was extracted from native xanthine dehydrogenase either by precipitation with trichloroacetic acid or by heat treatment [18]. Extracted flavin could be demonstrated fluorimetrically using a fluorescence spectrophotometer (Perkin-Elmer 204). Flavin isolated by heat treatment was identified as FAD by thin-layer chromatography. Riboflavin, FMN and FAD were used as references. Solutions of extracted flavin (FAD) were stored at 4°C in the dark. Absorption and difference spectra of the purified xanthine dehydrogenase and of the flavin extracts were monitored with a double beam double wavelength spectrophotometer (Perkin-Elmer Hitachi 556). The content of FAD was estimated from the difference in absorbances of the H_2O_2-oxidized and sodium dithionite-reduced forms at 448 nm (ϵ_{mM} 11.3).

Selenium, tungsten, molybdenum, zinc and iron contents were determined by neutron activation analysis [19] carried out by Dr. Kasparek, Kernforschungsanlage Jülich. Non-haem iron was also determined chemically (Merckotest, Fa. Merck, Darmstadt, F.R.G.). Acid-labile sulphur content of purified xanthine dehydrogenase was estimated colorimetrically with *p*-aminodimethylaniline (*N*,*N*-dimethyl-*p*-phenylenediamine) and Fe^{3+} in acidic solution [20].

ESR spectra were recorded on an E4 spectrometer (Varian Associates, Walton-on-Thames, U.K.), with an ESR9 liquid helium flow cryostat (Oxford Instruments, Oxford, U.K.). Spectra were recorded digitally and subtracted on a DL4000 signal processing system (Data Laboratories, Mitcham, U.K.). *g*-values were calibrated relative to 1,1-diphenyl-2-picrylhydrazyl.

Results

Purification of xanthine dehydrogenase from C. acidiurici

Since xanthine dehydrogenase and formate dehydrogenase are both affected by selenite and tungstate [4,6] a simultaneous purification of both enzymes was tried. After fractionation with ammonium sulphate, the protein solution containing formate dehydrogenase and xanthine dehydrogenase was passed through a Sepharose 6B column. Xanthine dehydrogenase and formate dehydrogenase were eluted in different protein peaks (Fig. 1). Formate dehydrogenase exhibited a molecular mass of more than 500000. Due to the extreme oxygen sensitivity of formate dehydrogenase, a further purification failed. Formate

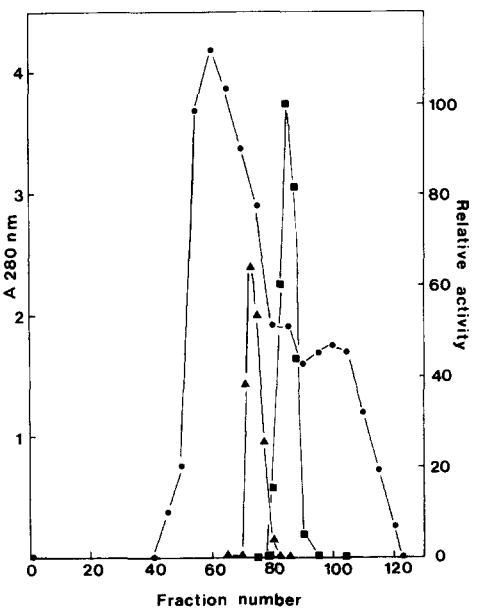

Fig. 1. Sepharose 6B column chromatography of xanthine dehydrogenase and formate dehydrogenase. 46 mg protein were applied to a column (2.5×100 cm) of Sepharose 6B equilibrated with 50 mmol/l potassium phosphate buffer (pH 7.5), 50 mmol/l $(NH_4)_2SO_4$ and 3 mmol/l 2-mercaptoethanol. Elution was performed with the same buffer solution and eluates were collected in 3.25 ml fractions at a flow-rate of 18 ml/h. ●, protein; ■, xanthine dehydrogenase; ▲, formate dehydrogenase.

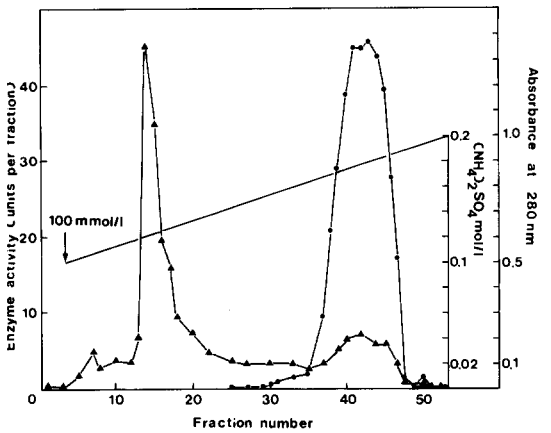

Fig. 2. DE-52 column chromatography of xanthine dehydrogenase. Chromatography was carried out on a column of DE-52 (1.0×15.0 cm) equilibrated with 50 mmol/l potassium phosphate buffer (pH 7.5). Elution of xanthine dehydrogenase (11 mg protein with 800 U) was performed with buffer containing 100–200 mmol/l $(NH_4)SO_4$ at a flow-rate of 12 ml/h. Eluates were collected in 2.25 ml fractions. ▲, protein; ●, xanthine dehydrogenase.

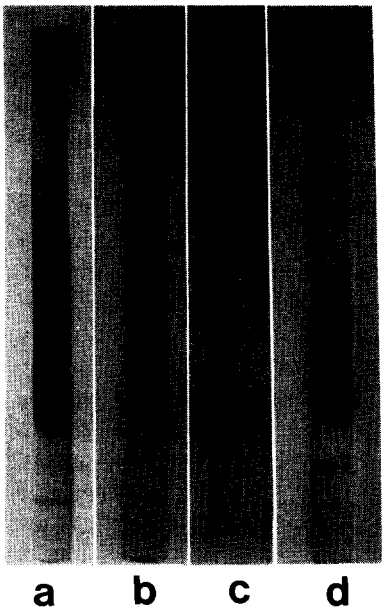

Fig. 3. Electrophoresis of the xanthine dehydrogenase at different purification steps. Conditions of electrophoresis are described in the text. (a) 40 μg xanthine dehydrogenase obtained from DE-52 chromatography; analyzed immediately after preparation; (b) homogenous enzyme, 20 μg protein; (c) activity stain of (b) immediately after electrophoresis, 35 μg protein; (d) 40 μg enzyme stored for 1 day anaerobically in the dark at 4°C.

dehydrogenase preparations obtained after Sepharose 6B column chromatography were completely inactivated at 1–3 h storage at 4°C in the dark under strictly anaerobic conditions. Both purified xanthine dehydrogenase and partially purified formate dehydrogenase lost their activity completely when stored in the frozen state below 0°C. Pooled peak fractions from Sepharose 6B chromatography with 85% of the xanthine dehydrogenase activity were concentrated, absorbed on a DE-52 column and eluted with a gradient of ammonium sulphate (Fig. 2). Investigation by polyacrylamide gel electrophoresis of pooled protein samples immediately after elution from DE-52 resulted in one major protein band accompanied by a smaller one. After storage for 3 days at 4°C in the dark, the identical enzyme preparation showed up to four protein bands by gel electrophoresis. The corresponding activity stains resulted in one activity band (Fig. 3).

TABLE I

PURIFICATION OF XANTHINE DEHYDROGENASE FROM *CLOSTRIDIUM ACIDIURICI*

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	329	1010	3.1	100	1
Resuspended sediment of 48–65% $(NH_4)_2SO_4$ fractionation	46	997	21.7	99	7
Sepharose 6B	11	860	78	85	25
DE-52	1.2	370	308	37	99
Preparative gel electrophoresis	0.13	50	385	5	124

Absorption spectra of xanthine dehydrogenase after chromatography on DE-52 led to a A_{280}/A_{450} ratio of 3.9–4.1, thus indicating a high degree of purity. Further purification of the material described above was carried out by preparative gel electrophoresis which yielded only one protein band after analytical electrophoresis. The specific activity of the 124-fold purified enzyme was calculated to be 385 units/mg protein. A summary of the purification procedure is given in Table I.

Molecular mass and subunit structure

The molecular mass of the enzyme was determined by gel filtration with Sepharose 6B and Sephacryl S-300 to be 224000. The number and molecular mass of subunits were estimated by SDS-polyacrylamide gel electrophoresis. The presence of five subunits with molecular masses of 110000, 83000, 56000, 53000 and 26000 was revealed.

Spectral characteristics and cofactor composition

Absorption spectra of oxidized and reduced native xanthine dehydrogenase are shown in Fig. 4. The oxidized enzyme exhibited an absorption maximum at 423 nm and a shoulder at 447 nm. The maximum of the difference spectrum was monitored at 448 nm, thus clearly indicating a flavin as a component of the native enzyme. Isolation of the flavin was performed by trichloroacetic acid extraction. The resulting spectrum showed one maximum at 448 nm which was eliminated by reduction with sodium dithionite. However, the presence of some other light-absorbing material is indicated. Trichloroacetic acid extracts of purified xanthine dehydrogenase were also investigated by fluorescence spectroscopy. If the excitation wavelength was fixed at 430 nm, the emission spectrum showed a maximum at 523 nm due to extracted flavin component. The flavin extracted from the enzyme gave the same R_f value as authentic FAD after thin-layer chromatography. From the difference spectrum of the oxidized-reduced forms, a molar ratio of 1.69 mol FAD per mol xanthine dehydrogenase was calculated. The content of non-haem iron and acid-labile sulphur was determined by chemical methods to be 7.7 g atom Fe and 7.5 gatom S^{2-} per mol of the enzyme (DE-52 step). Investigations by neutron activation analysis

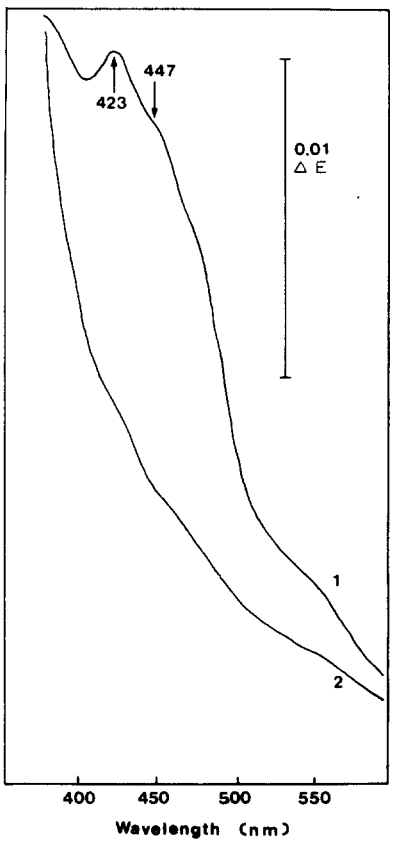

Fig. 4. Visible absorption spectra of purified xanthine dehydrogenase (1, oxidized; 2, reduced).

of the purified enzyme (last step of Table I) exhibited on the basis of the molecular weight of 225000 a molar content of 1.8 gatom molybdenum, 0.12 gatom tungsten and 0.13 gatom selenium. This enzyme preparation was also routinely analyzed for iron (6.6 gatom) and zinc (0.34 gatom). These data show that the xanthine dehydrogenase from *C. acidiurici* is at least a molybdenum-containing iron-sulphur flavoprotein as generally obtained.

Optimum pH and temperature for xanthine dehydrogenase activity

The effects of pH and temperature were studied with xanthine and methyl viologen as substrate and electron acceptor, respectively. The enzyme was found to be most active at 55°C and at pH 8.5 using Tris-HCl as buffer. However, all further investigations for enzyme activity were carried out

at 37°C at optimum pH. The activation energy was calculated from the Arrhenius plot to be 39 800 $J \cdot mol^{-1}$.

Substrate and electron acceptor specificity

The compounds listed in Table II were examined as substrates with methyl viologen as electron acceptor. In addition to xanthine, hypoxanthine derivatives, purine and 4-aminoimidazol-5-carboxamide were converted by the purified enzyme. The highest activity was monitored with xanthine, whereas methylated xanthine derivatives could not serve as substrates. Aldehyde-oxidizing activity was only observed in crude extract preparations. The activity of purified xanthine dehydrogenase with several artificial and physiological electron acceptors was tested using xanthine as substrate. The following reduction rates (%) could be measured in relation to methyl viologen (100%): benzyl viologen (52), methylene blue (87), DCIP + PMS (9), DCIP (2.5), potassium ferricyanide (99), *p*-nitroblue tetrazolium (32), oxygen (0.1). No activity was found with ferredoxin prepared from *C. pasteurianum*, and NAD(P). Among the physiological electron acceptors, only FMN (17%) and FAD (22%) were reduced to a significant extent. Xanthine dehydrogenase activity could also be measured with uric acid and reduced methyl viologen as substrates. Optimal assay conditions proved to be at pH 7.0 using triethanolamine-HCl buffer (100 mM). Under these conditions, 40% of the activity of the reverse reaction (xanthine to uric acid) was reached. No reduction of uric acid was observed with $FADH_2$ and $NAD(P)H_2$ even at high enzyme concentrations.

K_m values were calculated for xanthine ($1.35 \cdot 10^{-3}$ mol/l) and methyl viologen ($4.4 \cdot 10^{-4}$ mol/l). The enzyme exhibited normal Michaelis-Menten kinetics.

TABLE II

SUBSTRATE SPECIFICITY OF XANTHINE DEHYDROGENASE FROM *CLOSTRIDIUM ACIDIURICI*

Incubations contained in a total volume of 1.0 ml: 100 mmol/l Tris-HCl (pH 8.5), 3 mmol/l methyl viologen, 3.5 mmol/l substrate. Compounds which were also tested and found not to be substrates for xanthine dehydrogenase: 6-hydroxypterine (xanthopterine), 3-methyl xanthine, theophylline, theobromine, caffeine, xanthosine, 8-azaadenine, 8-azaguanine, guanosine, imidazole, allantoin, cytosine, cytidine, thymine, uracil, alloxan, orotic acid, thiamine, nicotinic acid, picolinic acid and salicyl aldehyde. 100% = 4.5 units/mg protein (crude extract) and 148 units/mg protein (purified enzyme).

Substrates	Relative activity (%)	
	Crude extract	Purified xanthine dehydrogenase
Purine	23	2
Allopurinol	30	8
Hypoxanthine	29	7
6-Mercaptopurine	58	9.5
Xanthine	100	100
Adenine	5	0
Guanine	0.1	0.3
4-Aminoimidazol-5-carboxamide	6	1.1
Acetaldehyde	45	0

Characterisation of redox centres by ESR spectroscopy

Effects of reductants. No ESR signal was detected in the oxidized xanthine dehydrogenase as isolated. Treatment of the xanthine dehydrogenase with xanthine induced a radical signal at $g = 2.004$, width 1.8 mT, possibly due to flavin, and signals around $g = 1.97$ characteristic of Mo(V) [18] (Fig. 5a). Reduction of the iron-sulphur clusters was not observed under these conditions. Despite the fact that NAD was not found to be an effective acceptor for the enzyme, NADH caused a gradual appearance of a radical signal from the enzyme (Fig. 5b) and a slight but detectable reduction of the molybdenum. This indicates that NAD(H) is capable of interacting with the flavin. Reduction with dithionite for a brief period (10 s at 0°C) caused little reduction of the iron-sulphur and molybdenum centres, but a considerable free radical signal (Fig. 5c). After prolonged reduction, the radical decreased, indicating reduction to $FADH_2$, and signals due to Mo(V) (Fig. 5d) and reduced iron-sulphur clusters (Fig. 6) appeared.

Iron-sulphur centres. After reduction with dithionite the xanthine dehydrogenase showed two signals that are characteristic of reduced iron-sulphur clusters. The first was detected without significant broadening at temperatures up to 55 K. This will be referred to a Fe-S I. The principal apparent g values (cf. Fig. 6a) are 2.034, 1.945,

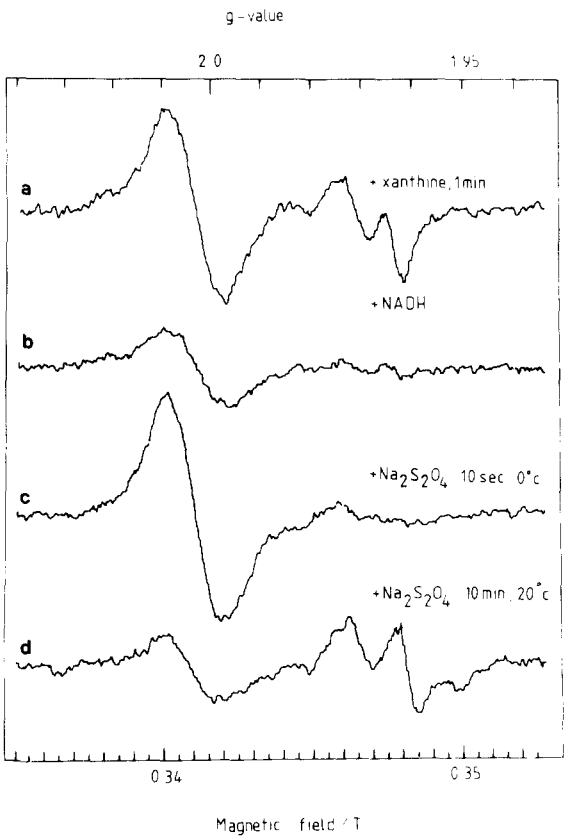

Fig. 5. ESR spectra of xanthine dehydrogenase, recorded at 130 K, with various conditions of reduction. (a) Sample reduced with 1 mM xanthine for 1 min at 20°C before freezing; (b) with 2.5 mM NADH for 1 min; (C) with 2 mM dithionite for 10 s at 0°C; (d) 2 mM dithionite for 10 min at 20°C. Instrument settings: microwave power 1 mW, frequency 9.18 GHz, modulation amplitude 0.5 mT.

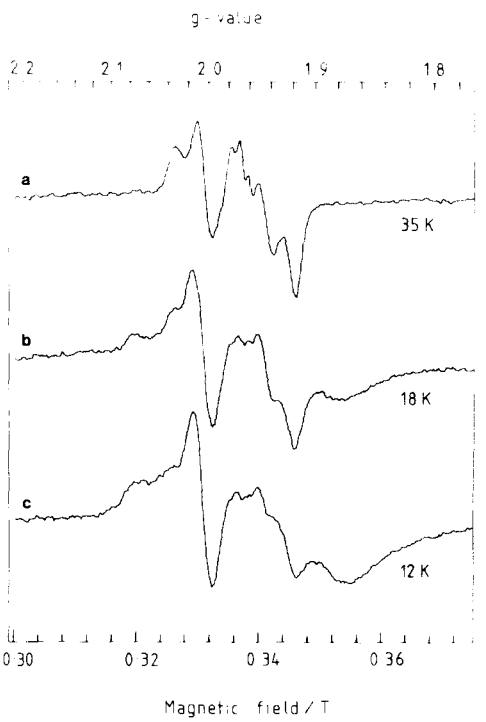

Fig. 6. ESR spectra of dithionite-reduced xanthine dehydrogenase, emphasizing the signals from reduced iron-sulphur clusters. Temperature of measurement: (a) 35 K, (b) 18 K, (c) 12 K. Other settings: microwave power 20 mW, frequency 9.18 GHz, modulation amplitude 1 mT.

1.918. The second cluster, Fe-S II, was only detected at temperatures below 25 K. Its spectrum was broader, with g values of 2.075, approx. 1.924, and 1.871 (Fig. 6b, c) (the central feature is broad and partly obscured by Fe-S I).

The two clusters are analogous to those found in eucaryotic xanthine dehydrogenase and xanthine oxidase [22–24]. By analogy with these, and in particular the temperature dependence of the ESR signals, it seems likely that Fe-S I and Fe-S II are both [2Fe-2S] clusters.

Molybdenum centre. The ESR parameters of the Mo(V) signals are somewhat different in the xanthine-reduced and dithionite-reduced samples. Both spectra show splittings attributable to strong interaction with a hydrogen nucleus. Superhyperfine splittings were estimated by inspection to be 1.2 mT for the xanthine-reduced signal and 1.6 mT for the dithionite-reduced signal. Although it is likely that neither represents a pure species, the former (Fig. 5a) resembles more closely the rapid Mo(V) signals of xanthine oxidase, while the latter (Fig. 5c) is more like the slow Mo(V) signal of desulpho xanthine oxidase and xanthine dehydrogenase [3,21]. The rapid signal would derive from active enzyme molecules which are reducible by xanthine. On prolonged reduction with dithionite, these would be reduced further to ESR-undetectable Mo(IV) and signals from desulpho Mo(V) would appear.

Further evidence for the presence of a desulphomolybdenum centre is provided by the detection of a desulpho-inhibited signal (nomenclature of Bray [21]) from the protein reduced in 9 M ethanediol. This signal represents a stable form

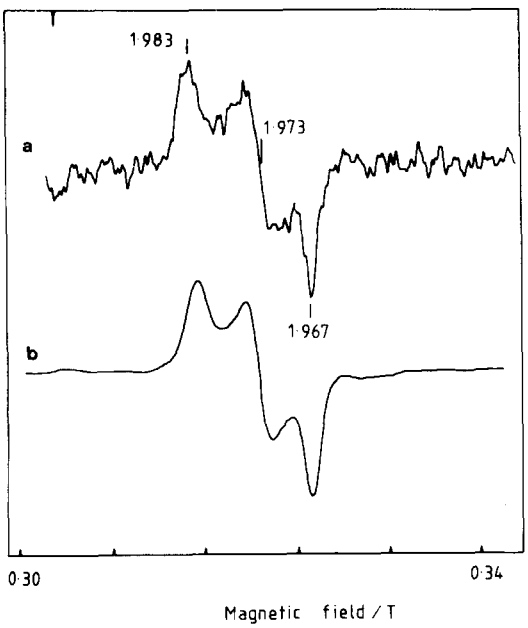

Fig. 7. ESR spectra of (a) *C. acidiurici* xanthine dehydrogenase, (b) desulpho xanthine oxidase from milk, reduced with dithionite in 9 M ethanediol. Conditions of measurement: temperature 150 K, microwave power 1 mW, frequency 9.26 GHz.

of Mo(V) with a very characteristic lineshape [25]. The shape of the spectrum in *C. acidiurici* xanthine dehydrogenase ($g = 1.979, 1.970, 1.964$) is similar to that in desulpho xanthine oxidase ($g = 1.978, 1.970, 19.64$) (Fig. 7). This indicates a similar if not identical coordination geometry in the two proteins. No evidence was obtained for substitution of selenium ligands, or for tungsten ESR signals which would be expected to have lower g values than molybdenum [26].

Discussion

The xanthine dehydrogenase of *C. acidiurici* resembled in its general properties the corresponding enzymes of procaryotes and eucaryotes [3]. The molar ratio of functional groups as iron, labile sulphur, flavin and molybdenum are essentially identical. It has been observed before that the stoicheiometry of these components may improve if more gentle enrichment procedures or protecting agents were employed to end up with a ratio of 4 : 4 : 1 : 1 for iron, sulphur, flavin and molybdenum [3]. Regarding these components the enzyme of *C. acidiurici* fits into the scheme, whereas the the enzyme of *C. cylindrosporum* lacks the full complement of molybdenum [10]. This is also indicated by the much lower starting and final activity obtained in that study, although they should not differ in cell extracts [4]. This might partly be due to a shortage of trace elements such as molybdenum and selenium which were deliberately not added to the growth media in former times [10]. Selenium in particular has a pronounced effect on the level of active enzyme in both bacteria [4]. Traces of selenium must have been present in those former studies, for neither bacterium will grow at all in its absence [7]. The purified xanthine dehydrogenase from *C. acidiurici* was found to contain only small amounts of selenium. Some support for the existence of selenium comes from a comparison with the xanthine dehydrogenase and nicotinic acid hydroxylase of *C. barkeri*, which likewise are affected by selenite in the growth medium [27]. *C. barkeri* xanthine dehydrogenase was found to contain 0.9 atom selenium per M_r 530000 [28]. The nicotinic acid hydroxylase contained 0.1–0.5 atom selenium per M_r 300000 [29], and immunologically behaved identically to the xanthine dehydrogenase (Rienhöfer, A and Andreesen, J.R., unpublished data). Although xanthine dehydrogenase activity in *C. barkeri* depends on selenium supplementation during growth, the high level of cross-reacting material is not influenced by selenium or molybdenum (Rienhöfer, A. and Andreesen, J.R., unpublished data), which excludes an action of selenium at the level of gene expression. These results support the view that selenium is a genuine constituent of those xanthine dehydrogenases which require selenium for activity.

Selenium has been found to be a constituent of a number of enzymes from anaerobic bacteria, and the amounts found are often less than stoicheiometric. Examples are glycine reductase of *C. sticklandii* (0.66 atom selenocysteine/mol) [30], the β-ketothiolase of *C. kluyveri* (up to 1 mol selenomethionine per tetramer) [31], hydrogenase (3.8 mol selenocysteine per octamer [32]) and formate dehydrogenase [33] of *Methanococcus vannielii*. The low content of selenium in *C. acidiurici* xanthine dehydrogenase implies that if selenium is a constituent of the active enzyme, most of it was

lost during the purification. The lability of the enzyme is indicated by the smearing of the proteins on polyacrylamide gel electrophoresis of protein after storage for 1 day. Possibly, more gentle purification techniques would produce a more active enzyme preparation with a higher specific activity. The possible loss of selenium may also be related to the lack of activity of the xanthine dehydrogenase with physiological electron acceptors.

Unlike the chicken xanthine dehydrogenase and bovine milk xanthine oxidase, the enzyme of *C. acidiurici* could be split into subunits by SDS. Considering that the subunit of 53 kDa might be a proteolytic product of the 56 kDa species, there seems to be some correlation in molecular mass of the subunits from the enzyme of *C. acidiurici* with the fragments obtained after limited proteolysis of chicken xanthine dehydrogenase [35] and milk xanthine oxidase [36]. The subunits of the *C. acidiurici* enzyme could be combined in the following order: a monomer of 110 kDa consists of subunits of 26 kDa and 84 kDa, the latter of 26 kDa and 56 kDa. The molecular mass of the dimer of 224 kDa is quite low compared to other enzymes [3] except from *Rhodopseudomonas capsulata* [37], *Enterobacter cloacae* [38] and *Arthrobacter* S-2 [39]. Thus, the ratio E_{280}/E_{450} is smaller than in other molybdenum iron-sulphur flavin hydroxylases [3] for it contains the full complement of flavin and iron/sulphur, but less protein.

The xanthine dehydrogenases of *C. cylindrosporum* [10] and *C. acidiurici* do not couple with NAD or NADP. In *Veillonella alcalescence* (*Micrococcus lactilyticus*), ferredoxin is an efficient electron acceptor besides many artificial dyes [40]. However, ferredoxin (of *C. pasteurianum*) did not react with the enzyme from *C. acidiurici*, although this could be anticipated by the redox potentials of both couples which are both quite low. The formate dehydrogenase of *C. acidiurici* is only active in electron transfer to NAD via ferredoxin using crude cell extracts, but no reaction occurred with purified preparations [41]. Therefore a protein subunit catalyzing an electron transfer to the natural acceptor might be lost too, indicated by the lower molecular weight of the dimer, or modified during enzyme purification as found for NAD − linked xanthine dehydrogenase [42]. Finally, an other carrier not tested, such as flavodoxin, might act as the natural acceptor.

Due to the presence of different redox-active groups, xanthine-oxidizing enzymes have been extensively studied by ESR spectroscopy. The lineshapes of the ESR signals of the two [2Fe-2S] clusters of the enzyme of *C. acidiurici* were similar to those of mammalian milk xanthine oxidase [22–24] and liver xanthine dehydrogenase [43], despite the considerable evolutionary distance between these proteins. Thus, the *C. acidiurici* xanthine dehydrogenase differs from the enzyme from *Veillonella alcalescences* [44], in which the signal of only one type of cluster was detected. The molybdenum centre also appears to be remarkably similar to that in xanthine oxidase.

Because of the instability of the enzyme, the midpoint potentials of the centres have not been determined. However, the lack of reduction of iron-sulphur clusters by xanthine suggests that the potentials of the clusters are more negative than the xanthine/urate couple (−400 mV at pH 7.5) [45]. It may be noted that a protein, of unknown function, isolated from the anaerobic bacterium *Desulfovibrio gigas* was also found to contain molybdenum and two types of [2Fe-2S] clusters with similar g values to *C. acidiurici* xanthine dehydrogenase, but no flavin [50].

The rather large free radical signals observed from flavin are characteristic of xanthine dehydrogenases, in which the semiquinone form of flavin tends to be stabilized [39]. During reduction by dithionite, the radical appeared before the iron-sulphur and Mo(V) signals. Since this was not done under rapid-freeze conditions, and intramolecular electron transfer is presumably relatively fast, this indicates that the FAD/FADH couple has a less negative potential that the iron-sulphur clusters.

Analogy with other xanthine dehydrogenases, together with the analytical data, suggest that the xanthine dehydrogenase of *C. acidiurici* is a dimer, with one FAD, one Mo and two [2Fe-2S] clusters per 110 kDa monomer unit. Regarding the selenium, no information could be obtained by ESR spectroscopy. However, this would only be the case if selenium is subject of a redox reaction during catalysis. However, xanthine dehydrogenase of *C. acidiurici* and *C. barkeri* [29] are

affected by selenium and contain it is non-stoicheiometric portions. They are by far the most active xanthine-oxidizing enzymes known. In case of carbon monoxide oxidase of *Pseudomonas carboxydovorans*, selenium activates the enzyme reaction; however, it is ESR-silent and not part of the molybdenum cofactor, but of the protein [47]. The latter enzyme also contains some zinc. The presence of trace of tungsten within the purified xanthine dehydrogenase of *C. acidiurici* might result from its presence in the growth media [4] and its specific incorporation into the formate dehydrogenase protein fraction [48]. However, molybdenum enzymes can generally incorporate tungsten and form by that inactive enzyme species [8,26].

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