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Xanthine Dehydrogenase and 2-Furoyl-Coenzyme A Dehydrogenase from *Pseudomonas putida* Ful: Two Molybdenum-Containing Dehydrogenases of Novel Structural Composition

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The constitutive xanthine dehydrogenase and the inducible 2-furoyl-coenzyme A (CoA) dehydrogenase could be labeled with [^{185}W]tungstate. This labeling was used as a reporter to purify both labile proteins. The radioactivity cochromatographed predominantly with the residual enzymatic activity of both enzymes during the first purification steps. Both radioactive proteins were separated and purified to homogeneity. Antibodies raised against the larger protein also exhibited cross-reactivity toward the second smaller protein and removed xanthine dehydrogenase and 2-furoyl-CoA dehydrogenase activity up to 80 and 60% from the supernatant of cell extracts, respectively. With use of cell extract, Western immunoblots showed only two bands which correlated exactly with the activity stains for both enzymes after native polyacrylamide gel electrophoresis. Molybdate was absolutely required for incorporation of ^{185}W, formation of cross-reacting material, and enzymatic activity. The latter parameters showed a perfect correlation. This evidence proves that the radioactive proteins were actually xanthine dehydrogenase and 2-furoyl-CoA dehydrogenase. The apparent molecular weight of the native xanthine dehydrogenase was about 300,000, and that of 2-furoyl-CoA dehydrogenase was 150,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of both enzymes revealed two protein bands corresponding to molecular weights of 55,000 and 25,000. The xanthine dehydrogenase contained at least 1.6 mol of molybdenum, 0.9 ml of cytochrome *b*, 5.8 mol of iron, and 2.4 mol of labile sulfur per mol of enzyme. The composition of the 2-furoyl-CoA dehydrogenase seemed to be similar, although the stoichiometry was not determined. The oxidation of furfuryl alcohol to furfural and further to 2-furoic acid by *Pseudomonas putida* Ful was catalyzed by two different dehydrogenases.

Pseudomonas putida Ful was isolated from an enrichment culture with 2-furoic acid as the sole source of carbon and energy (24). The formation of 2-furoyl-coenzyme A (CoA) is the first reaction step in 2-furoic acid degradation. The second enzymatic step is catalyzed by a dehydrogenase that introduces a hydroxyl group to form 5-hydroxy-2-furoyl-CoA (23, 24, 49, 50). Other substrates for *P. putida* Ful were furfuryl alcohol and furfural, which were degraded via 2-furoic acid and 2-furoyl-CoA (Fig. 1). In contrast, the anaerobic degradation of furfural by *Desulfovibrio furfuralis* does not involve the respective CoA ester (6, 12).

P. putida Ful is also able to grow on xanthine as the sole carbon and energy source. Unlike the 2-furoyl-CoA dehydrogenase (FCoADH), the xanthine dehydrogenase (XDH) is a constitutive, separate enzyme that also can carry out an FCoADH reaction (24). Although a FCoADH from a different strain of *P. putida* was described as containing supposedly copper but no iron or flavin (23), the reactions catalyzed by this enzyme seem to be quite analogous to those catalyzed by molybdenum-containing hydroxylases (2, 9, 15). In all of these cases, the hydroxyl group derives from water (2, 23; W. C. Evans, Biochem. J. **103**:1p–3p, 1967). Xanthine oxidases and dehydrogenases from different sources have been widely studied, and numerous methods of purification have been described (9). Generally, the eucaryotic xanthine oxidase has a native molecular weight of about 300,000. This large protein can be dissolved into two inactive subunits of approximately 150,000 daltons (Da) (9). Each subunit contains one atom of molybdenum, one molecule of FAD, and four nonheme iron-sulfur groups (9, 16). Under proteolytic conditions, this large subunit can be cleaved into three domains with molecular weights of about 90,000, 42,000, and 20,000 (10, 36). The 90,000-Da fragment contains the molybdenum cofactor and the iron-sulfur center, whereas the FAD is located on the 42,000-Da fragment (10, 37). Several bacterial enzymes are known which can be dissociated by sodium dodecyl sulfate (SDS) into a similar subunit structure and cofactor content. The XDH from *Clostridium acidiurici* (53), the aldehyde oxidase-dehydrogenase (39, 40), nicotine dehydrogenase (13), the nicotinate dehydrogenase (11, 28; M. Nagel, Ph.D. thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1989), and the carbon monoxide dehydrogenase (22, 31) belong to this group.

Some atypical XDHs and nicotinate dehydrogenases that do not seem to fit into the general scheme outlined above have been described but not extensively purified. The XDHs from different *Pseudomonas* species exhibit a molecular weight of about 270,000 and seem to contain two nonidentical subunits (42, 45, 57). Furthermore, these molybdoenzymes apparently lack flavin but possess heme (42, 58). The nicotinate dehydrogenase of *Pseudomonas ovalis* Chester also contains a cytochrome (21). Heme is a regular constituent of the molybdoenzyme sulfite oxidase (40). The enzyme from liver is a homodimer of 55,000 Da with a native molecular weight of 115,000. Each subunit contains a heme and one atom of molybdenum; no iron-sulfur center and no flavin are detected (8). Despite their structural diversity, all

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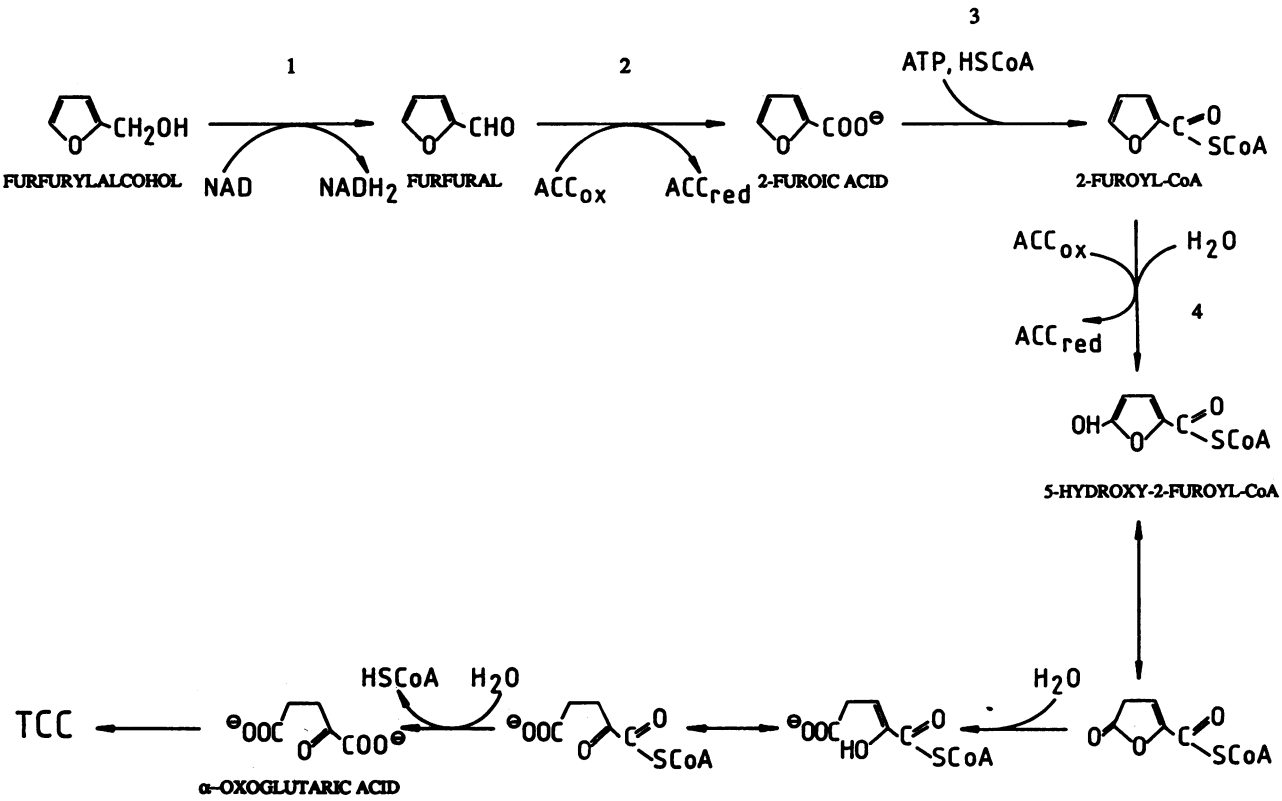

FIG. 1. Pathway of furfuryl alcohol oxidation by *P. putida* Ful. 1, Furfuryl alcohol dehydrogenase; 2, furfural dehydrogenase; 3, 2-furoyl-CoA synthetase; 4, FCoADH. ACC, Acceptor; TCC, tricarboxylic acid cycle.

of these molybdoenzymes seem to have a similar molybdenum cofactor (17).

Molybdenum and especially its competitive inhibitor tungsten (32) exhibited a strong influence on growth, 2-furoic acid consumption and XDH and FCoADH activities (24). To prove that both enzymes actually belong to the class of molybdenum-containing dehydrogenases, both labile enzymes were purified to homogeneity by incorporation of [^{185}W]. We now report that the formation of XDH and FCoADH from *P. putida* Ful is specifically induced by molybdate. These enzymes are unique in their structural composition and content of redox-active groups.

MATERIALS AND METHODS

Organisms. *Arthrobacter oxidans* P-34 (DSM 419), *Arthrobacter picolinophilus* (DSM 20665^T), *Bacillus niacini* (DSM 2923), and *Clostridium barkeri* (DSM 1223) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Federal Republic of Germany). *P. putida* N-9 (ATCC 14674) was obtained from the American Type Culture Collection (Rockville, Md.). *P. putida* Ful (24), *P. putida* Chin IK, *Alcaligenes* sp. strain DPK 1, *Mycobacterium* sp. strain INA 1, *Arthrobacter* sp. strain P1, and the gram-negative rod strain KNA 2 were isolated in our laboratory.

Culture media and preparation of cell extracts. Cells were usually grown in 250-ml baffled flasks containing 50 ml of medium at 30°C as described previously (24). If indicated, the growth medium contained 10 mM furfuryl alcohol or furfural as the sole source of carbon and energy. To determine a molybdenum requirement for growth, a trace element solution without molybdate and tungstate was used (24). Mass cultures for purification of FCoADH were usually grown in 2-liter baffled flasks containing 500 ml of medium with 20 mM 2-furoic acid as the carbon and energy source containing 2×10^{-8} M molybdate and 2×10^{-7} M [^{185}W]tungstate (1.2 MBq). For purification of XDH, *P. putida* Ful was cultured on Luria broth containing 2×10^{-7} M [^{185}W]tungstate (1.2 MBq). Cells were harvested and disrupted as described before (24).

Enzyme assays. Furfuryl alcohol dehydrogenase was measured by determining NAD reduction at 340 nm. Reaction mixtures contained (in a final volume of 1 ml) 800 μl of 50 mM potassium phosphate buffer (pH 8.0), 100 μl of 50 mM furfuryl alcohol, 50 μl of 30 mM NAD, and various amounts of protein.

The assay for furfural dehydrogenase consisted of 800 μl of 50 mM potassium phosphate buffer (pH 7.0), 100 μl of 50 mM furfural, 50 μl of acceptor mix (5 ml of 10 mM phenazine ethosulfate in H_2O plus 1 ml of 5% 2,6-dichlorophenolindophenol in ethanol), and various amounts of protein to give a final volume of 1 ml. Reduction of the blue dye was measured by decrease of A_{522} ($\varepsilon = 8.6\ mM^{-1}\ cm^{-1}$).

Activity of 2-furoyl-CoA synthetase and FCoADH were measured as described previously (24).

XDH was measured by determining the reduction of thiazolyl blue [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) at 590 nm, using an absorption coefficient of $\varepsilon = 8.8\ mM^{-1}\ cm^{-1}$. Reaction mixtures contained (in a final volume of 1.3 ml) 900 μl of 50 mM potassium phosphate buffer (pH 7.8) containing 1% Triton X-100, 200 μl of 5 mM xanthine, 50 μl of 10 mM thiazolyl blue, 50 μl of

10 mM phenazine ethosulfate, and various amounts of protein. Several other electron acceptors were also tested in these enzyme assays (final millimolar concentration): benzyl viologen, 10; brilliant cresyl blue, 0.15; ferricyanide, 1; methylene blue, 0.15; methyl viologen, 8; nitroblue tetrazolium chloride, 0.2; and thionine, 0.15.

Specific activities were expressed as micromoles of substrate transformed per minute per milligram of protein at 30°C. Protein was measured by the method of Bradford (5).

HPLC. High-performance liquid chromatography (HPLC) of furfuryl alcohol, furfural, and 2-furoic acid was performed by using a Kontron system with a model 420 pump, an injector, an RP-18 reverse-phase column (4 by 250 mm; 10-μm particle size), and a model 430 UV detector. Isocratic elution was performed with 60% H_2O and 40% methanol containing 5 mM tetrabutylammonium hydrogen sulfate (pH 7.5) (adjusted with 1 M dipotassium hydrogen phosphate) at a flow rate of 0.3 ml/min. The detection was set at 220 nm.

Breakdown of furfuryl alcohol and furfural. Cells were grown on furfuryl alcohol or furfural and harvested by centrifugation; 0.1 g of cells was suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 0.05% Triton X-100. This suspension had to be frozen for at least 12 h at 20°C (33). The cells were thawed and incubated with the corresponding substrate. After several times, a 100-μl sample was taken and centrifuged, and the supernatant was analyzed by HPLC.

Determination of α-oxoglutaric acid. Cells were grown on 2-furoic acid and harvested by centrifugation. Then 1 g of cells was incubated in 25 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM 2-furoic acid and 1 mM arsenite. Within 24 h of incubation, substrate concentration and α-oxoglutaric acid formation were measured by NADH oxidation at 340 nm catalyzed by glutamate dehydrogenase.

α-Oxoglutaric acid was further determined by its conversion into the dinitrophenylhydrazone and its thin-layer chromatography on silica gel plates (43).

Purification of the smaller ^{185}W-labeled protein. *P. putida* Ful cells labeled with ^{185}W were mixed with nonradioactive cells before preparation of the cell extract. The purification procedure started with 800 mg of protein in the cell extract containing 10 kBq of radioactivity. The extract was applied to a column (5.0 by 40 cm; bed volume, 40 ml) of Q-Sepharose Fast Flow that was previously equilibrated with 50 mM potassium phosphate buffer (pH 7.5). After being washed with 300 ml of this buffer, proteins were eluted with a gradient of 0 to 1 M KCl in 500 ml of the same buffer at a flow rate of 30 ml/h. FCoADH activity was eluted at approximately 0.5 M KCl, pooled, concentrated by ultrafiltration (Ultrafilter SM 145 49; Sartorius, Göttingen, Federal Republic of Germany) to a final volume of 4 ml, and applied to a column (1.6 by 100 cm; bed volume, 190 ml) of Sephacryl S-300 HR previously equilibrated with 50 mM potassium phosphate buffer (pH 7.5). The proteins were separated by using the latter buffer at a flow rate of 18 ml/h. Fractions with FCoADH activity were pooled, dialyzed at 4°C against 10 mM potassium phosphate buffer (pH 7.0), and applied to a column (2.6 by 40 cm; bed volume, 6 ml) of hydroxylapatite (Bio-Gel HTP) that was equilibrated with dialysis buffer. After being washed with this buffer (20 ml), a stepwise gradient was applied of 20 ml each of 0.05, 0.1, 0.25, 0.5, 0.75, and 1 M potassium phosphate (pH 7.0) at a flow rate of 12 ml/h. The pooled enzyme fraction were directly applied to a column (2.6 by 40 cm; bed volume, 5 ml) of phenyl-Sepharose previously equilibrated with 1 M potassium phosphate buffer (pH 7.5). After being washed with this buffer (20 ml), a stepwise gradient was applied of 20 ml each of 0.5, 0.1, 0.05, 0.01, and 0.005 M potassium phosphate buffer (pH 7.5). The pooled fractions were concentrated by ultrafiltration to a final volume of 5 ml and then applied in 1-ml portions to an HPLC gel filtration column (Bio-Rad TSK-400; 0.75 by 30 cm) previously equilibrated with 100 ml of potassium phosphate buffer (pH 7.5). The proteins were separated by using this buffer with a flow rate of 18 ml/h.

Purification of the larger ^{185}W-labeled protein. Radioactive and nonradioactive *P. putida* Ful cells were suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM iodoacetamide and 100 mM ammonium sulfate as stabilizers (23) before cells were disrupted by sonic treatment. The purification procedure started with 1,160 mg of protein in the extract containing 40.8 kBq of radioactivity. The extract was applied to a column of Q-Sepharose Fast Flow as described above. XDH activity was eluted at approximately 0.5 M KCl. The pooled enzyme solution was concentrated by ultrafiltration to a final volume of 4 ml and applied to a column of Sephacryl S-300 HR as described above. Fractions with XDH activity were pooled and directly applied to a column (2.6 by 40 cm; bed volume, 10 ml) of folate-Sepharose 4B previously equilibrated with 50 mM potassium phosphate buffer (pH 7.5). Folate-Sepharose 4B was prepared as described previously (38). After being washed with 100 ml of the equilibration buffer, a gradient was applied consisting of 0 to 0.5 mM hypoxanthine in equilibration buffer and a further gradient of 0 to 1 M KCl. Fractions with radioactivity were pooled, dialyzed at 4°C against 10 mM potassium phosphate buffer (pH 7.0), and applied to a column containing hydroxylapatite as described above.

Electrophoresis. Fractions containing enzyme activity were tested for purity by SDS-polyacrylamide gel electrophoresis (29) by using 12% polyacrylamide gels and a molecular weight marker kit (MW-SDS-70L; Sigma, Deisenhofen, Federal Republic of Germany) in a Minigel apparatus (0.1 by 10 by 10 cm; Biometra, Göttingen, Federal Republic of Germany). Linear polyacrylamide gradient gel electrophoresis was performed as described previously with a 4 to 27.5% polyacrylamide gradient (24). Electrophoresis was carried out at 100 V for 15 h at 4°C with 5 mM Tris–38 mM glycine buffer. FCoADH and XDH activities were demonstrated in polyacrylamide gradient gels by using 2-furoyl-CoA (0.3 mM) for FCoADH and xanthine (1 mM) for XDH as the substrates. Nitroblue tetrazolium chloride (0.05%) was used as an electron acceptor. The gel was incubated in the staining solution in the dark for approximately 30 min.

Molecular weight determination. The molecular weights of furfural dehydrogenase, homogenous XDH, and FCoADH were determined by gel filtration chromatography on an HPLC gel filtration column (TSK-400; 0.75 by 30 cm) by using ferritin (M_r 450,000), bovine liver catalase (M_r 240,000), rabbit muscle aldolase (M_r 158,000), bovine serum albumin (M_r 66,000), and chicken serum albumin (M_r 45,000). The native molecular weights of XDH and FCoADH were determined by gel filtration on Sepharose CL-6B, using the molecular weight markers mentioned above.

Native gradient polyacrylamide gel electrophoresis in the presence of molecular weight marker proteins was done to estimate the native molecular weight of the furfuryl alcohol dehydrogenase and the furfural dehydrogenase by staining the lanes for activity. The staining solution for the furfuryl alcohol dehydrogenase contained in 10 ml of 50 mM potassium phosphate buffer (pH 7.5), 0.2 ml of 2.5% nitroblue tetrazolium chloride, 0.3 ml of 30 mM NAD, and 5 ml of 50

mM furfuryl alcohol. The staining solution for the furfural dehydrogenase contained in 10 ml of 50 mM potassium phosphate buffer (pH 7.5), 0.2 ml of 2.5% nitroblue tetrazolium chloride, and 5 ml of 50 mM furfural. The gels were incubated in the staining solutions in the dark for approximately 30 min.

Determination of protein spectra and radioactivity. Protein spectra were obtained at room temperature with a double-beam spectrophotometer (Uvikon 810; Kontron Instruments, Eching, Federal Republic of Germany). Samples were measured against a reference containing the sample buffer.

Radioactivity was determined in a liquid scintillation counter (model LS 75,00; Beckman, Munich, Federal Republic of Germany) by using Opti-Fluor (Packard, Frankfurt, Federal Republic of Germany) as the scintillation fluid and was expressed as counts per minute.

Identification of the molybdenum cofactor. Molybdoenzymes oxidized by KI and I_2 showed the typical blue fluorescence of the oxidized pterin as part of the molybdenum cofactor (23). XDH or FCoADH solution (500 μl; 0.5 mg/ml) was adjusted to pH 2.5 with 15% phosphoric acid. A 2% KI–1% I_2 solution was added to the sample, and both were heated for 30 min in a boiling water bath, cooled, and centrifuged at 20,000 × *g* for 10 min. The supernatant was adjusted to 1 N NH_4OH. The fluorescence spectra were obtained at room temperature with a spectrofluorometer (SFM 23/3 with power supply SFM 23; Kontron). The relative fluorescence was obtained at 360 nm (excitation) and 480 nm (emission).

Analysis of cytochrome. Low-temperature spectrophotometry is used to detect differences in the absorption spectra of hemoproteins (19). The spectra were obtained with a double-wavelength spectrophotometer (Sigma ZWS II; Biochem, Munich, Federal Republic of Germany). The heme content was quantitated as the pyridine hemochromogen (14). Cytochrome *b* was stained after SDS-polyacrylamide gel electrophoresis by 3,3′,5,5′-tetramethylbenzidine (19).

Determination of molybdenum, iron, and sulfur. The analysis for molybdenum (7), iron (51), and sulfur (3) was done as described.

Immunological techniques. Antiserum was raised by subcutaneous injection of 400 μg of the purified larger ^{185}W-labeled protein together with Freund adjuvants into a 3-month-old rabbit. A booster injection with 300 μg of antigen was given 2 weeks later. Ten days later the rabbit was bled. Preimmune serum was taken before immunization, and no cross-reaction could be detected. Immunoglobulin G (IgG) specific against the larger radioactive protein from the resulting serum was purified on protein G-Sepharose CL-6B (41). Antibody titer against the larger radioactive protein was determined by double-immunodiffusion tests. The specificity of antibodies raised against the enzyme was demonstrated by anti-rabbit IgG alkaline phosphatase labeling of blotted proteins (48), and the larger ^{185}W-labeled protein in extracts was quantitated by rocket immunoelectrophoresis (54). For analysis of relationships, extracts from several other bacteria were tested by double-immunodiffusion tests, rocket immunoelectrophoresis, and the Western immunoblot technique.

Inhibition of XDH and FCoADH activity was tested after incubation with various amounts of antibodies raised against the larger radioactive protein at 30°C for 15 min, followed by low-speed centrifugation.

Chromatography on IgG-Sepharose. Protein G-Sepharose-purified antibodies raised against the larger ^{185}W-labeled protein (15 mg) were covalently bound to CNBr-activated Sepharose 4B (1 g) as described previously ("Affinity Chromatography, Principles and Methods," Pharmacia Fine Chemicals, Uppsala, Sweden, 1983). Cell extract of *P. putida* Fu1 grown on Luria broth or xanthine or partially purified XDH (up to 500 mg) was applied to the antibody column (5 ml) at a flow rate of 6 ml/h, followed by a washing step with 50 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 100 mM NaCl to remove unspecific bound proteins. The XDH was eluted from the IgG-Sepharose by electroelution with a Biotrap BT 1000 (Schleicher & Schüll, Dassel, Federal Republic of Germany) with 5 mM Tris–38 mM glycine buffer (pH 8.3) at 100 V for 12 h.

Chemicals. Enzymes and coenzymes were obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany), anti-rabbit IgG alkaline phosphatase, molecular weight markers, and NAD were from Sigma, Sepharose CL-6B, CNBr-activated Sepharose 4B, Sephacryl S-300 HR, Q-Sepharose Fast Flow, and protein G-Sepharose 4B were obtained from Pharmacia (Freiburg, Federal Republic of Germany), benzyl viologen, methyl viologen, nitroblue tetrazolium chloride, and thiazolyl blue were obtained from Serva (Heidelberg, Federal Republic of Germany), and hydroxylapatite Bio-Gel HTP and the TSK-400 HPLC column were from Bio-Rad Laboratories (Munich, Federal Republic of Germany). ^{185}W was obtained from Amersham Buchler (Braunschweig, Federal Republic of Germany). All other chemicals were of the highest purity available from commercial sources.

RESULTS

Breakdown of furfuryl alcohol and furfural. *P. putida* Fu1 grew also on furfuryl alcohol or furfural as substrate, as became evident during this study. Furfuryl alcohol, furfural, and 2-furoic acid could be separated by HPLC. Permeabilized cells of *P. putida* Fu1 which were grown on furfuryl alcohol were incubated with this component as substrate. Furfuryl alcohol was first converted to furfural, which then resulted in 2-furoic acid (Fig. 1), as indicated by the appearance or disappearance of the respective peaks during HPLC analysis. 2-Furoic acid was transformed by the 2-furoyl-CoA synthetase into 2-furoyl-CoA (24). 2-Furoyl-CoA was converted into α-oxoglutaric acid by resting cells, which were incubated with 2-furoic acid and arsenite. Under these conditions, one molecule of 2-furoic acid gave rise to one molecule of α-oxoglutaric acid (Fig. 1).

Furfuryl alcohol dehydrogenase and furfural dehydrogenase. Growth of *P. putida* Fu1 on furfuryl alcohol and furfural was similarly affected by molybdate and tungstate, respectively, as described for 2-furoic acid (24). To exclude a possible involvement of the molybdoenzyme aldehyde oxidase in both reactions, the respective enzymes involved were partially characterized.

Furfuryl alcohol dehydrogenase activity was catalyzed in extracts by a soluble enzyme which exhibited a specific activity of 0.02 U/mg of protein. The furfuryl alcohol dehydrogenase was active only with NAD as natural electron acceptor. A pH optimum was observed at 8.0 in potassium phosphate buffer. Michaelis-Menten-type saturation kinetics were obtained with furfuryl alcohol. The apparent K_m was determined from Lineweaver-Burk plot to be 0.14 mM. The native molecular weight was determined to be 170,000 (±20,000) by activity stain in a native gradient gel. After gel filtration chromatography on a TSK-400 column (even in the

presence of Zn^{2+} ions), no enzyme activity could be recovered. Ethanol was converted by extracts with a specific activity of about 0.06 U/mg.

Furfuryl alcohol degradation was catalyzed by furfural dehydrogenase to form 2-furoic acid. Specific activities of about 0.15 U/mg of protein were measured in crude extracts. A pH optimum was observed at pH 7.0. Michaelis-Menten-type saturation kinetics were obtained with furfural, and the apparent K_m was determined to be 0.16 mM. The approximate molecular weight of the native enzyme was 100,000 (±20,000), as determined by gel filtration chromatography and activity stain in a native gradient gel. Acetaldehyde and benzaldehyde were also converted by these extracts, with specific activities of 0.15 U/mg of protein for acetaldehyde and 0.3 U/mg of protein for benzaldehyde. The furfural dehydrogenase reaction was active with NAD as well as with dichlorophenolindophenol plus phenazine ethosulfate.

Both enzymes mentioned above were inducible, for no enzyme activities were detected in cells grown on 2-furoic acid or on Luria broth. The activities of the soluble enzymes furfuryl alcohol dehydrogenase, furfural dehydrogenase, and 2-furoyl-CoA synthetase were not affected by growing *P. putida* Ful in mineral salts medium on 10 mM furfuryl alcohol in the presence of molybdate and tungstate, unlike FCoADH (24). As observed previously for FCoADH (24) the activity of XDH decreased in the presence of 10^{-6} M tungstate to just 20% of the activity after growth in the presence of 10^{-6} M molybdate (0.3 U/mg of protein = 100%).

Incorporation of ^{185}W. *P. putida* was grown on mineral salts medium (24) with 10 mM 2-furoic acid and 2×10^{-8} M molybdate and 2×10^{-7} M [^{185}W]tungstate (1.2 MBq). More than half of the radioactivity added was recovered in the soluble part of the cell extract. The radioactive extract was mixed with nonradioactive cell extract. This extract was applied to a gel filtration column, and the labeling patterns of the soluble proteins were monitored after separation by gel chromatography. The first ^{185}W radioactivity peak eluted with the void volume. The second radioactivity peak eluted at a position corresponding to proteins of a native molecular weight of approximately 300,000. The third radioactivity peak corresponded to proteins of 150,000 Da. A fourth radioactivity peak probably represented unbound tungstate. The radioactivity peaks coeluted with the XDH activity at a molecular weight of 300,000 (±20,000) and with the FCoADH activity at a molecular weight of about 300,000 and 150,000 (Fig. 2). The FCoADH activity coeluting with XDH was due to an unspecificity of the latter constitutive enzyme (24), for extracts of cells grown on Luria broth or xanthine exhibited no FCoADH activity at a molecular weight of 150,000 but showed activity at a molecular weight of 300,000. Fluorescence peaks after KI-I_2 oxidation coeluted with both enzyme activities. Growth of *P. putida* Ful on 2-furoic acid in the absence of molybdate showed only one ^{185}W radioactivity peak which eluted at the void volume, which indicates a direct role of molybdenum in the formation of both ^{185}W-labeled proteins.

Purification of the smaller ^{185}W-labeled protein (FCoADH). After cell breakage and removal of cell debris, more than 90% of FCoADH activity and 50% of radioactivity were found in the supernatant. During the first three purification steps, FCoADH activity coeluted perfectly with radioactivity. Thereafter, no enzyme activity could be detected because of the instability of the FCoADH activity. The specific radioactivity increased during purification. This protein of *P. putida* Ful was obtained with 21-fold-increased specific radioactivity and 9% recovery (Table 1).

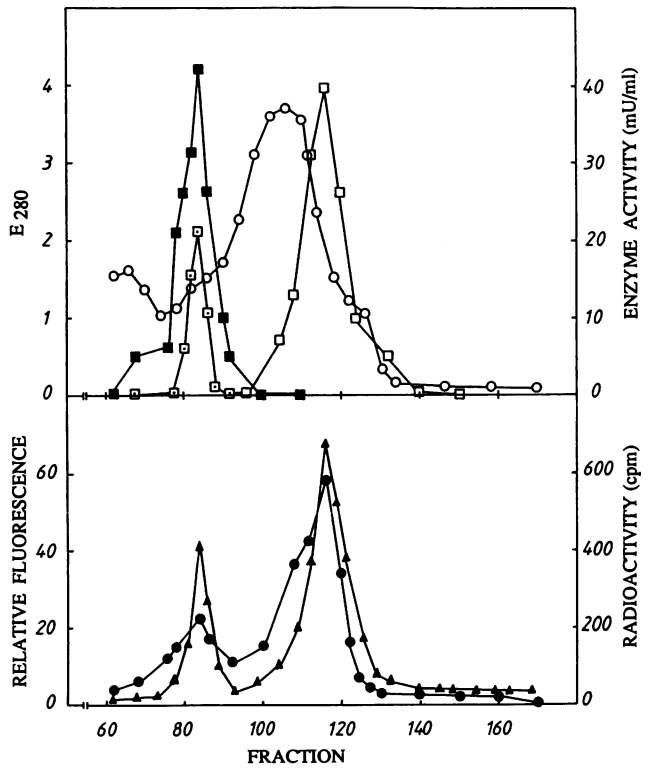

FIG. 2. Chromatography on Sepharose CL-6B of ^{185}W-labeled extract of *P. putida* Ful grown on 2-furoic acid. Symbols: ■, activity with xanthine; □, activity with 2-furoyl-CoA; ○, ε_{280}; ●, relative fluorescence; ▲, radioactivity.

Purification of the larger ^{185}W-labeled protein (XDH). After cells were broken and centrifuged, more than 90% of XDH activity and 50% of radioactivity were found in the supernatant. The XDH activity was extremely unstable and could be measured only until the third purification step. During these steps, XDH activity and radioactivity coeluted perfectly. The specific radioactivity increased during purification. This

TABLE 1. Purification of FCoADH (smaller ^{185}W-labeled protein) and XDH (larger ^{185}W-labeled protein) from *P. putida* Ful

Purification step	Total protein (mg)	Total radioactivity (10^3 cpm)	Specific radioactivity (cpm/mg)	Recovery (%)	Purification (fold)[a]
FCoADH					
Cell extract	800	600	750	100	1
Q-Sepharose	64	304	3,753	51	5
Sephacryl S-300	20	210	10,630	35	15
Hydroxylapatite	14	164	11,720	27	16
Phenyl-Sepharose	5.2	96	12,800	16	17
TSK 400	3.2	50	15,700	9	21
XDH					
Cell extract	1,120	2,447	1,451	100	1
Q-Sepharose	63	215	3,419	9	2
Sephacryl S-300	18	188	9,385	8	6
Folate-Sepharose	5	94	18,840	5	13
Hydroxylapatite	4.5	10	21,978	4	15

[a] Based on counts per minute per milligram of protein.

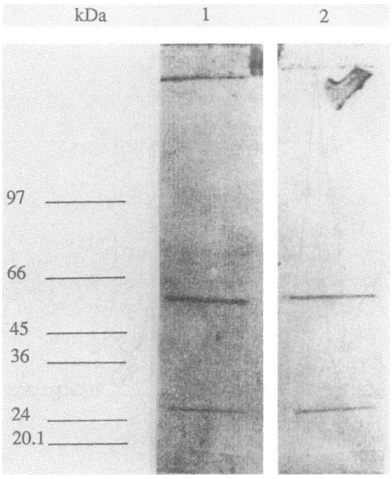

FIG. 3. SDS-polyacrylamide gel electrophoresis of the larger (lane 1) and the smaller (lane 2) ^{185}W-labeled proteins in the presence of molecular weight marker proteins.

protein of *P. putida* Fu1 was obtained with 15-fold-increased specific radioactivity and 4% recovery (Table 1).

Stability of enzyme activities. Both enzyme activities were stable over a long time period when cell extract was frozen at −20°C; however, during all purification steps tried, enzyme activity was finally completely lost. When the IgG-Sepharose column was used, the larger protein was purified to homogeneity in only one step, but again no activity was left. Electroelution of the bound larger protein to IgG-Sepharose fully inactivated the enzyme.

To stabilize the enzyme activities, several procedures were tested. Addition of glycerol, 2-furoic acid, and xanthine had no effect. The presence of saccharose (10%) in the buffers stabilized XDH activity to some extent when the antibody column was used. Addition of 100 mM ammonium sulfate and 1 mM iodoacetamide (23) before cells were broken stabilized XDH during several purification steps. Incubation of inactivated XDH with 25 μM FAD or FMN, 2.5 mM Mg^{2+} ions, 5 mM molybdate, 50 μM cytochrome *b*, or heme for 30 min at 30°C did not affect enzyme activity.

Determination of purity and molecular mass. Electrophoresis of the purified larger and smaller ^{185}W-labeled proteins on SDS-polyacrylamide gels resulted in both cases in two protein bands with molecular weights of 55,000 and 25,000 (Fig. 3). Molecular mass estimations done by native gradient polyacrylamide gel electrophoresis under nondenaturing conditions and gel filtration chromatography resulted in molecular weights of about 300,000 (±20,000) and 150,000 (±10,000), respectively, for both radioactive proteins. Compared with the molecular masses of the SDS-denatured proteins, these proteins thus consisted of two nonidentical subunits of $\alpha_4\beta_4$ and $\alpha_2\beta_2$ composition, respectively.

Substrate and electron acceptor usage. Because of the instability of XDH and FCoADH activities, all tests using different substrates and electron acceptors could be done only with partially purified preparations of both ^{185}W-labeled proteins, which still contained enzyme activities. 2-Furoyl-CoA was the only substrate for the smaller radioactive protein, whereas the larger protein converted hypoxanthine (83%) and 2-furoyl-CoA (51%) with a high rate (when xanthine was set to be 100%). Michaelis-Menten-type saturation kinetics were obtained with the smaller ^{185}W-labeled protein for 2-furoyl-CoA and with the larger protein for xanthine and 2-furoyl-CoA. The apparent K_m values were determined from Lineweaver-Burk plots to be 0.05 mM for 2-furoyl-CoA (smaller radioactive protein), 0.14 mM for xanthine, and 0.25 mM for 2-furoyl-CoA (larger radioactive protein).

In the case of the XDH-containing preparation, enzyme activity was determined with thiazolyl blue (set to be 100%), nitroblue tetrazolium chloride (97%), NAD(P) (79%), dichlorophenolindophenol plus phenazine ethosulfate (67%), ferricyanide (63%), and methylene blue (51%). No activity was detected with oxygen, methyl viologen, or benzyl viologen. FCoADH-containing fractions were active with tetrazolium salts, methylene blue, and dichlorophenolindophenol plus phenazine ethosulfate as electron acceptors, whereas no activity was found with NAD(P), methyl viologen, or benzyl viologen, as observed before for crude extracts (24). The XDH activity with NAD(P) might have been due to the presence of an additional protein(s), for the activity with these acceptors was lost more rapidly.

Spectra and cofactor content. The absorption spectrum of the homogeneous larger ^{185}W-labeled protein (containing in former purification steps XDH activity) exhibited a defined maximum at 416 nm (Fig. 4). When the protein was reduced with dithionite, the maximum of the Soret band shifted from 416 to 422 nm, and maxima of the α and β bands were obtained at 556 and 528 nm. Low-temperature spectrophotometry at −190°C revealed an α band at 556 nm, as described for cytochrome *b* (19). Fluorescence spectra after oxidation of this protein with KI-I_2 showed excitation and emission maxima at 385 and 462 nm (Fig. 4), respectively, indicative for bactopterin (35).

The absorption spectrum of the homogeneous smaller ^{185}W-labeled protein (containing previously FCoADH activity) exhibited absorbance only in the range of 400 nm and low absorbance at 550 nm, as is known for certain iron-sulfur-containing proteins. An absorption spectrum similar to that depicted in Fig. 4 was obtained before the last purification step, indicating that the cytochrome moiety was finally separated. Fluorescence spectra still showed excitation and emission maxima at 383 and 455 nm, respectively, indicative for the presence of bactopterin in this enzyme also (35).

The cofactor content was determined for the larger radioactive protein after its purification to homogeneity by using an IgG-Sepharose column. It was at least 1.6 mol of molybdenum, 0.9 mol of cytochrome *b*, 5.8 mol of iron, and 2.4 mol of labile sulfur per mol of enzyme. No chemical analysis was done for the smaller radioactive protein, for that protein could not be selectively purified by that method.

SDS-polyacrylamide gels that were stained with 3,3′,5,5′-tetramethylbenzidine, for localization of the cytochrome *b* showed a band at a molecular weight of 25,000 but none at 55,000, indicative of localization on the small subunit.

Immunological studies. The specificity of the isolated antibodies against the larger ^{185}W-labeled protein was proved by the Western immunoblot technique after native polyacrylamide gel electrophoresis. By that method, cell extract showed only two bands which exactly correlated with the bands obtained after activity staining for XDH and FCoADH, respectively. Two immunolabeled bands with subunit molecular weights of about 55,000 and 25,000 were detected after SDS-polyacrylamide gel electrophoresis of cell extract or of a homogeneous preparation of the larger radioactive protein. After separation of the homogeneous smaller ^{185}W-labeled protein by SDS-polyacrylamide gel electrophoresis, only one band with a molecular mass of about 55,000 Da reacted with the antibodies directed against

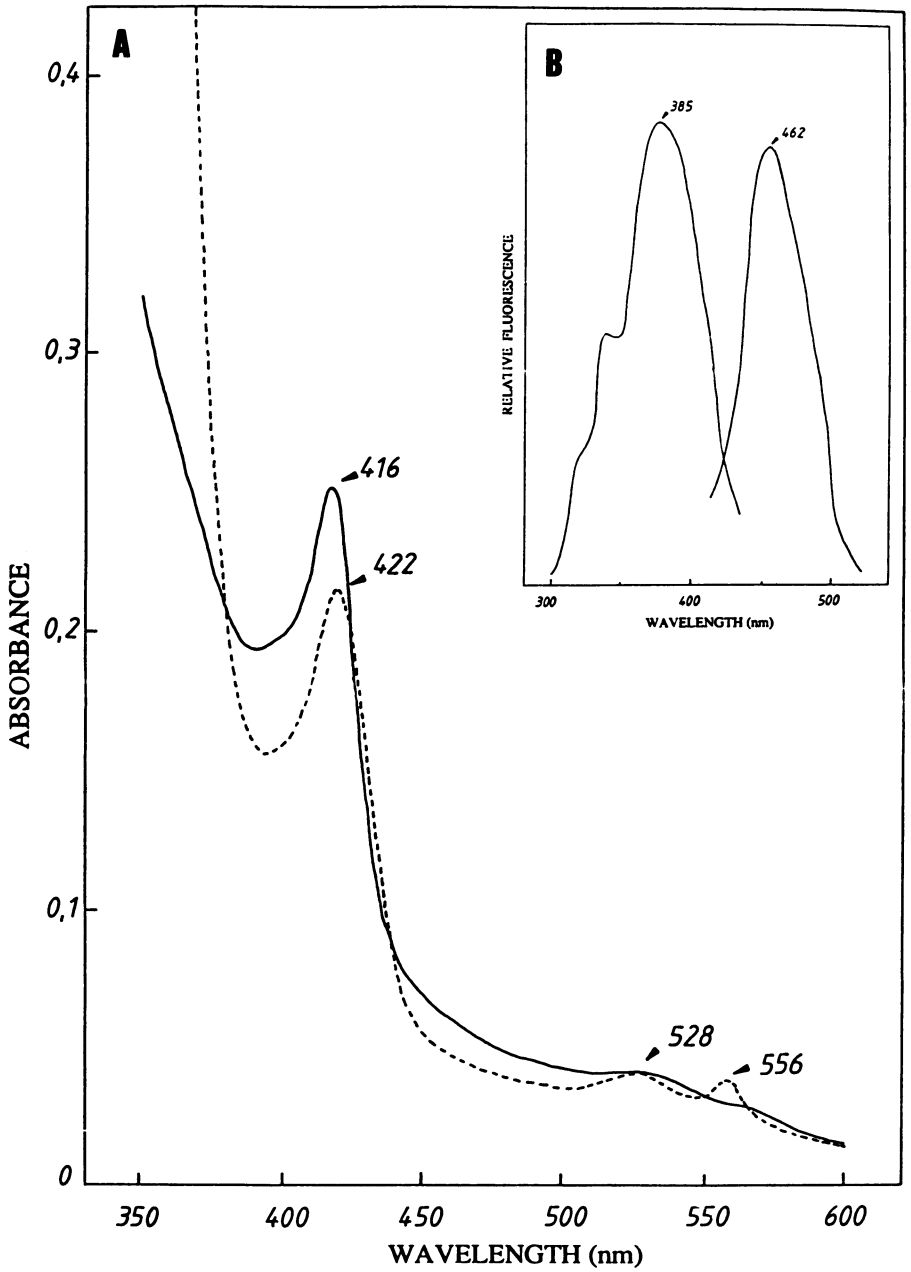

FIG. 4. Spectra of the purified larger ^{185}W-labeled protein. (A) Oxidized (——) and reduced (– – –) enzyme; (B) excitation and emission fluorescence spectra of the KI-I_2-oxidized protein.

the larger radioactive protein, indicating a specificity of the antibodies to react with the large subunit.

After incubation of cell extract with up to 150 μg of IgG per mg of protein, 80% of the XDH activity initially present in cell extract was obtained in the pellet and 12% was left in the supernatant. The FCoADH activity was decreased to 60% in an analogous experiment (Fig. 5).

The amount of cross-reacting material was quantitated by rocket immunoelectrophoresis in cell extracts of *P. putida* Ful grown on xanthine or on 2-furoic acid by using a standard titration with both purified proteins (1 to 8 μg). The height of the rockets obtained by reaction of the antibodies with the smaller ^{185}W-labeled protein was about 20 to 30% that of the large ^{185}W-labeled protein. The contents were calculated to be 2% of the soluble extract of *P. putida* Ful grown on xanthine and 0.8 to 1.8% of the extract of cells grown on 2-furoic acid, taking into account the different reactivities of the antibodies against both proteins (Fig. 6).

Proteins that coeluted with XDH and FCoADH activity were labeled by ^{185}W only if molybdate was supplied (Fig. 2). To investigate the role of molybdenum in enzyme formation, extracts of *P. putida* Ful grown on 2-furoic acid in the presence of molybdate or tungstate were tested for the amount of cross-reacting material by rocket immunoelectrophoresis and for XDH and FCoADH activities. The enzyme activities measured in extracts of cells grown in the presence of different amounts of molybdate and tungstate correlated with the measured and calculated amount of cross-reacting

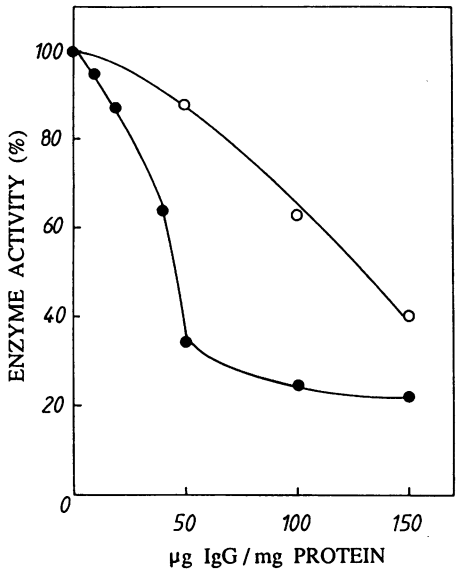

FIG. 5. Inhibition of XDH (●) and FCoADH (○) activities with antibodies raised against the larger ^{185}W-labeled protein in crude extract; 100% was set to be 180 mU/mg of protein for XDH and 55 mU/mg of protein for FCoADH.

material (Fig. 6). Without addition of both trace elements, some cross-reacting material and enzyme activities could be detected, which might have been due to contamination of the components of the media by molybdate. Addition of 10^{-8} M tungstate totally suppressed both formation of both enzyme activities and cross-reacting material. In its presence, at least 10^{-10} M molybdate was necessary to induce formation of cross-reacting material and of XDH and FCoADH. This pattern strongly supports the view that both proteins purified by using ^{185}W as label were genuine molybdoproteins, for their expression was under the control of molybdate.

Extracts from various aerobic and anaerobic bacteria listed under Materials and Methods were screened for similar proteins by cross-reactivity in double immunodiffusion tests, rocket immunoelectrophoresis, and Western blot after growth on the respective heterocyclic compound and on nutrient broth. Three gram-negative bacteria (*P. putida* N-9, *Alcaligenes* sp. strain DPK 1, and *P. putida* Chin IK) utilizing nicotinic acid, dipicolinic acid, and quinoline, respectively, showed cross-reaction with antibodies raised against the larger radioactive protein. These cross-reactions were also detected when the organisms were grown on nutrient broth, indicating the presence of a constitutive protein as observed in *P. putida* Ful. The antibodies did not cross-react with nicotinate dehydrogenase of *B. niacini* (DSM 2923), milk xanthine dehydrogenase, and liver sulfite oxidase.

DISCUSSION

The ability of aerobic and anaerobic bacteria to utilize furane derivatives has been demonstrated previously (6, 12, 24, 49, 50). Furfuryl alcohol was degraded in *P. putida* Ful by an NAD-dependent furfuryl alcohol dehydrogenase which seems to be a regular alcohol dehydrogenase, for it too used ethanol as a substrate. The native molecular weight of alcohol dehydrogenases differs from 55,000 to 200,000 (46). With a size of about 170,000 Da, the enzyme of *P. putida* Ful falls into the upper range. Furfural was oxidized to 2-furoic acid and then converted to 2-furoyl-CoA. Anaerobic degradation of furfural by *D. furfuralis* does not involve the formation of the respective CoA ester (6, 12). The furfural dehydrogenase activity of *P. putida* Ful was inducible, converted acetaldehyde and benzaldehyde, and was not CoA dependent. Aldehyde dehydrogenases appear to be generally tetrameric enzymes with molecular weights of 200,000 to 260,000. They have very broad substrate specificities, and the K_ms for most aldehydes are in the micromolar range (55). The approximate molecular weight of the furfural dehydrogenase was about 100,000. Thus, it could be a dimer. The molybdenum–iron-sulfur–flavoprotein aldehyde oxidase is another aldehyde-oxidizing enzyme exhibiting a molecular weight of about 270,000 (9). The enzyme activity of furfural dehydrogenase of *P. putida* Ful was not influenced by the availability of molybdenum or tungsten during growth; therefore, it is not likely that the furfural dehydrogenase reaction is catalyzed by an aldehyde oxidase.

Aerobic degradation of 2-furoic acid involves the formation of 2-furoyl-CoA followed by hydroxylation to form 5-hydroxy-2-furoyl-CoA (23, 24, 50). The hydroxyl group derives from water rather than from molecular oxygen (23). The hydroxylation of 2-furoyl-CoA was reported to be catalyzed in *P. putida* F2 by a large membrane-associated enzyme, which should contain copper but no iron or flavin (23), whereas the activity of the enzyme studied in *P. putida* Ful was dependent on the availability of molybdate (24). Close inspection of the data (23) indicates that the enzyme exhibits a small peak at 411 nm indicative of a cytochrome, which also might have been lost. Formation of the hydroxylated intermediate seems to be quite similar to the hydroxylation of N-heterocyclic compounds such as purines (4), nicotine (13), nicotinic acid (11, 34), or picolinic acid (44), as noted before (23). A relationship of the two reactions is indicated by the observed unspecificity of XDH for 2-furoyl-CoA, as observed in our strain *P. putida* Ful (24).

The inhibitory effects of tungstate on xanthine and 2-furoic acid utilization and on the corresponding enzymatic dehydrogenase reactions (24) point to an involvement of a molybdoenzyme instead of a copper-containing enzyme (23). Tungstate exerts in almost all organisms an antagonism with molybdenum-containing enzymes because of its chemical similarity (30). Incorporation of ^{185}W into proteins has been described previously (52, 56). [^{185}W]tungstate was incorporated into XDH- and FCoADH-containing fractions only in the presence of small amounts of molybdate, as observed for sulfite oxidase (20). The simultaneous presence of both trace elements was essential for obtaining radioactively labeled enzymes.

The observed strict coelution of ^{185}W and of XDH and FCoADH enzyme activities during the first purification steps and the lack of other highly labeled proteins already pointed to a possible identity of both purified ^{185}W-labeled proteins with XDH and FCoADH. However, a formal proof could be drawn only from immunological studies. The antibodies raised against the larger ^{185}W-labeled protein inhibited XDH and FCoADH activities in crude extracts. Western blots of cell extract after native polyacrylamide gel electrophoresis showed a labeling pattern which corresponded exactly to the positions by activity stains for XDH and FCoADH. Furthermore, the amount of cross-reacting material and the activities of both enzymes after culture of *P. putida* Ful in the presence of different amounts of molybdate and tungstate correlated exactly. Formation of both cross-reacting material and enzyme activities was regulated by the presence of molybdate in culture medium, emphasizing that the ^{185}W-

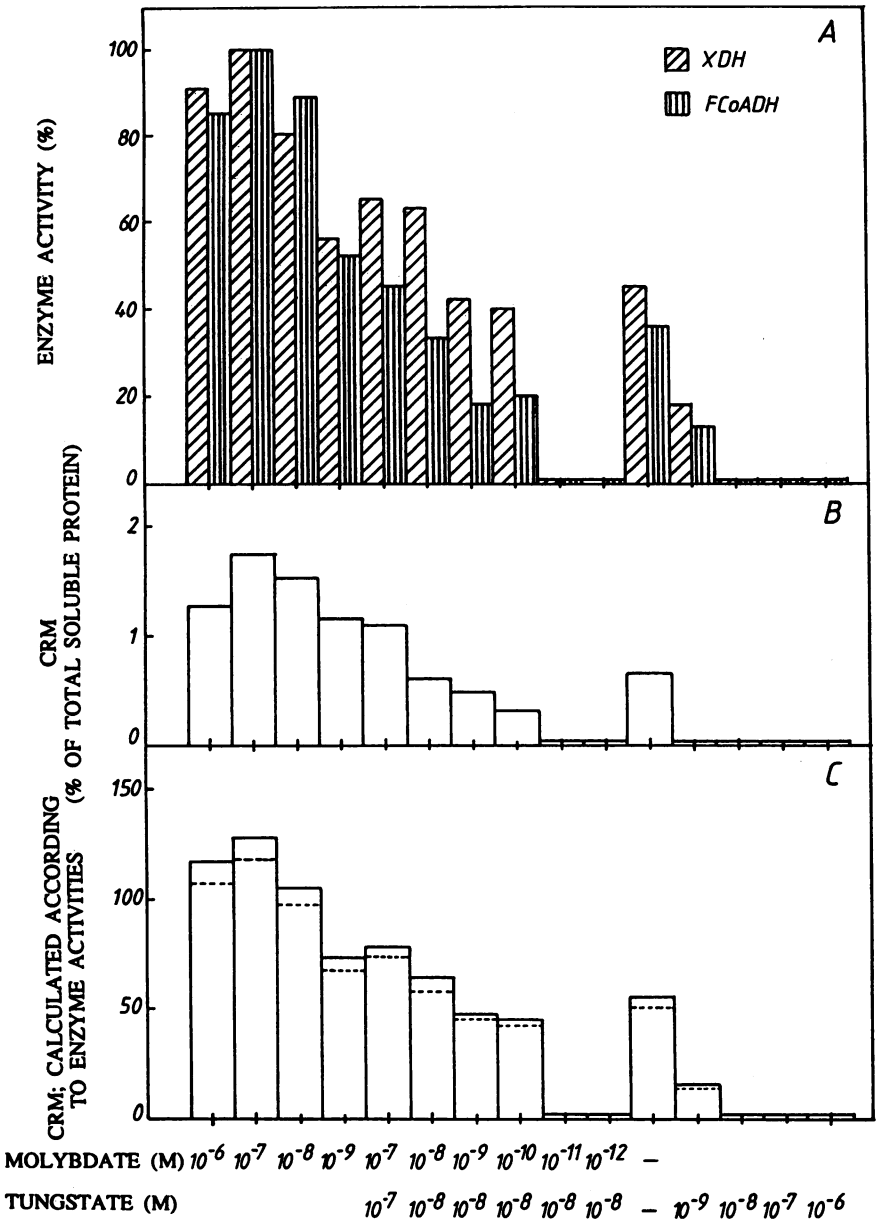

FIG. 6. Comparison of enzyme activities and amount of cross-reacting material (CRM) after culture of *P. putida* Fu1 in the presence of various amounts of molybdate and tungstate. (A) Enzyme activities of XDH and FCoADH (100% was set to be 75 and 110 mU/mg of protein, respectively. (B) Amount of cross-reacting material quantitated with rocket immunoelectrophoresis. (C) Amount of cross-reacting material calculated according to enzyme activities measured; the amount was calculated according to the intensity of cross-reaction of XDH and FCoADH. The latter enzyme showed only between 20% (– – –) and 30% (——) of cross-reaction with the antibodies raised against the larger radioactive protein.

labeled proteins are true molybdoproteins which can be labeled by ^{185}W only under appropriate conditions. Therefore, the proteins purified by the ^{185}W label can be considered to be XDH and FCoADH, although both homogeneous proteins lost enzymatic activities during the purification procedures. This phenomenon has previously been observed for XDH (53).

Heterocyclic aromatic compounds are usually converted by molybdoenzymes such as xanthine dehydrogenase-oxidase (4, 9, 53) and the closely related aldehyde dehydrogenase-oxidase (39, 40), which represent the prototypes of this class of enzymes (9, 25), for both enzymes convert a broad spectrum of heterocyclic compounds (25), in contrast to nicotine dehydrogenase (13), nicotinate dehydrogenase (11; Nagel, Ph.D. thesis, 1989), and quinoline dehydrogenase (M. Blaschke, Diplom thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1990). They all contain molybdenum, flavin, and nonheme iron-sulfur as redox-active centers, and all are composed of three subunits after SDS-polyacrylamide gel electrophoresis or proteolytic treatment with molecular weights of about 90,000, 42,000, and 20,000, exhibiting mostly a subunit structure of $L_2M_2S_2$ (Table 2). An enzymatic active LMS form is still the exception, having been found only for one aldehyde dehydroge-

TABLE 2. Comparison of XDH and FCoADH from *P. putida* Ful with XDH and liver sulfite oxidase

Enzyme	Determination						
	Molecular size (kDa)		Structure	Redox-active component			
	Whole protein	Subunits or domains		Mo	FAD	Cytochrome *b*	Fe-S
XDH generally *P. putida* Ful	300	90, 42, 20	$\alpha_2\beta_2\gamma_2$	+	+	−	+
XDH	300	55, 25	$\alpha_4\beta_4$	+	−	+	+
FCoADH	150	55, 25	$\alpha_2\beta_2$	+	−	+	+
Liver sulfite oxidase	115	55	α_2	+	−	+	−

nase (39), nicotine dehydrogenase (13), and 6-hydroxy nicotinate dehydrogenase (Nagel, Ph.D. thesis, 1989).

As found in this study, XDH and FCoADH of *P. putida* Ful are members of a new class of molybdenum-containing dehydrogenases with a unique mixture of redox-active components and subunit structure so far unknown to be involved in the metabolism of heterocyclic compounds (Table 2). The results confirm the reports (23, 42, 45, 57) that *Pseudomonas* species contain different types of XDH. The deviation from the known pattern in *P. putida* Ful is not due to a loss of cofactor or subunit during enzyme purification, as indicated by analysis of the immunoprecipitate which lacks flavin and a possible third subunit of different molecular weight. The formate dehydrogenase of *Methanobacterium formicicum* is a molybdo-iron-sulfur protein which loses flavin during purification under reducing conditions in the absence of salts (1). Molybdenum, iron-sulfur, and heme are redox-active compounds of nitrate reductase and formate dehydrogenase in *Escherichia coli* (47) and *Wolinella succinogenes* (26). In those organisms, cytochrome *b* is located on a small subunit of 20,000 Da. However, both enzymes consist of three subunits and are very tightly bound to the membrane, unlike the enzymes studied in *P. putida* Ful. In addition, the α subunit, which carries the molybdenum and iron-sulfur center, is generally much larger (110,000 to 150,000 Da) (47), thus excluding a high similarity to the enzymes reported here. The eucaryotic sulfite oxidase represents another class of molybdoenzyme (Table 2) that is composed of two identical 55,000-Da subunits which contain both one heme (cytochrome *b*) and one atom of molybdenum but no iron-sulfur (8, 40). In contrast, XDH of *P. putida* Ful contained the cytochrome on the smaller 25,000-Da subunit.

The replacement of flavin by cytochrome *b* represents an important change that should have a drastic effect on the reversibility of the enzyme reaction (4), which no longer should take place because of the change in redox potential from −350 to −250 mV for flavins to about 0 mV characteristic for cytochrome *b* (16). The stoichiometries obtained for the redox-active compounds of the purified XDH of *P. putida* Ful represent minimal values as a result of losses generally experienced with molybdoenzymes (9). This might also be one reason for the loss of enzymatic activity. Thus, it seems to be reasonable that the native XDH contains four molybdenum, four cytochrome *b*, eight nonheme iron, and eight labile sulfur. The final preparation of FCoADH did not contain a cytochrome *b* moiety. It might have been lost, for that heme is not covalently bound to proteins. It is of interest that the anti-XDH IgG cross-reacted only with the large subunit of FCoADH, indicating a somewhat different epitope structure of the small subunit despite the same molecular mass.

As suggested by the partial immunological identity of XDH and FCoADH and the high degree of structural similarity (Table 2), these enzymes might have been derived from a common progenitor. Cross-reactions were also observed between anti-XDH IgG and crude extracts of some gram-negative bacteria which utilized nicotinic acid, dipicolinic acid, or quinoline. This result might indicate the presence of polypeptide domains, similar to the type of XDH reported here that is composed differently from the well-studied form (9). According to our study, two different types of XDH have evolved. In both types, XDH is a molybdoprotein of about 300,000 Da in its native size (Table 2) and shows a broader substrate spectrum than the proteins, such as nicotinate dehydrogenase (11) or FCoADH (24), that might have derived from it. Both types contain certain enzymes that are active as 150,000 Da form.

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