APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1989, p. 1829–1834
0099-2240/89/071829-06$02.00/0

Vol. 55, No. 7

Molybdenum Involvement in Aerobic Degradation of 2-Furoic Acid by *Pseudomonas putida* Ful

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Received 5 December 1988/Accepted 20 April 1989

An organism identified as *Pseudomonas putida* was isolated from an enrichment culture with 2-furoic acid as its sole source of carbon and energy. The organism contained a 2-furoyl-coenzyme A (CoA) synthetase to form 2-furoyl-CoA and a 2-furoyl-CoA dehydrogenase to form 5-hydroxy-2-furoyl-CoA as the first two enzymes involved in the degradation. Tungstate, the specific antagonist of molybdate, decreased growth rate and consumption of 2-furoic acid but had no influence on growth with succinate. Correspondingly, the 2-furoyl-CoA dehydrogenase activity decreased when the organism was grown on 2-furoic acid in the presence of increasing amounts of tungstate. The addition of molybdate reversed the negative effect on 2-furoyl-CoA dehydrogenase activity, which points to the involvement of a molybdoenzyme in this reaction. Both enzymes studied were inducible. No plasmid was detected in this organism.

Furan derivatives are widespread in nature (32). They are also formed by the Maillard reaction and have been detected in sewage water (2). In previous papers, the degradation of 2-furoic acid by *Pseudomonas putida* F2 was reported (31, 32). All heterocyclic intermediates are esters of coenzyme A (CoA). Therefore, formation of 2-furoyl-CoA (FCoA) is the first reaction step. The second enzyme is a dehydrogenase and catalyzes a hydroxylation reaction to form 5-hydroxy-FCoA (Fig. 1). It appears that the oxygen of the hydroxyl group is derived from water (32) and that the hydroxylating enzyme copurifies with copper but not with iron (19). The spectrum exhibits no absorbance above 273 nm, which excludes an iron-sulfur flavoprotein. However, the hydroxylation reaction by FCoA dehydrogenase (FCoADH) seems to be quite analogous to those catalyzed by other bacterial molybdenum-containing hydroxylases (3, 10) such as xanthine dehydrogenase (34), aldehyde oxidase (27), nicotine dehydrogenase (9), nicotinic acid dehydrogenase (5, 6, 23), and carbon monoxide dehydrogenase (25). Studies with ^{18}O revealed that the oxygen of the hydroxyl group is derived from water but not from O_2 (14, 17; W. C. Evans, 1967, Mechanism of microbial hydroxylations, Biochem. J., **103:** 1p–3p). To test the hypothesis that molybdenum might also play a role in 2-furoic acid metabolism, we isolated a new strain of *P. putida* from enrichment culture by using 2-furoic acid as the sole carbon and energy source in the presence of molybdenum as a micronutrient. We now report that the two initial degradation steps are identical to those obtained before; however, the trace element molybdenum and especially its competitive inhibitor tungsten (13) exhibited a strong influence on growth, 2-furoic acid consumption, and FCoADH activity.

MATERIALS AND METHODS

Enrichment and isolation. The organism used in this study was isolated from soil samples. Inocula were suspended in flasks with 5 ml of a mineral salts medium. This medium consisted of 0.18 mM $CaCl_2$, 0.14 mM $MnSO_4$, 5.6 mM NH_4Cl, 0.85 mM NaCl, 10 mM potassium phosphate buffer (pH 7.2), and 10 mM 2-furoic acid or succinate as the carbon and energy source. The final pH was adjusted to 7.2. After sterilization, 5 ml of filter-sterilized vitamin solution (11) and 1 ml of sterile trace element solution SL 10 (35) were added per liter. The flasks were aerobically shaken at 30°C. After 2 weeks, serial dilutions were plated on mineral agar (mineral salts medium containing 2% agar) and incubated at 30°C. Several colonies were isolated and again were transferred onto mineral salts medium. After incubation, two different organisms were isolated, of which one, designated strain Ful, was studied in detail. During the studies, this strain was maintained on nutrient agar plates containing (per liter of demineralized water) 10 g of tryptone, 5 g of yeast extract, 2 g of succinate, and 20 g of agar, and did not lose the ability to grow on 2-furoic acid, whereas the second isolate, a coryneform bacterium, lost it.

Growth conditions. Usually, cells were grown in 250-ml baffled flasks containing 50 ml of medium. Mass cultures were grown in 2-liter baffled flasks containing 500 ml of medium with 20 mM 2-furoic acid and 20 mM succinate as carbon and energy sources. To determine a molybdenum requirement for growth, the trace element solution without molybdate and tungstate was used. The latter were added separately as indicated. Bacterial growth was measured at 600 nm (1-cm light path).

Identification of bacteria. Identification of the isolate was based on the scheme described earlier (26). API test strips 20 NE and 20 E (bioMérieux, Nürtingen, Federal Republic of Germany) were used for rapid identification of the strain Ful. In addition, *Pseudomonas* F agar, acetamide-Cetrimide-glycerol-mannit agar, Cetrimide agar, glutamate-starch-phenol red agar, or deoxycholate-citrate-lactose-saccharose agar was used as the selective medium according to the instructions of the 1987 Merck Nährböden-Handbuch.

Preparation of cell extracts. Cells were harvested by centrifugation, washed once with 0.9% sodium chloride solution, and suspended in 50 mM potassium phosphate buffer, pH 7.5. Cells were disrupted in a Schoeller sonifier with eight cycles of 1 min each at maximum output with a cooling interval of 1 min. The resulting extract was centrifuged for 50

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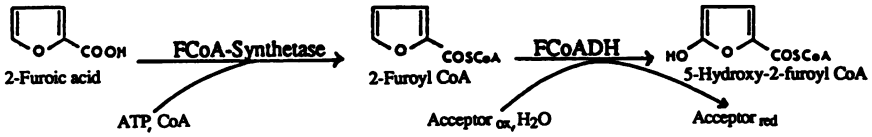

FIG. 1. Proposed initial steps in the degradation of 2-furoic acid.

min at 20,000 × *g*. The pellet was routinely discarded, and the supernatant fluid was frozen at −18°C until used.

Enzyme assays. FCoA synthetase activity was measured in a coupled enzyme assay by determining NADH oxidation at 340 nm. Reaction mixtures contained (in 880 μl) 350 μl each of 100 mM glycylglycine buffer (pH 7.8) and of 10 mM $MgCl_2$, 10 μl each of 4 mM CoA, of 5 mM ATP, and of 8 mM phosphoenolpyruvate, 0.75 U each of pyruvate kinase and of lactate dehydrogenase, 25 μl of 30 mM NADH, 100 μl of 50 mM 2-furoic acid, and various amounts of protein.

The FCoADH was measured by determining the reduction of Nitro Blue Tetrazolium chloride at 535 nm with an absorption coefficient (ε) of 18.3 mM^{-1} cm^{-1}. Reaction mixtures contained (in 1,100 μl) 850 μl of 50 mM potassium phosphate buffer, pH 7.5, with 0.15% (wt/vol) Nitro Blue Tetrazolium chloride and 1% Triton X-100, 100 μl of 1.5 mM FCoA, and various amounts of protein. Several other electron acceptors were tested in the enzyme assay, and final concentrations (in mM) were the following: benzyl viologen, 10; brilliant cresyl blue, 0.15; dichlorophenol-indophenol plus phenazine ethosulfate, 0.16 plus 0.12; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 0.15; ferricyanide, 1; methylene blue, 0.15; methyl viologen, 8; NAD(P), 0.5; 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 (1).

Preparation of FCoA. To prepare FCoA, the FCoA synthetase had to be partially enriched. Therefore, the crude extract was ultracentrifuged for 1 h at 100,000 × *g*. The supernatant was applied to a DEAE-Sephacel column (1.6 × 40 cm) equilibrated with 50 mM glycylglycine buffer, pH 7.8, containing 5 mM $MgCl_2$ (enrichment-buffer). The column was washed with enrichment-buffer, and the enzyme was eluted by a linear KCl gradient (0 to 1 M KCl) at approximately 0.3 M KCl. To the pooled FCoA synthetase-containing fractions, solid ammonium sulfate was added to give 60% saturation. After centrifugation, the pellet was suspended in enrichment-buffer and lyophilized. To produce FCoA, 10 ml of enrichment-buffer contained 3 mM each of CoA, ATP, phosphoenolpyruvate, NADH, and 2-furoic acid, 0.75 U each of pyruvate kinase and lactate dehydrogenase, and 0.4 U of lyophilized FCoA-synthetase. After incubation for about 4 h at room temperature, the reaction was complete. The resulting FCoA was detected and quantified by high-pressure liquid chromatography (HPLC) and was stored at 4°C.

Determination of the molecular weight of the FCoADH. Estimation of the native molecular weight was performed by gel filtration chromatography on Sephacryl S-200 HR with alcohol dehydrogenase (M_r, 150,000), hexokinase (M_r, 100,000), bovine serum albumin (M_r, 66,000), and peroxidase (M_r, 45,000) as markers.

Native gradient polyacrylamide gel electrophoresis was done by using a gradient from 4 to 27.5% acrylamide in the presence of molecular weight marker proteins (24). The estimation of the native molecular weight was done by staining the lane containing FCoADH for enzymatic activity. The staining solution contained, in 10 ml of 50 mM potassium phosphate buffer (pH 7.5), 0.2 ml of 2.5% Nitro Blue Tetrazolium chloride, and 2 ml of 1.5 mM FCoA. The polyacrylamide gel was incubated in the staining solution in the dark for approximately 30 min.

HPLC. HPLC of 2-furoic acid was performed by using a Kontron system with a pump model 420, an injector, a RP-18 reverse-phase column (4 by 250 mm, 10-μm particle size), and a detector model 430. Isocratic elution was performed with 60% 2-propanol and 40% H_2O, pH 2.9 (with formic acid), at a flow rate of 1 ml/min. The detection was set at a 244-nm wavelength.

Formation of FCoA was quantitated by HPLC with the same configuration as that described above, except that an Ultrasphere C_8 column (4.6 by 220 mm, 5-μm particle size) was used. Isocratic elution was performed with 60% methanol, 35% H_2O, and 5% acetonitrile, pH 5.0, at a flow rate of 1 ml/min. The detection was set at 244 nm.

Chemicals. All chemicals used were of highest commercially available grade and obtained from Aldrich-Chemie, Steinheim, Merck, Darmstadt, Serva, Heidelberg, or Sigma-Chemie, Deisenhofen, Federal Republic of Germany. All enzymes used in enzyme assays were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany.

RESULTS

Isolation and characterization of the strain Ful. Biodegradation of 2-furoic acid was detected after 2 weeks of enrichment culture in the presence of 10 mM 2-furoic acid. A pure culture was obtained by repeated subculture on minimal salts agar plates. One of the two isolated bacteria, strain Ful, was a gram-negative rod which was motile by means of one to three polar flagella. It was oxidase and catalase positive and nonfermentative. No denitrification was observed. The organism was able to grow on arabinose, glucose, mannose, and xylose but not on *N*-acetylglucosamine, adipate, cellobiose, dulcitol, fructose, lactose, maltose, or mannitol. Strain Ful was able to grow on selective media such as *Pseudomonas* F agar, glutamate-starch-phenol red agar, deoxycholate-citrate-lactose-saccharose agar, acetamide-Cetrimide-glycerol-mannit agar, MacConkey agar, and Cetrimide agar. During growth, the production of a green-blue fluorescent pigment was detected. The method of Holmes and Quickley (16) did not furnish evidence for the presence of a plasmid in strain Ful. The ability to utilize 2-furoic acid was not lost during culture on nonselective media. On the basis of the characteristics mentioned above in combination with the results of the API test strips, the organism was tentatively classified as *P. putida*.

Growth of *P. putida* Ful on 2-furoic acid and other heterocyclic compounds. During growth of *P. putida* Ful on 2-furoic acid, the pH increased from 7.2 to 7.5. The organism exhibited a doubling time of 2 h when grown on 2-furoic acid (Fig. 2A). For 2-furoic acid, 10 mM was found to be the optimal substrate concentration, but even higher concentrations of up to 20 mM did not inhibit growth. *P. putida* Ful grew on 2-furoic acid in the pH range of 6.5 to 8.5; the optimal pH was 7.2.

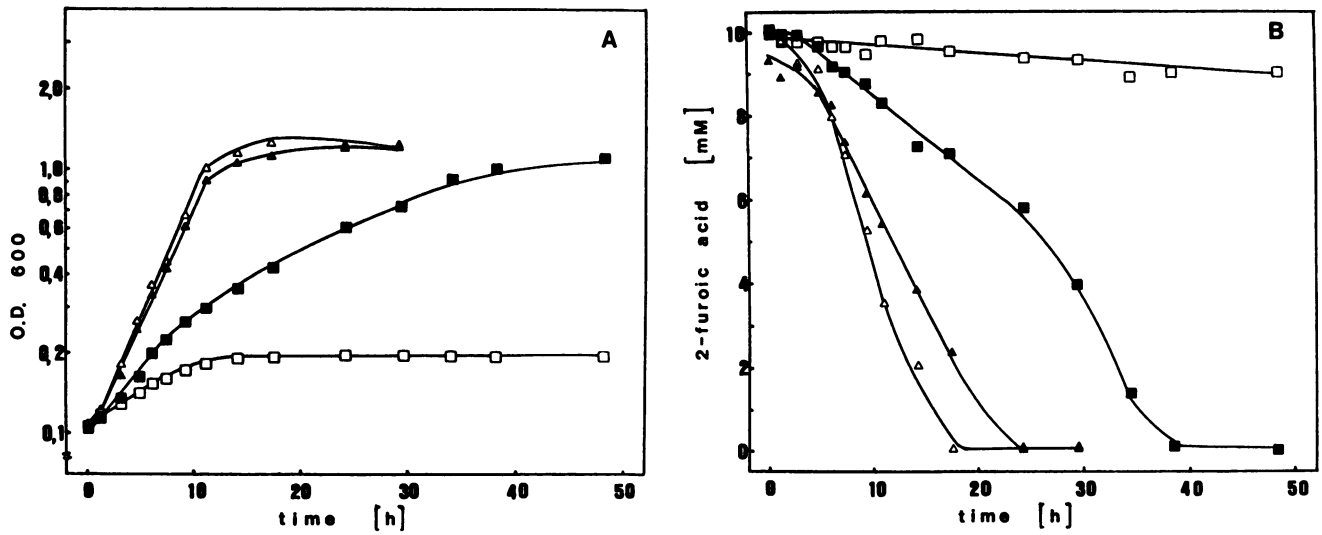

FIG. 2. Influence of molybdenum and tungsten on growth (A) and 2-furoic acid utilization (B) by *P. putida* Ful. Symbols: △, 10^{-6} M molybdate added to the growth medium; ▲, without addition of molybdate and tungstate; ■, addition of 10^{-6} M each of molybdate and tungstate; □, addition of 10^{-6} M tungstate.

Growth of *P. putida* Ful was also tested on some other heterocyclic compounds: it did not grow on 2 mM 2-thiophenic acid nor on 2 mM 2-pyrrolic acid; however, the organism grew on 5 mM xanthine.

2-Furoic acid was utilized by glucose-grown cells in the absence, but not in the presence, of 0.6 mM chloramphenicol. Thus, enzymes required for 2-furoic acid utilization had to be formed de novo.

Influence of molybdenum and tungsten. Addition of molybdate had no influence on growth of *P. putida* Ful on 2-furoic acid (Fig. 2A). However, the presence of equimolar amounts of molybdate and tungstate in the growth medium inhibited the growth of *P. putida* Ful as indicated by the increased (twofold) doubling time (Fig. 2A). If the growth medium was only supplemented with 10^{-6} M tungstate, the specific inhibitor of molybdate, no growth nor 2-furoic acid consumption could be measured. No stimulation or inhibition of growth on succinate as the sole carbon and energy source could be detected by the addition of various amounts of molybdate or tungstate. Therefore, the inhibition by tungstate seems to be specific for 2-furoic acid.

FCoA synthetase. Crude extracts contained a soluble enzyme which catalyzed the formation of FCoA (Fig. 1) and which exhibited a specific activity of 0.2 U/mg. A broad pH optimum was observed between pH 8.5 and 9.5 with glycylglycine buffer. Michaelis-Menten-type saturation kinetics were obtained with 2-furoic acid. The apparent K_m was determined from the Lineweaver-Burk plot to be 0.75 mM. The estimation of the native molecular weight by gel filtration chromatography on Sephacryl S-200 HR resulted in two activity peaks eluting at molecular weights of 40,000 and 85,000. In order to produce FCoA as a substrate for the proposed second enzyme (Fig. 1), the FCoA synthetase was partially enriched by chromatography on DEAE-Sephacel and by ammonium sulfate precipitation. No FCoADH activity was observed in this preparation. The formation of FCoA was monitored by HPLC and depended strictly on the presence of FCoA synthetase, 2-furoic acid, CoA, ATP, and Mg^{2+}.

FCoADH. The second step of 2-furoic acid degradation was catalyzed by FCoADH, which forms 5-hydroxy-FCoA (Fig. 1). Specific activities of about 0.09 U/mg were measured for crude extracts. A distinct pH optimum was observed at pH 7.5. Michaelis-Menten-type saturation kinetics were obtained with FCoA or with 2-furoic acid when a coupled assay with ATP, CoA, and FCoA synthetase was used. The apparent K_m was determined from Lineweaver-Burk plots to be 0.9 mM for 2-furoic acid in the coupled assay; however, for FCoA, the FCoADH exhibited a high affinity of 50 μM as the apparent K_m. The approximate molecular weight of the native enzyme was 100,000 (±10,000) as determined by gel filtration chromatography and activity stain in a native gradient gel.

FCoADH was active with the electron acceptors (compared with Nitro Blue Tetrazolium chloride set to be 100%) MTT (58%) and methylene blue (64%). No activity was found with ferricyanide, methyl viologen, benzyl viologen, or NAD(P). With 2,6-dichlorophenol-indophenol plus phenazine ethosulfate, brilliant cresyl blue, or thionine, a rapid reaction was observed which, however, lacked a dependence on substrate addition. Xanthine, *N*-methylnicotinamide, acetaldehyde, nicotine, nicotinic acid, and some purine compounds were tested as substrates in the FCoADH assay with crude cell extracts of cells grown on 2-furoic acid. Although the highest activity was monitored with FCoA, all other substances listed also served as substrates in the Nitro Blue Tetrazolium chloride assay. Xanthine, in particular, was also converted at a high rate (51%), compared with that for FCoA (Table 1). However, after gel filtration chromatography on Sephacryl S-200 HR, the conversion of xanthine by the FCoADH-containing fraction was reduced to 5%. In crude extracts of cells grown on xanthine, FCoA served to a low degree as substrate, compared with xanthine. In native 7.5% polyacrylamide gels, two activity bands with different R_f values could be detected after staining with FCoA or with xanthine (Table 2). FCoA, preferentially stained at the higher R_f value, and the FCoADH were detectable in all extracts tested. The xanthine-converting enzyme had a lower R_f value than that for the FCoADH; the activity band appeared in all extracts of cells tested but not in the partially enriched FCoADH preparation, where a substantial decrease in activity with xanthine was to be measured with

TABLE 1. FCoA- and xanthine-converting enzymes in cell extracts of differentially grown cells and a partially enriched FCoADH

Substrate in enzyme assay	Sp act (U/mg of protein) in cells grown on:			
	Luria broth	Xanthine	2-Furoic acid	FCoADH[a]
FCoA	0.012	0.007	0.080	0.060
Xanthine	0.060	0.060	0.040	0.003

[a] Partially enriched FCoADH was derived from 2-furoic acid-grown cells.

FCoA. Thus, two separate enzymes were present in *P. putida* Ful; both converted FCoA but only one converted xanthine. *N*-Methylnicotinamide served as a substrate in xanthine-grown cells only at an alkaline pH of 9.0 (0.01 U/mg of protein), not at pH 7.5, the typical pH for a xanthine dehydrogenase (12).

Influence of molybdenum and tungsten on FCoADH activity. The activity of FCoADH was affected by the growth of *P. putida* Ful in mineral salts medium on 10 mM 2-furoic acid in the presence of various amounts of molybdate and tungstate (Fig. 3). The highest specific activity of the FCoADH was observed in the presence of 10^{-6} M molybdate (100%). Without the addition of molybdate and tungstate to the growth medium, the activity was only 77%, whereas the specific activity was decreased to 65% after the cells were cultured in the presence of equimolar concentrations (10^{-6} M) of both trace elements. Tungstate 10^{-6} M caused a decrease in FCoADH activity to just 20%. Thus, this pattern is indicative of molybdoenzymes (9, 13, 34).

Cyanide can inactivate molybdoenzymes by removing the sulfur from the molybdenum cofactor as thiocyanate (4). Of the total FCoADH activity, 50% was lost after anaerobic incubation of the crude extract for 1 h with 0.3 mM cyanide. No activity was detected after 60 min of preincubation with 10 mM cyanide; the time course of the inactivation showed a 50% loss in activity after 10 min. Arsenite and methanol are inhibitors of molybdoenzymes (3). With crude extracts, no inactivation by concentrations up to 1 M methanol or 1 mM arsenite could be observed.

DISCUSSION

The ability of bacteria of the genus *Pseudomonas* to utilize 2-furoic acid has been demonstrated before (18, 19, 31). In *P. putida* F2, the degradation of 2-furoic acid is initiated by activation to FCoA followed by hydroxylation to yield 5-hydroxy-FCoA (32). The hydroxylation of FCoA seems to

TABLE 2. Separation of xanthine- and FCoA-oxidizing enzymes by native polyacrylamide gel electrophoresis and visualization by activity staining

R_f of activity stain	Substrate	Bands[a] after growth on:			
		Luria broth	Xanthine	2-Furoic acid	2-Furoic acid–FCoADH[b]
0.16	Xanthine	++	+++	++	–
	FCoA	+	+	+	–
0.22	Xanthine	–	–	–	–
	FCoA	+	+	+++	+++

[a] Symbols: +, weak; ++, good; +++, strong activity band visible; –, band undetected.
[b] Partially enriched FCoADH.

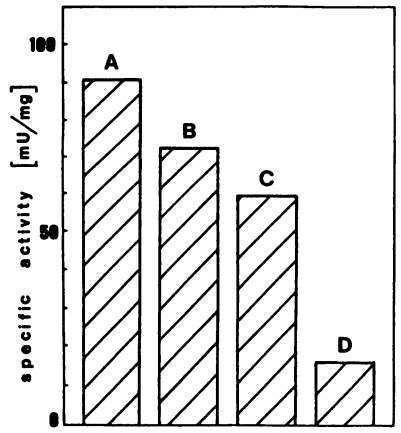

FIG. 3. Influence of molybdate and tungstate on the FCoADH activity after growth of *P. putida* Ful on 2-furoic acid in the presence of 10^{-6} M molybdate in the growth medium (A), the absence of molybdate and tungstate (B), the presence of 10^{-6} M molybdate and tungstate (C), or the presence of 10^{-6} M tungstate (D).

be similar to that of *N*-heterocyclic compounds such as nicotine (7, 9, 15), nicotinic acid (8), or picolinic acid (29, 30). During hydroxylation of heterocyclic compounds, the oxygen of the hydroxyl group is derived from water. This was also anticipated for the FCoADH isolated from a different strain of *P. putida* (19). However, the enzyme isolated from that organism exhibited quite different properties, for it forms very large aggregations of M_r 3.3×10^6 and is not an iron-sulfur flavoprotein as are most of the hydroxylating enzymes (3, 10). Copper was found to be selectively enriched during enzyme purification (19), whereas molybdenum is part of most of the other hydroxylating enzymes (3, 10). In contrast to the results obtained with *P. putida* F2 (19), the FCoADH activity obtained from our strain Ful was not affected by the copper-chelating compound cuprizone. However, the FCoADH activity corresponded to the amount of molybdenum in the growth medium, as reported for the other hydroxylating enzymes mentioned above. The effect of molybdate was selectively observed for growth and substrate utilization of 2-furoic acid, as evidenced by the strong inhibition of tungstate, which was not observed for succinate, a later intermediate of 2-furoic acid decomposition (31, 32). In addition, the activity of FCoADH was likewise effected by molybdate and tungstate. A similar effect can also be found for utilization of nicotinic acid and 6-hydroxy nicotinic acid by *Bacillus* sp. (DSM 2923) (25; M. Nagel, diplom thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1986). Growth of *Arthrobacter oxidans* on nicotine and nicotine dehydrogenase activity both depend on molybdate and also are inhibited by tungstate (9). The composition of this enzyme as a molybdenum-containing iron-sulfur flavoprotein was verified after purification to homogenity (9).

The native FCoADH exhibited a molecular weight of 100,000 (±10,000). This corresponds to molecular weights of a few other molybdoenzymes like a special aldehyde oxidase (33), nicotine dehydrogenase (9), and 6-hydroxy nicotinic acid dehydrogenase (Nagel, diplom thesis). In contrast to these enzymes, the other molybdoenzymes like xanthine dehydrogenase contain two, instead of one, protomers, each of a molecular weight of about 150,000 (3, 10). FCoADH was inactivated by cyanide, thus resembling other molybdoen-

zymes with a sulfur-containing ligand (4) in the bactopterin (23). Further studies are necessary for verification that FCoADH is a new member of the class of molybdenum-containing hydroxylases.

Xanthine was converted at a high rate by crude cell extracts of *P. putida* Ful after growth on 2-furoic acid. However, the ability to convert xanthine was reduced after gel filtration chromatography in FCoADH-containing preparations. The xanthine-converting enzyme was not inducible and separate from the FCoADH. Concomitant with the low FCoADH activity detected in xanthine- and Luria broth-grown cells, the activity stains revealed the presence of two enzymes. At the R_f value of the xanthine dehydrogenase, FCoA also gave rise to a weak activity band, thus pointing to an existing unspecificity of the xanthine dehydrogenase for FCoA. Xanthine dehydrogenases and aldehyde oxidases are quite similar enzymes that show remarkable unspecificities towards their substances (21, 22). As suggested by their structural similarities, these enzymes might have been derived from a common progenitor by gene duplication (20), but now they differ in their substrate spectra. The conversion of *N*-methylnicotinamide is an important reaction for a differentiation (21, 22). An aldehyde oxidase converts this substance already at neutral pH, whereas a xanthine dehydrogenase hydroxylates *N*-methylnicotinamide only at alkaline pH (12, 28). Since the enzyme of *P. putida* Ful converted *N*-methylnicotinamide only at alkaline pH, this enzyme has to be classified as xanthine dehydrogenase. The comparison of FCoADH and xanthine dehydrogenase to show possible interrelationships awaits further purification and characterization.

ACKNOWLEDGMENTS

We thank the Bundesministerium für Forschung und Technologie and the Fonds der Chemischen Industrie for financial support.

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