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Molybdenum-dependent degradation of quinoline by *Pseudomonas putida* Chin IK and other aerobic bacteria

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Abstract. Eighteen different aerobic bacteria were isolated which utilized quinoline as sole source of carbon, nitrogen, and energy. Attempts were unsuccessful at isolating anaerobic quinoline-degrading bacteria. The optimal concentration of quinoline for growth was in the range of 2.5 to 5 mM. Some organisms excreted 2-hydroxyquinoline as the first intermediate. Hydroxylation of quinoline was catalyzed by a dehydrogenase which was induced in the presence of quinoline or 2-hydroxyquinoline. Quinoline dehydrogenase activity was dependent on the availability of molybdate in the growth medium. Growth on quinoline was inhibited by tungstate, an antagonist of molybdate. Partially purified quinoline dehydrogenase from *Pseudomonas putida* Chin IK indicated the presence of flavin, iron-sulfur centers, and molybdenum-binding pterin. M_r of quinoline dehydrogenase was about 300 kDa in all isolates investigated.

Key words: 2-Hydroxyquinoline – Molybdenum-binding pterin – Quinoline – Quinoline dehydrogenase

The aromatic *N*-heterocyclic compound quinoline is known to be a pollutant of soil and water (Southworth and Keller 1984; Pereira et al. 1987, 1988; Aislabie et al. 1990). Quinoline and its derivatives naturally occur in coal tar and different oils (Grant and Al-Najjar 1976), and they were found to be carcinogenic to humans (Tada et al. 1980).

Several aerobic and some anaerobic bacteria were isolated from different sources, which are able to degrade quinoline and its derivatives (Shukla 1986, 1989; Dembek and Lingens 1988; Pereira et al. 1988; Schwarz et al. 1988; Brockmann et al. 1989; Kuhn and Suflita 1989; Röger and Lingens 1989; Schwarz et al. 1989; Tibbles et al. 1989). Two different catabolic pathways were described which both involve the formation of 2-hydroxyquinoline as the first intermediate (Shukla 1986; Schwarz et al. 1989). Hydroxylation of heterocyclic compounds adjacent to the heteroatom was also described for furan-2-carboxylate (Trudgill 1969; Koenig and Andreesen 1989), isonicotinate (Ensign and Rittenberg 1965), nicotinate (Ensign and Rittenberg 1964; Nagel and Andreesen 1989), nicotine (Hochstein and Rittenberg 1959; Freudenberg et al. 1988), purines (Vogels and van der Drift 1976; Dürre and Andreesen 1982; Wagner et al. 1984; Berry et al. 1987), and thiophene-2-carboxylate (Cripps 1973). The oxygen atom introduced into the ring always derives from water and not from molecular oxygen (Shukla 1986; Pereira et al. 1988). Many of the enzymes catalyzing this kind of hydroxylation reaction were shown to be molybdenum-containing dehydrogenases (Coughlan 1980; Dilworth 1983; Krüger et al. 1987; Freudenberg et al. 1988; Koenig and Andreesen 1989; Nagel et al. 1989; Hinton and Dean 1990).

In order to investigate whether degradation of quinoline is also initiated by a molybdenum-dependent dehydrogenase reaction, several different bacteria were isolated which were capable of utilizing quinoline as sole source of carbon, nitrogen, and energy. The type of enzyme reaction initiating the breakdown of quinoline and the influences of molybdate and tungstate on the activities of these enzymes were studied.

Materials and methods

Isolation and characterization of bacteria

Quinoline degrading bacteria were isolated using the mineral medium described by Nagel and Andreesen (1989) but omitting ammonium and Tris. Quinoline was autoclaved separately as a suspension in 50 mM potassium phosphate buffer pH 7.5 (0.3% v/v). After sterilization the quinoline suspension was added to the medium to give a final concentration of 0.03% v/v (~ 2.5 mM). Using quinoline

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Abbreviations: APS, ammonium peroxodisulfate; DCPIP, 2,6-dichlorophenol-indophenol; EEO, electroendosmosis; MTT, thiazolyl blue; PES, phenazine ethosulfate; TEMED, *N,N,N′,N′*-tetramethyl-ethylenediamine

as sole source of carbon, nitrogen, and energy, 5-ml portions of this mineral medium were inoculated with a small sample and incubated on a rotary shaker at 30°C. After appearance of visible turbidity an aliquot of the culture was transferred to the same medium and incubated under the same conditions. Subsequently, a sample of the latter culture was plated on quinoline mineral agar. Finally, single colonies from this agar were streaked on nutrient agar to check for purity of the cultures. Bacteria were stored on quinoline mineral agar at 4°C or by adsorption to silica gel as described by Nagel and Andreesen (1991). For anaerobic incubation, quinoline-containing mineral medium (Nagel and Andreesen 1989) was degassed by boiling, flushed with N_2 and filled into Hungate tubes, which additionally contained 0.05% (w/v) cystein-HCl and resazurin (1 mg/l). All isolates were characterized by a variety of tests which included gram-stain, KOH test, catalase, oxidase, motility, occurrence and insertion of flagella, production of acid from several carbohydrates, production of indole, gelatinase, lecithinase, degradation of casein, formation of nitrite from nitrate, and growth on MacConkey agar, GSP agar, cetrimid agar, *Pseudomonas* F agar, *Pseudomonas* P agar, and King's medium B. Aminopeptidase reaction was detected by test strips (Merck, Darmstadt, FRG). Pure cultures of gram-negative bacteria were characterized by API 20 NE test system (API Bio mérieux, Nürtingen, FRG).

Detection of plasmids

Isolation of plasmids was performed according to Kado and Liu (1981). DNA was separated using agarose gels (1% w/v, standard EEO, Serva, Heidelberg, FRG) of 7.6 × 2.5 × 0.2 cm for 4 h at 30 V.

Detection of quinoline and its derivatives

Absorption spectra were recorded using a Uvikon 810 Spectrophotometer (Kontron, Eching, FRG). Standards were dissolved in ethanol.

Identification and quantification of quinoline and 2-hydroxyquinoline were performed using HPLC. Equipment consisted of two pumps model 420, a UV/VIS detector model 430, and a personal computer with software MT450 (Kontron, Eching, FRG). A Kontrosorb RP-18 column (3.5 × 220 mm, 5 µm) was used to separate samples at a flow rate of 0.3 ml/min. Eluent was 2-propanol/H_2O (60:40). Quinoline and 2-hydroxyquinoline were eluted at about 12 min and 9 min, respectively. Detection occurred at 220 or 270 nm. Identification was supported by using dual wavelength and snap shot mode.

Detection of dehydrogenase activity

Standard assay for detection of quinoline dehydrogenase was performed in a disposable cuvette and contained (µl): 25 mM phosphate buffer pH 8.0 including 1% (v/v) Triton X-100, 900; 10 mM MTT, 50; 10 mM PES, 10; enzyme solution, 10. Reaction was started by the addition of 100 µl of a suspension of 10 mM quinoline in reaction buffer. The extinction coefficient of reduced MTT at 590 nm was determined to be $\varepsilon_{590} = 14.0\ mM^{-1}\ cm^{-1}$. Other electron acceptors tested were used as described by Dietrichs et al. (1990) and Nagel and Andreesen (1990). Enzyme activity is expressed as µmol of quinoline converted per minute at 30°C.

Column chromatography

Gel filtration was performed on Sephacryl S-200 HR (2.6 × 80 cm) and Superdex 200 PG (1.6 × 60 cm) (Pharmacia-LKB, Freiburg, FRG). The eluent used was 50 mM phosphate buffer pH 7.5 and flow rate was 0.5 ml/min.

For anion exchange chromatography on DEAE-Sephacel (Pharmacia-LKB, Freiburg, FRG) the gel material was equilibrated with 25 mM phosphate buffer pH 7.5. 80 ml of crude extract from *Pseudomonas putida* Chin IK (protein content was 44 mg/ml according to Bradford 1976) was bound to about 40 ml of DEAE-Sephacel. After washing with equilibration buffer (10-fold bed volume) the protein was desorbed by a linear KCl gradient from 0 to 1.0 M (400 ml total volume) at a flow rate of 0.5 ml/min. Quinoline dehydrogenase activity eluted at about 300 mM KCl.

Characterization of enzyme

Absorption spectra were recorded by a Uvikon 810 Spectrophotometer. KI/I_2 oxidation and fluorescence spectra were performed as described by Freudenberg et al. (1988).

Electrophoretic and immunological techniques

Non-denaturing gradient PAGE was prepared using 10 × 10 cm glass plates (Biometra, Göttingen, FRG) with 1 mm spacers. A gradient between 4 and 27.5% acrylamide was established from a light (L) and a heavy (H) solution (3.0 ml each) in a gradient mixer. L solution: acrylamide, 8.0 g; bisacrylamide, 0.4 g; TEMED, 1.2 µl; gel buffer, ad 200 ml. H solution: acrylamide, 52.25 g; bisacrylamide, 2.75 g; glycerol, 17.2 ml; TEMED, 1.2 µl; gel buffer, ad 200 ml. Gel buffer was 0.25 M Tris-HCl pH 8.5. Polymerization was initiated by addition of 5.2 µl 10% (w/v) APS to each of the acrylamide solutions. The stacking gel was composed of: L solution, 2.5 ml; TEMED, 2.0 µl; 10% (w/v) APS, 8.5 µl. Gels were run at 100 V for about 12 h at 4°C. Electrode buffer contained (g/l): Tris, 0.6; glycine, 2.88. They were stained for activity using a reaction buffer similar to that used for photometrical enzyme tests in cuvettes.

SDS-PAGE and semi-dry blotting was done as described by Nagel and Andreesen (1990). Subsequent treatment of nitrocellulose membranes was performed according to a protocol of Jagus and Pollard (1988). Cross-reactions were detected by phosphatase-labeled anti-IgG antibodies (Sigma, Deisenhofen, FRG) according to the supplier's instruction. Electroelution was carried out as described by Nagel and Andreesen (1990).

Chemicals

Gel filtration molecular weight markers were obtained from Bio-Rad Laboratories (München, FRG). Quinoline and 2-hydroxyquinoline were purchased from Sigma (Deisenhofen, FRG). Other heterocyclic compounds were from Aldrich (Weinheim, FRG). Nitrocellulose membranes were purchased from Sartorius (Göttingen, FRG). All other chemicals were of highest purity available and obtained from Merck (Darmstadt, FRG).

Results

A variety of aerobic bacteria was isolated from different sources which were able to use quinoline as sole source of carbon, nitrogen, and energy (Table 1). Attempts to isolate anaerobic quinoline degrading bacteria were not successful. The optimal concentration of quinoline in the growth medium was 2.5 mM for all bacteria although many of them tolerated much higher amounts of substrate (*Pseudomonas acidovorans* CH 1: up to 15 mM). However, addition of more than 2.5 mM quinoline resulted in retardation of growth rate, but final optical densities still increased. The ability of quinoline degra-

Table 1. Isolated aerobic bacteria capable of quinoline degradation

Source	Organisms as identified
Inflow of sewage plant	*Pseudomonas acidovorans* CH 1
	Pseudomonas alcaligenes EL I
	Strain EL II (Gram-positive coccus, oxidase +, catalase +)
Activated sludge	*Pseudomonas putida* Chin IK
	Strain Chin I (Gram-negative rod, oxidase +, catalase +)
Compost	*Pseudomonas* sp. CH 2
	Rhodococcus sp. CH 5
	Strain CH 6 (gram-positive coryneform, oxidase −, catalase +)
	Strain Komp 3 (gram-positive coccus, oxidase −, catalase +)
Horse dung	Strain PK 3 (gram-positive coccus, oxidase −, catalase +)
Running water	*Pseudomonas* sp. CH 3
	Pseudomonas sp. CH 4
	Strain CH 7 (gram-positive coccus, oxidase −, catalase +)
	Strain CH 8 (gram-positive coccus, oxidase −, catalase +)
	Strain CH 9 (gram-positive coccus, oxidase −, catalase +)
	Strain CH 10 (gram-positive coccus, oxidase −, catalase +)
	Strain CH 11 (gram-positive coccus, oxidase −, catalase +)
Pond	Strain TW (gram-negative rod, oxidase +, catalase +)

dation was stable for all isolates. Strains CH 1, CH 4, CH 5, and CH 6 were checked for the presence of plasmids but only strains CH 1 and CH 4 were found to be positive for this character. Growth of all isolates on quinoline was markedly inhibited by the addition of tungstate (10^{-7} M final concentration), a specific antagonist of molybdate, to the growth medium while growth on succinate was not influenced by the addition. Thus, it seems likely that quinoline degradation generally involves a molybdenum-dependent reaction.

More detailed investigations were carried out using *Pseudomonas putida* Chin IK and *Rhodococcus* sp. CH 5. Both organisms grew best at an initial pH of 7.5 to 8.0. Optimal concentration of quinoline was 2.5 mM for both bacteria. Except of quinoline some other heterocyclic compounds were utilized (Table 2). In contrast to quinoline, degradation of 2-hydroxyquinoline by *P. putida* Chin IK was not inhibited by the addition of tungstate to the medium. During growth on quinoline, 2-hydroxyquinoline was first excreted into the medium and subsequently reutilized (Fig. 1). Formation of 2-hydroxyquinoline was also observed during growth of *Pseudomonas acidovorans* CH 4 and *Rhodococcus* sp. CH 5.

Resting cells of *P. putida* Chin IK grown on glucose did not degrade quinoline in the presence of chloramphenicol while cells grown on quinoline were able to metabolize it without any lag. Growth of strain Chin IK did not show diauxic behavior in mineral medium containing 10 mM glucose plus 2.5 mM quinoline or 10 mM succinate plus 2.5 mM quinoline. Quinoline was

Table 2. Heterocyclic substrates which in addition to quinoline served as sole source of carbon for growth of *Pseudomonas putida* Chin IK and *Rhodococcus* sp. CH 5

Substrate[a]	Strain Chin IK	Strain CH 5
2-Carboxyquinoline	−	+
4-Carboxyquinoline	−	+
2-Hydroxyquinoline	+	+
4-Hydroxyquinoline	−	+
2-Methylquinoline	−	+
Coumarin	+	−
Furan-2-carboxylate	+	−
Furfural	+	−
Furfuryl alcohol	+	n.d.

[a] Nicotinate, nicotine, 2-pyrrolecarboxylate, xanthine, and 2-thiophenecarboxylate were not degraded by both organisms. These compounds were added to the medium to give a final concentration of 2.5 mM. n.d., not determined

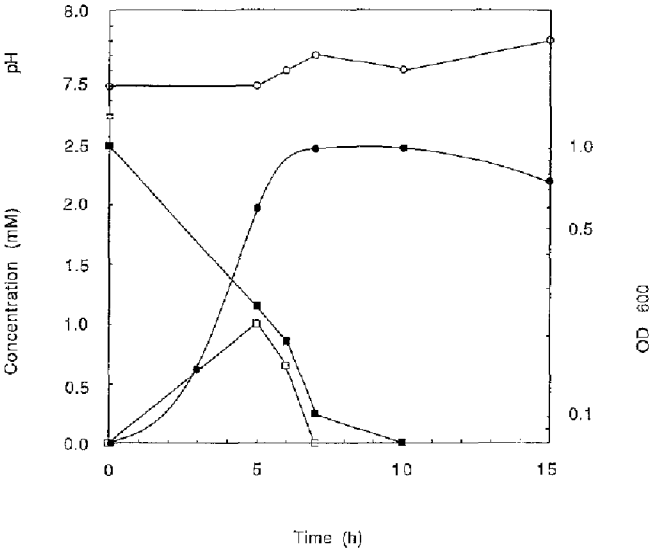

Fig. 1. Growth of *Pseudomonas putida* Chin IK in mineral medium containing 2.5 mM quinoline as sole source of carbon, nitrogen, and energy. ●, Optical density at 600 nm; ■, quinoline; □, 2-hydroxyquinoline; ○, pH

co-metabolized in the presence of glucose and succinate as well as in the presence of complex components like peptone and beef extract.

In order to investigate whether a dehydrogenase was involved in the initial breakdown of quinoline, a variety of electron acceptors was tested under anaerobic conditions with respect to their substrate-dependent reduction (Table 3). In crude extracts of all organisms investigated a reduction of some artificial electron acceptors of high redox potential, like DCPIP, methylene blue, MTT, and thionin, was found. For the dehydrogenase activity of *P. putida* Chin IK was most stable compared to the activity of other strains, further studies on this enzyme were carried out with that strain. Best results were obtained using PES + MTT as electron acceptors in the presence of 0.1 to 1.5% (v/v) Triton X-100. The reaction rate was dependent on both the concentration

Table 3. Electron acceptors used for the detection of quinoline dehydrogenase activity in extracts of some isolates

Organism	MTT + PES [mU/mg]	% Activity[a] compared to MTT + PES		
		DCPIP + PES	Methylene blue	Thionin
Pseudomonas acidovorans CH 1	810	71	21	22
Pseudomonas sp. CH 4	300	161	99	86
Rhodococcus sp. CH 5	340	97	130	89
Strain CH 6	370	226	94	65
Strain CH 7	290	187	93	75
Strain CH 9	50	88	119	72
Strain TW	370	168	81	65
Pseudomonas putida Chin IK[b]	250	58	32	25

[a] Ferricyanide and DCPIP alone gave high reaction rates without addition of quinoline in case of all organisms and no acceleration was detected in assay with substrate included. NAD was negative for all organisms listed

[b] Cytochrome *c*, FAD, FMN, and viologens were only tested for *P. putida* Chin IK but found to be negative

of PES and MTT. Optimal pH was 11.0 independent of the buffer system used. Activity at pH 8 was about 80% of the activity of optimal pH. K_m for quinoline was determined to be 6.3×10^{-5} M. Dehydrogenase activities of *P. putida* Chin IK and *Rhodococcus* sp. CH 5 were not inhibited by methanol (up to 1.0 M), but 2 mM of either arsenite or cyanide resulted in more than 50% inactivation of initial activity after 15 min of incubation. The M_r of the quinoline dehydrogenases from strains Chin IK, CH 1, CH 4, CH 5 to 7, CH 9, and TW was determined to be about 300 kDa by means of non-denaturing gradient PAGE and subsequent staining for activity. R_f values differed only slightly. Gel filtration on Sephacryl S-200 HR of the enzyme from *P. putida* Chin IK gave a similar result. Strains CH 1, CH 5, CH 6, CH 7, TW, and Chin IK also exhibited xanthine dehydrogenase activity at positions of quinoline dehydrogenase activity as evidenced by staining for the respective enzyme activity after non-denaturing PAGE. Strains CH 6, CH 7, CH 9, and TW additionally contained a band exhibiting only xanthine dehydrogenase activity whereas a separate enzyme was not detected in strains CH 1, CH 5, and Chin IK.

The expression and activity of quinoline dehydrogenase was dependent on the substrate combination used for cultivation of *P. putida* Chin IK. No activity was detected in extracts of cells grown on succinate, proving its inducible nature. The addition of 2.5 mM quinoline to 10 mM succinate resulted in about 10% the activity determined in cells grown on quinoline as sole carbon and nitrogen source, whereas the quinoline dehydrogenase activity was about 80% in cells supplied with ammonia as additional nitrogen source. Cells grown on 2-hydroxyquinoline as sole substrate contained about 40% of the activity detected with quinoline as substrate.

Quinoline dehydrogenase activity was influenced by the presence of molybdate and tungstate, an antagonist

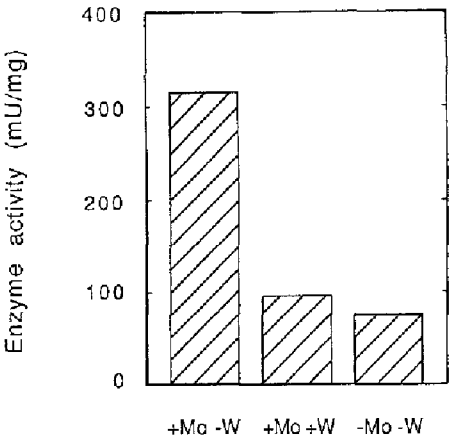

Fig. 2. Dependence of quinoline dehydrogenase activity (*Pseudomonas putida* Chin IK) on the presence of molybdate and tungstate (10^{-7} M each) during growth

of molybdate, in the medium. Equimolar concentrations (10^{-7} M each) of molybdate plus tungstate resulted in 30% of enzyme activity compared with a medium containing molybdate alone (Fig. 2). Very low activity was detected in extracts of cells of *P. putida* Chin IK grown in the presence of 10^{-7} M tungstate, consistent with the poor growth and quinoline degradation by these cells.

Partial purification of the enzyme from strain Chin IK involved anion exchange chromatography on DEAE-Sephacel, two gel filtration steps on Sephacryl S-200 HR and Superdex 200 PG, and preparative non-denaturing PAGE (5%) followed by electroelution using a Biotrap chamber. The absorption spectrum of this partially purified fraction exhibited maxima at 450 and 550 nm indicating the presence of flavin and Fe/S centers. The quotients of absorbance at different wavelengths were: 280/450 = 18.3 and 450/550 = 2.1. The fluorescence spectrum after oxidation of the same sample with KI/I_2 showed maxima for excitation and emission at 390 and 460 nm, respectively, indicating the presence of a molybdenum-binding pterin.

A possible immunological relationship of the dehydrogenase from *P. putida* Chin IK to other molybdenum-containing dehydrogenases was tested. No immunological cross-reaction was observed with antibodies raised against nicotinate dehydrogenase from *Bacillus niacini* (Nagel and Andreesen 1990) or xanthine dehydrogenase from *Pseudomonas putida* Fu1 (Koenig and Andreesen 1990) by means of double immunodiffusion and Western blotting.

Discussion

Many of the organisms isolated in this study were shown to belong to the genus *Pseudomonas*. This is in agreement with previous investigations where it appeared to be the dominant genus among quinoline degrading bacteria (Grant and Al-Najjar 1976; Bennett et al. 1985; Shukla 1986; Schwarz et al. 1988; Aislabie et al. 1990). Concentrations of quinoline exceeding 0.03% (~ 2.5 mM) in-

hibited growth as also described by Shukla (1986). 2-Hydroxyquinoline was metabolized by all the isolates tested. Excretion of this compound into the medium seems to happen quite often and was observed even under anaerobic conditions (Bennett et al. 1985; Brockman et al. 1989; Kuhn and Suflita 1989). This indicates that hydroxylation of quinoline at position 2 of the pyridine moiety is the generally favored reaction initiating its degradation and does not involve oxygen (Beedham 1985; Shukla 1986; Schwarz et al. 1988, 1989). There is some evidence that *Pseudomonas putida* Chin IK metabolizes quinoline via the coumarin pathway (Shukla 1989) because the latter compound served as carbon source for this organism, too. Although the degradation of unsubstituted coumarin by quinoline degrading bacteria has not been reported, hydroxylation of the former compound would lead to 8-hydroxycoumarin, an intermediate of quinoline catabolism typical for *Pseudomonas* species, but not for *Rhodococcus* (Shukla 1989; Schwarz et al. 1989).

The similarity of the initial reaction during aerobic and anaerobic degradation of quinoline to hydroxylation reactions involved in catabolism of other heterocyclic compounds like purines (Vogels and van der Drift 1976; Dürre and Andreesen 1982; Berry et al. 1987) and pyridines (Shukla 1984; Freudenberg et al. 1988; Nagel and Andreesen 1989) suggested that the first enzyme which attacks the pyridine moiety of quinoline might be a molybdenum-containing dehydrogenase as described for similar enzymes (Krüger et al. 1987; Freudenberg et al. 1988; Nagel and Andreesen 1989; Nagel et al. 1989; Koenig and Andreesen 1990; Siegmund et al. 1990; Nagel and Andreesen 1990). This is supported by our results that degradation of quinoline was specifically inhibited by tungstate, an antagonist of molybdate (Mills and Bremner 1980). Further evidence for the involvement of molybdenum in the first hydroxylating enzyme of strain Chin IK and strain CH 5 was obtained by the inhibitory effect of cyanide and arsenite on both enzymes. Both inhibitors attack the cyanolyzable sulfur atoms of the molybdenum cofactor (Coughlan 1980; Coughlan et al. 1980). Partially purified enzyme preparations from *P. putida* Chin IK exhibited fluorescence spectra characteristic for a molybdenum-binding pterin (Johnson and Rajagopalan 1982). Although the enzyme preparation was not pure, as indicated by the absorption quotients at 280, 450, and 550 nm (Coughlan 1980) and non-denaturing PAGE, it seemed to be reasonable that these contaminating proteins did not contain chromophores as can be deduced from the 450/550 nm quotient. The absorption spectrum obtained for the enzyme from *P. putida* Chin IK was typical for a molybdenum-containing dehydrogenase with flavin and iron-sulfur centers present. Three dominant proteins stained at about 85, 30, and 18 kDa after SDS-PAGE. A similar pattern was found for many bacterial enzymes belonging to this class (Wagner et al. 1984; Freudenberg et al. 1988; Nagel and Andreesen 1990). The M_r of about 300 kDa as estimated for quinoline dehydrogenases from several different isolates also supports the relationship to molybdoenzymes of the xanthine dehydrogenase/oxidase-type (Coughlan 1980; Rajagopalan 1988). A further strong indication is the coincidence of the bands stained for activity after non-denaturing PAGE using xanthine and quinoline as a substrate. A variety of azaheterocyclic compounds like quinoline and isoquinoline are oxidized by the molybdenum-containing enzyme aldehyde oxidase from liver (Krenitsky et al. 1972; Stubley et al. 1979; Bunting et al. 1980; Beedham 1985). Thus, all the evidence presented indicates the presence of a molybdenum-containing quinoline dehydrogenase exhibiting similarities to xanthine dehydrogenase.

Further studies on quinoline dehydrogenases will concentrate on the relationship of the subunits and nature of the molybdenum cofactor. Bacterial molybdoenzymes tested so far contain a molybdenum-binding pterin presumably different from that of eukaryotes (Krüger and Meyer 1986; Hinton and Dean 1990). The higher M_r of the bacterial cofactor is due to the attachment of GDP to the alkyl chain of the cofactor in case of dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* (Johnson et al. 1990).

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