Quinoline Oxidoreductase from *Pseudomonas putida* 86: An Improved Purification Procedure and Electron Paramagnetic Resonance Spectroscopy†

Barbara Tshisuaka,‡ Reinhard Kappl,§ Jürgen Hüttermann,§ and Franz Lingens*,‡

Fachrichtung Biophysik und Physikalische Grundlagen der Medizin, Universität des Saarlandes, Klinikum Bau 76, 66421 Homburg/Saar, Germany, and Institut für Mikrobiologie der Universität Hohenheim, Garbenstrasse 30, 70593 Stuttgart, Germany

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ABSTRACT: Quinoline oxidoreductase, an iron–sulfur molybdenum flavoprotein containing flavin adenine dinucleotide and molybdopterin cytosine dinucleotide, was purified from *Pseudomonas putida* 86 to homogeneity. The various electron-transfer centers of the purified enzyme were examined by electron paramagnetic resonance spectroscopy. Quinoline deuterated at position 2 was prepared by deuterodecarboxylation of 2-quinolinecarboxylic acid. Quinoline added to the enzyme elicited the Mo(V) "rapid" type Q signal arising from the complex of enzyme and substrate, whereas in oxidized quinoline oxidoreductase a Mo(V) "resting" signal was observed. EPR spectroscopy at helium temperatures below 70 K revealed the existence of two types of iron–sulfur centers, Fe-S I and Fe-S II. An organic free radical appeared upon reduction with sodium dithionite. Inactivation of the enzyme by cyanide led to the inactive desulfo quinoline oxidoreductase, which yielded another Mo(V) signal designated "slow" type Q upon reduction with dithionite. Desulfo quinoline oxidoreductase was partially reactivated by incubation with sulfide.

Quinoline oxidoreductase is an enzyme induced by aerobic growth of *Pseudomonas putida* 86 on mineral salts medium containing quinoline (2,3-benzopyridine) as the sole source of energy, carbon, and nitrogen. This enzyme initiates the degradation of quinoline by hydroxylating the carbon atom adjacent to the nitrogen (Schwarz et al., 1989). Quinoline is thus converted to 2-oxo-1,2-dihydroquinoline, the prevailing tautomeric form of 2-hydroxyquinoline. Quinoline oxidoreductase from *P. putida* 86 was purified and characterized. It was shown that the oxygen atom incorporated into the product of the enzymic reaction is derived from water. The enzyme consisting of three nonidentical subunits is an iron–sulfur molybdenum flavoprotein containing flavin adenine dinucleotide (Bauder et al., 1990). In contrast to bovine milk xanthine oxidase (Kramer et al., 1987) and xanthine dehydrogenase from *P. putida* 86, the molybdenum in quinoline oxidoreductase is not associated with molybdopterin as the organic part of the molybdenum cofactor, but with molybdopterin cytosine dinucleotide (Hettrich et al., 1991). This variant of molybdopterin was only recently discovered in carbon monoxide dehydrogenase from *Pseudomonas carboxydoflava* (Johnson et al., 1990). Apart from its presence in the quinoline oxidoreductases of *P. putida* 86 and *Rhodococcus* sp. B1, molybdopterin cytosine dinucleotide was also found in quinaldine oxidoreductase from *Arthrobacter* sp. Rü 61a (de Beyer & Lingens, 1993) and in 4-quinolinecarboxylic acid oxidoreductase from *Agrobacterium* sp. 1B (Bauer & Lingens, 1992). Since the internal electron transport chain of quinoline oxidoreductase is composed of four redox active centers detectable by EPR[1] spectroscopy, this method is well suited for the study of the enzyme. The molybdenum center as the site of substrate binding and catalysis is of special interest, because until now the coincidence of the Mo(V) signal and the interaction between enzyme and substrate could not be demonstrated for a molybdenum-containing hydroxylase with molybdopterin cytosine dinucleotide.

MATERIALS AND METHODS

Bacterial Growth and Purification of Quinoline Oxidoreductase. Pseudomonas putida 86 was cultured aerobically in a 100-L fermenter at 30 °C in medium containing (g/L) 4.33 Na_2HPO_4, 2.65 KH_2PO_4, 0.4 $MgSO_4 \cdot 7H_2O$, 1.0 NaCl, 0.1 $FeSO_4 \cdot 7H_2O$, and 0.01 $Na_2MoO_4 \cdot 2H_2O$. The pH was adjusted to 7.3 with NaOH. After sterilization, 0.5 mL/L quinoline was added. Bacterial growth and concentrations of quinoline and 2-oxo-1,2-dihydroquinoline were measured according to Bauder et al. (1990). When neither quinoline nor 2-oxo-1,2-dihydroquinoline was detectable in the medium during bacterial growth, an additional portion of 0.5 mL/L quinoline was added. After a fermentation time of 40 h, the cells were separated by centrifugation. Fifty grams of cells (wet weight) was suspended in 75 mL of buffer (100 mM Tris-HCl, pH 8.5), disrupted by sonification at 0 °C, and centrifuged for 40 min at 50000*g* (4 °C). The supernatant was fractionated by ammonium sulfate precipitation (0.8 to 1.5 M). The precipitate was dissolved in buffer, heated to 58 °C in a water bath, and maintained at this temperature for 10 min. After centrifugation, ammonium sulfate (300 mM) was added to the supernatant before it was loaded onto a phenyl-Sepharose CL-4B column (2.8 × 17 cm) equilibrated in buffer containing 300 mM ammonium sulfate. The column was washed with 1 L of buffer containing 100 mM ammonium sulfate and eluted at 1 mL/min with 50 mM Tris-HCl, pH 8.5. Active fractions were pooled and applied to a DEAE-Sephacel column (2.8 × 17 cm) equilibrated in 50 mM Tris-HCl, pH 8.0. The column was washed with 1 L of 200 mM Tris-HCl, pH 8.0, and eluted at 1 mL/min with a linear gradient from 250 to 900 mM Tris-HCl, pH 8.0. Active fractions were pooled, concentrated, and washed with 100

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‡ Institut für Mikrobiologie der Universität Hohenheim.

§ Universität des Saarlandes.

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[1] Abbreviations: *E*, extinction coefficient; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; INT, *p*-iodonitrotetrazolium violet.

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mM Tris-HCl, pH 8.0, in an ultrafiltration cell equipped with an XM 100A membrane (Amicon). For gel filtration the concentrate was loaded in portions of 0.5 mL onto a HiLoad 16/60 Superdex 200 prep grade column equilibrated in 100 mM Tris-HCl, pH 8.0. The column was run at 1.5 mL/min, and the active fractions were pooled, concentrated as above, and stored at −80 °C after glycerol (final concentration: 10%) had been added. The purified enzyme had a specific activity of 19 units/mg. Protein was determined according to Lowry et al. (1951) with ovalbumin as standard or by using the molar extinction coefficient of quinoline oxidoreductase (E_{450nm} = 64.5 × 10^3 M^{-1} cm^{-1}).

UV/Vis Spectroscopy. The absorption spectra of quinoline oxidoreductase (0.95 mg in 1.0 mL 50 mM Tris-HCl, pH 8.0) were recorded with a Uvicon 810 spectrophotometer connected to a Uvicon 21 recorder (Kontron Analytic GmbH). Ten microliters of a freshly prepared 10% aqueous $Na_2S_2O_4$ solution or 10 μL of quinoline, isoquinoline, quinaldine, or 1-oxo-1,2-dihydroquinoline (all 17 mM in 2-propanol) was added to the enzyme as isolated, and spectra were taken immediately after the samples were mixed and were completed within 5 min. For the difference spectrum the spectrum of active quinoline oxidoreductase (21 units/mL) was subtracted from that of inactive desulfo enzyme (0.1 units/mL).

Assay of Quinoline Oxidoreductase Activity. Quinoline oxidoreductase was assayed by following the quinoline-dependent reduction of the monotetrazolium salt *p*-iodonitrotetrazolium violet (INT) to its formazan as described previously (Bauder et al., 1990) except that 5 mM INT was used. One unit of enzyme activity was defined as 1 μmol of formazan formed per minute at 25 °C.

Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed as described by Hames (1981) using the high- and neutral-pH nondissociating discontinuous buffer systems with 10% acrylamide in the resolving gel. Tricine–SDS–polyacrylamide gel electrophoresis (Schägger & von Jagow, 1987) separated the enzyme's subunits under dissociating and reducing conditions and was also used to check homogeneity.

Inactivation of Quinoline Oxidoreductase by Cyanide. Quinoline oxidoreductase (6.9 units/mL) was incubated with KCN (30–70 mM) at room temperature. Aliquots were taken from the incubation mixture and assayed for enzyme activity by quinoline-dependent reduction of INT.

Reactivation of Cyanide-Inactivated Quinoline Oxidoreductase by Sulfide. Quinoline oxidoreductase (5.9 units/mL) was inactivated with 70 mM KCN until activity decreased to less than 0.01 units/mL. After the enzyme was rinsed with buffer (0.1 M Tris-HCl, pH 8.0) by ultrafiltration (Centricon 100, Amicon), an incubation with 10 mM Na_2S followed for 2 and 4 h at 45 °C in a water bath. The enzyme was again ultrafiltered before enzyme activity was determined. Thiocyanate was determined in the ultrafiltrate according to Sörbo (1957).

Synthesis of Deuterated Quinoline. Quinoline-*2-d* was prepared by adapting the method of deuterodecarboxylation described by Zoltewicz et al. (1968). 2-Quinolinecarboxylic acid (1.75 g) was dissolved in 3 mL of D_2O by boiling and placed in a small distillation apparatus. The solution was heated slowly in a silicon oil bath. After D_2O had been distilled, formation of CO_2 was observed as foaming. Finally quinoline-*2-d* was distilled by raising the temperature of the silicon oil bath to 260 °C. The yield was 1 g of quinoline-*2-d*. ^{1}H-NMR spectra of deuterated and nondeuterated quinoline were compared (data not shown). Deuteration at position 2 was obvious due to a lower signal intensity (2%) for 2-H (=8.9 ppm) and a simplified multiplet (doublet) for 3-H (=7.4 ppm) proving that deuteration was nearly complete (98%).

EPR Spectroscopy. EPR spectra at X-band frequencies were recorded on a Bruker ER 420 or ESP 300 spectrometer equipped with a continuous helium flow cryostat (ESR 900) for the temperature range from 4 to 60 K or with a quartz dewar for measurements at liquid nitrogen temperatures. The magnetic field and the microwave frequency were determined with an NMR gaussmeter and a microwave counter, respectively. The modulation amplitude for recording of spectra was 0.5 mT if not stated explicitly.

Preparation of EPR Samples. For reduction, 1 μL of quinoline (340 mM in ethanol) or 10 μl of 200 mM $Na_2S_2O_4$ was added to 31 nmol of native quinoline oxidoreductase (approximately 300 units) in 200 μL of 100 mM Tris-HCl, pH 8.0, and the samples were transferred into quartz tubes and frozen in liquid nitrogen within 1 min. The dithionite-reduced sample was repeatedly thawed under a nitrogen atmosphere, incubated at room temperature, frozen, and reexamined by EPR. Desulfo quinoline oxidoreductase was prepared analogously to desulfo xanthine oxidase according to Massey and Edmondson (1970) and Gutteridge et al. (1978b). Quinoline oxidoreductase (90 nmol/mL) was incubated for 180 min at room temperature with KCN at a final concentration of 12.5 mM. The enzyme was thoroughly washed with buffer and concentrated by ultrafiltration. The samples (31 nmol in 200 μL of buffer), less than 0.5% functional, were reduced with quinoline and dithionite as described above and frozen in nitrogen within 1 min. After the EPR spectra were recorded, the samples were thawed, further incubated at room temperature for 10 min, reinvestigated by EPR, and so on, so that finally EPR spectra for 1, 10, 20, 30, and 45 min after addition of reductant were obtained.

RESULTS

Growth of Bacteria and Purification of Quinoline Oxidoreductase. Pseudomonas putida 86 was cultured in a mineral salts medium of a different composition compared to that used by Bauder et al. (1990). The phosphate content was especially augmented, and the supply of iron and molybdenum was improved. Furthermore, the fermentation time was extended to 40 h, and quinoline was added up to six times. So quinoline oxidoreductase was better induced as judged by the specific activity, which increased from 0.26 to 0.58 unit/mg in the extract. Apart from minor variations concerning hydrophobic interaction and anion-exchange chromatography, the purification of quinoline oxidoreductase was improved mainly by introducing a heat step and by using Superdex 200 for gel filtration. The yield amounted to 35% instead of 21%, and the specific activity was 19 instead of 13.2 units/mg, in comparison to the previously published procedure. The enzyme's purity was checked by gel electrophoresis under native and dissociating conditions (Figure 1) and further confirmed by its UV/vis spectrum (Figure 2). As depicted in Figure 1, the quinoline oxidoreductase of *P. putida* 86 (native molecular mass: 300 kDa) consists of three subunits of different sizes (85, 30, and 20 kDa). An $(\alpha\beta\gamma)_2$ composition of the enzyme is assumed. Table I lists data concerning purification of quinoline oxidoreductase from *P. putida* 86.

UV/Vis Spectra of Quinoline Oxidoreductase. The UV/vis spectrum of quinoline oxidoreductase (Figure 2) is characteristic for a molybdenum-containing hydroxylase. The ratios E_{280nm}/E_{450nm} and E_{450nm}/E_{550nm} were 4.0 and 3.0,

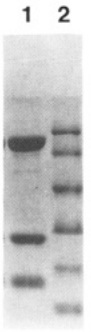

FIGURE 1: SDS–PAGE of (1) purified quinoline oxidoreductase from *Pseudomonas putida* 86 (native molecular mass: 300 kDa) dissociated into its three subunits (85, 30, and 20 kDa) and (2) standard proteins phosphorylase b (94 000 Da), bovine serum albumin (67 000 Da), ovalbumin (43 000 Da), carbonic anhydrase (30 000 Da), trypsin inhibitor (20 100 Da), and α-lactalbumin (14 400 Da).

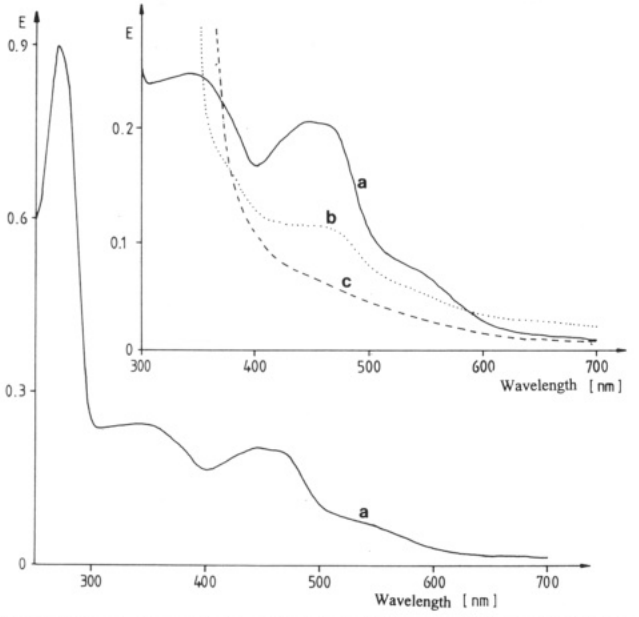

FIGURE 2: 2. UV/vis spectra of quinoline oxidoreductase from *Pseudomonas putida* 86 (a) in the oxidized form (as isolated), (b) reduced with quinoline, and (c) reduced with dithionite.

Table I: Purification of Quinoline Oxidoreductase from *Pseudomonas putida* 86

fraction	units	protein (mg)	sp act. (units/mg)	purification (*x*-fold)	yield (%)
extract	3000	5184	0.58	1	100
ammonium sulfate precipitation	2884	3605	0.81	1.4	96
heat step	2640	432	6.1	10.5	88
phenyl-sepharose	1488	91.5	16.2	28	50
DEAE-Sephacel	1165	*a*	*a*	*a*	39
Superdex 200	1054	55.6	19	33	35

[a] The high Tris-HCl concentration interferes with the Lowry protein determination.

respectively. The inset in Figure 2 shows UV/vis spectra of oxidized (trace a), substrate-reduced (trace b), and dithionite-reduced quinoline oxidoreductase (trace c). Addition of the substrate quinoline effected a decrease in absorbance in the region from 360 to 580 nm, whereas above 600 nm a slight increase was observed. Dithionite caused a stronger bleaching of the enzyme's chromophores in the entire range from 370 to 700 nm. Isoquinoline, quinaldine, and 2-oxo-1,2-dihydroquinoline, as substrates, did not bring about any change in absorbance.

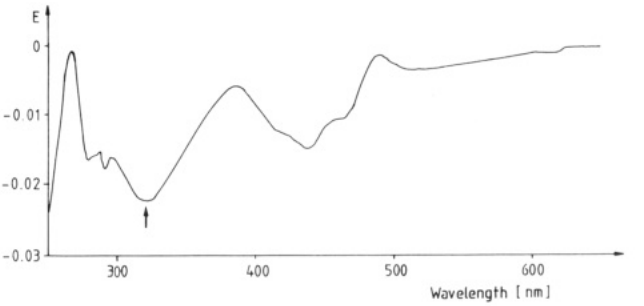

FIGURE 3: UV/vis difference spectrum between desulfo quinoline oxidoreductase and active quinoline oxidoreductase.

Inactivation of Quinoline Oxidoreductase by Cyanide. Potassium cyanide induced a concentration- and time-dependent inactivation of quinoline oxidoreductase. The enzyme lost 50% of its activity in 8.5 min when incubated in 30 mM KCN, in 5.0 min with 40 mM KCN, in 3.5 min with 50 mM KCN, and in 2.5 min with 70 mM KCN. The action of cyanide on quinoline oxidoreductase changed the UV/vis spectrum. Figure 3 shows the difference spectrum, which is negative and characterized by a trough at 320 nm. Thiocyanate was detectable in the ultrafiltrate of cyanide-inactivated quinoline oxidoreductase.

Reactivation of Cyanide-Inactivated Quinoline Oxidoreductase by Sulfide. The inactivated enzyme was not reactivated by merely eliminating the inhibitor. However, incubation with Na_2S caused a reactivation of 26% after 2 h and 63% after 4 h relative to the control experiment with active enzyme submitted to the same conditions (data not shown).

Mo(V) Signals. The EPR spectrum of the oxidized enzyme (as isolated) recorded at 77 K shows a line pattern with rhombic symmetry (Figure 4a) that resembles the "resting" signal known from xanthine dehydrogenases, aldehyde oxidase, and carbon monoxide oxidases (Dalton et al., 1976; Barber et al., 1976; Turner et al., 1987; Bray et al., 1983). There are some weaker and rather broad lines apparent which may arise from hyperfine interaction with the ^{95}Mo and ^{97}Mo isotopes, both with nuclear spin $I = {}^5/_2$ and with natural abundances of 15.9% and 9.6%, respectively. Due to the low signal intensity and resolution of the resting signal a complete assignment of the Mo hyperfine lines along the three *g* turning points could not be achieved. The apparent *g*-factors of the prevailing ^{96}Mo isotope (74.5% natural abundance) with zero nuclear spin are listed in Table II. Upon reduction of quinoline oxidoreductase with its substrate quinoline an intense EPR spectrum of a Mo(V) species was readily observable (Figure 4 b). It is designated "rapid" type Q to characterize its origin and its similarity with the "rapid" signals of milk xanthine oxidase (Bray, 1975). In contrast to the corresponding rapid signals of milk xanthine oxidase, the Mo(V) rapid type Q exhibits axial symmetry and the presence of only one strongly coupled proton. The spectral pattern remained unchanged when quinoline-*2-d* was used for reduction. Exchange of H_2O with D_2O in the protein sample leads to a clear loss of the proton hyperfine splitting, revealing an axial powder type spectrum due to *g*-anisotropy (Figure 4c). Apart from the intense signals of the Mo isotopes without a nuclear spin, weaker signals arising from ^{95}Mo and ^{97}Mo nuclei are discernible in the quinoline-reduced species. In the sample with D_2O buffer (Figure 4c) two of the expected six hyperfine lines are resolved at higher fields, which are split by about 3.06 mT. The stick diagram indicates the positions of other poorly resolved lines, which are centered around $g_\perp$ of the

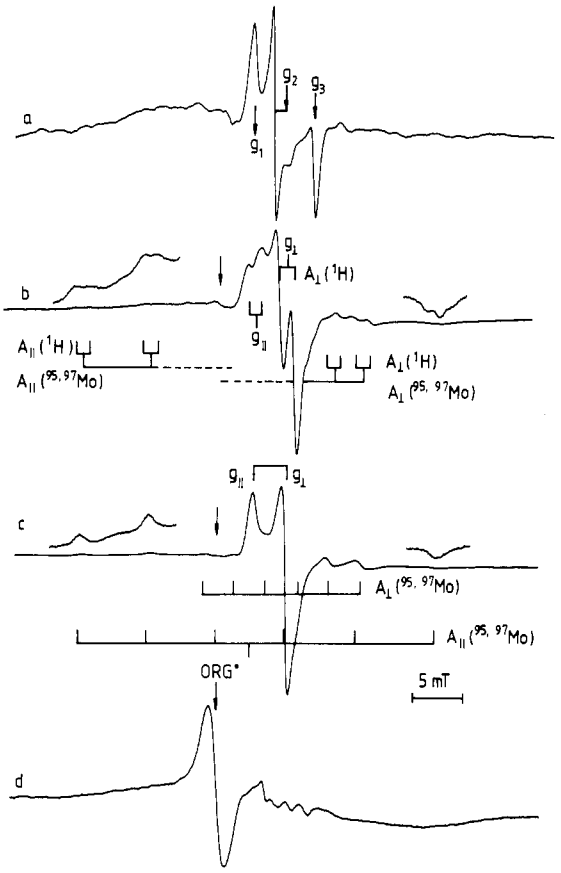

FIGURE 4: EPR spectra of quinoline oxidoreductase from *Pseudomonas putida* 86 (a) as isolated, (b) reduced with quinoline in nondeuterated solvent, and (c) reduced with quinoline in deuterated buffer. Spectrum d was obtained upon addition of an excess of dithionite to the native enzyme. The stick diagrams indicate the hyperfine splittings of the ^{95}Mo and ^{97}Mo isotopes and of an exchangeable proton. The position of the organic radical (ORG•) is marked with arrows. The spectra were recorded at 77 K.

major component. These $A_\perp$ features are additionally split by the proton hyperfine interaction in the H_2O sample, as is inferred from the corresponding lines at higher fields (Figure 4b). The measured proton hyperfine splitting is identical to that of the major component (1.54 mT). For other Mo hyperfine lines the splitting is not well resolved, overlapping severely with the signals of the major component. The rather broad $A_\parallel$ hyperfine lines of ^{95}Mo and ^{97}Mo nuclei show a splitting of about 6.66 mT (Figure 4c). These resonances are also split by a proton interaction (about 1.2 mT) for the quinoline-reduced enzyme in H_2O (Figure 4b), as indicated in the enlarged parts of the spectrum. Provisional EPR parameters of the Mo(V) signals, directly inferred from spectra, are compiled in Table II and compared with the set of parameters from spectral simulations of isotopically enriched xanthine oxidase (George & Bray, 1988). Another Mo(V) species, the "slow" type Q signal, was observed when cyanide-inactivated (desulfo) quinoline oxidoreductase was reduced with dithionite. The EPR spectra at 77 K, obtained 1 and 45 min after addition of dithionite, show the time-dependent increase of this species (Figure 5 a,b). It is superimposed on broad, unresolved, and time-independent signals of reduced Fe–S centers (see below) and reached maximal intensity approximately 30 min after addition of dithionite. The difference between the spectra yields the line pattern of the Mo(V) slow type Q signal (Figure 5c), which is interpreted as having rhombic *g*-symmetry together with an additional splitting of a proton, as suggested by the stick diagram. In the absence of isotopic substitution the proton assignment has to be considered as tentative. The apparent *g*-values and splittings are listed in Table II. Addition of quinoline to cyanide-inactivated quinoline oxidoreductase did not produce the Mo(V) rapid type Q signal. The low-intensity resting Mo(V) signal was detected in the cyanide-inactivated enzyme with quinoline added and without any reductant.

Organic Radical Species. Upon reduction of the native enzyme with dithionite instead of quinoline no Mo(V) rapid signals were present because molybdenum was thus converted to the EPR-silent tetravalent oxidation state. However, under these conditions a free radical signal with a *g*-factor of 2.0034 and a line width of 1.91 mT was obtained (Figure 4d; Table II). In contrast, using quinoline as reducing agent, only very small amounts of the free radical species may contribute to the Mo and proton hyperfine features in Figure 4b,c (indicated by arrows). Besides the organic radical, some weak resonances are visible toward higher field. The signals of the resting species are identified by spectral comparison. There remain two more weak lines which, however, are not identical to the isolated slow type Q signal of the reduced desulfo enzyme. Their origin is not clear presently.

Signals from Iron–Sulfur Centers. In the temperature range below 60 K distinct rhombic EPR signals of two Fe-S centers were resolved in quinoline oxidoreductase reduced with dithionite or the substrate quinoline. By comparison with the findings for xanthine oxidase, and according to the respective convention in the literature, the rhombic Fe-S signals with the smaller *g*-anisotropy are designated Fe-S I, and those with the larger *g*-anisotropy, Fe-S II, respectively (Bray, 1975; Bray et al., 1991). In the case of the dithionite-reduced enzyme, for which the Mo(V) signal is largely suppressed, the Fe-S centers dominate the EPR spectra recorded at different temperatures and microwave powers (Figure 6a,b). The position of the organic radical signal, which is easily saturated for higher microwave power (Figure 6b), is indicated by an arrow. The signals of the Fe-S I center are generally more intense with a smaller line width at various microwave powers compared to Fe-S II. The corresponding EPR parameters are listed in Table III.

When the enzyme is reduced with quinoline, two rhombic Fe-S centers are again visible in the EPR spectrum. The low-field signals of both Fe-S centers exhibit comparable intensities at different temperatures and incident microwave powers, as is inferred from Figure 6c,d. In contrast, the intermediate **g**-tensor component of Fe-S II is very broad and shows a large intensity, which is not expected from the relative intensities of the low-field lines. This is related to the superposition with an incompletely saturated, broad signal of the Mo(V) rapid type Q species, for which at lower microwave powers some structure of the signal is weakly discernible. As a consequence, the intermediate *g*-factors cannot be determined precisely. It is noteworthy that the *g*-factors of the high- and low-field tensor components differ slightly from those of the dithionite-reduced enzyme (Table III). At higher temperatures (about 40 K) the signals of Fe-S I show a larger intensity relative to the Fe-S II signals.

DISCUSSION

The improved purification method for quinoline oxidoreductase of *P. putida* 86 yielded enzyme of highest purity, as displayed by the E_{280nm}/E_{450nm} ratio of 4.0. This value is the best that has been reported so far for an iron–sulfur molybdenum flavoprotein. Until now the value of 5.0 was regarded as a criterion of purity for molybdenum-containing

Table II: Parameters of EPR Signals in Quinoline Oxidoreductase from *Pseudomonas putida* 86 in Comparison with Simulation Parameters of Xanthine Oxidase

organic radical	g, 2.0034	line width,[a] 1.91	
Mo(V) rapid type Q[b]	$g_{\|}$, 1.987 (g_1, 1.9901)	$g_{\perp}$, 1.967 (g_2, 1.9710)	(g_3, 1.9666)
	$A_{\|}(^1H)$,[a] 1.18 (A_1, 1.23/0.46)	$A_{\perp}(^1H)$,[a] 1.54 (A_2, 1.26/0.17)	(A_3, 1.31/0.16)
	$A_{\|}(^{95,97}Mo)$,[a] 6.66 (A_1, 6.65)	$A_{\perp}(^{95,97}Mo)$,[a] 3.06 (A_2, 2.68)	(A_3, 2.78)
Mo(V) resting	g_1, 1.991	g_2, 1.976	g_3, 1.954
Mo(V) slow type Q[c]	g_1, 1.987 (1.9719)	g_2, 1.965 (1.9671)	g_3, 1.959 (1.9551)
	A_1,[a] 1.41 (1.66/0.16)	A_2,[a] 1.66 (1.66/0.16)	A_3,[a] 1.81 (1.56/0.16)

[a] Values are given in mT. [b] Values in parenthesis for Mo(V) rapid type 1 in xanthine oxidase are from George and Bray (1988). [c] Values in parenthesis for Mo(V) slow in desulfo xanthine oxidase are from Bray (1980) and Gutteridge et al. (1978b).

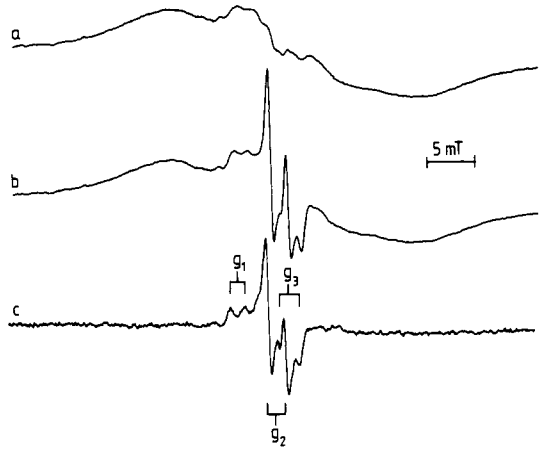

FIGURE 5: EPR spectra of desulfo quinoline oxidoreductase reduced with dithionite (a) 1 min and (b) 45 min after addition of dithionite; (c) difference spectrum, yielding the Mo(V) slow type Q signal. The spectra were recorded with identical spectrometer settings at 77 K.

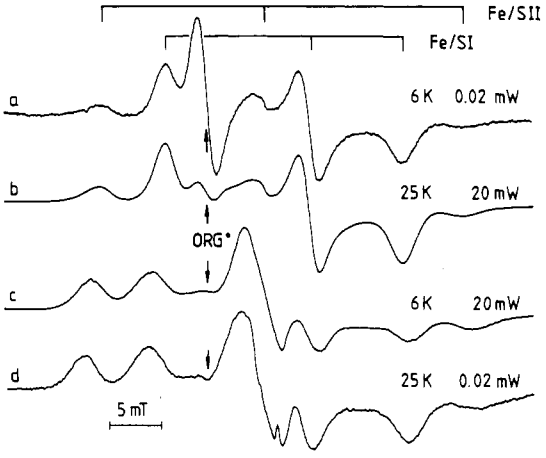

FIGURE 6: EPR spectra of the Fe-S centers in quinoline oxidoreductase. Spectra a and b were obtained on a sample reduced with dithionite, showing two different Fe-S species and the organic radical. Spectra c and d were recorded after reduction by quinoline. The recording temperature and microwave power are given with each spectrum.

hydroxylases and was believed to indicate the absence of other colorless proteins (Coughlan, 1980). Polyacrylamide gel electrophoresis under native and denaturing conditions further confirmed the homogeneity of quinoline oxidoreductase. An E_{450nm}/E_{550nm} ratio of 3.0 is indicative of the presence of flavin and iron–sulfur in a 1:4 ratio (Rajagopalan & Handler, 1964). This was substantiated by chemical analysis (Bauder et al., 1990). Inactivation of quinoline oxidoreductase with cyanide, reactivation of inactive enzyme with sulfide, the presence of thiocyanate following cyanide inactivation, and the characteristic decrease in the absorbance at 320 nm prove that cyanide abstracts the so-called cyanolyzable sulfur from the molybdenum center. In this respect quinoline oxidoreductase exhibits the same behavior as the well-known eukaryotic iron–sulfur molybdenum flavoproteins (Massey & Edmondson, 1970; Coughlan et al., 1969; Bray, 1975). In catalytically active quinoline oxidoreductase from *P. putida* 86 the molybdenum center is thus of the monoxo–monosulfido type, whereas in the inactive desulfo form it is of the dioxo type. According to the scheme of Wootton et al. (1991), quinoline oxidoreductase has to be classified as a molybdenum-containing hydroxylase, the only family of enzymes with pterin molybdenum cofactors that consists of members of prokaryotic and eukaryotic origin.

For the examination of quinoline oxidoreductase from *P. putida* 86 by EPR spectroscopy we used enzyme of the highest purity. The paramagnetic species, characterized by EPR spectroscopy of the oxidized and reduced forms of quinoline oxidoreductase, show similarities to other iron–sulfur molybdenum flavoproteins investigated so far. They are to some extent comparable with those of eukaryotic origin, like milk xanthine oxidase, as well as with those of prokaryotic origin, like xanthine dehydrogenases from *Clostridium acidiurici* (Wagner et al., 1984) and *Veillonella alcalescens* (Dalton et al., 1976) and carbon monoxide oxidases from pseudomonads (Bray et al., 1983). Until now molybdopterin cytosine dinucleotide as part of the pterin molybdenum cofactor has only been found in carbon monoxide oxidase (Johnson et al., 1990) and quinoline oxidoreductases (Hettrich et al., 1991). Bray et al. (1983) studied carbon monoxide oxidases from two different *Pseudomonas* strains by EPR spectroscopy, but they were not able to demonstrate the catalytic relevance of the Mo(V) signals. In contrast to these results, quinoline oxidoreductase of *P. putida* 86 produced a Mo(V) EPR signal, referred to as rapid type Q, instantly after addition of quinoline. Since the corresponding signal in bovine milk xanthine oxidase was attributed to the functional form of the enzyme (Bray, 1975; Gutteridge & Bray, 1980), and since only quinoline was able to provoke this signal, it is highly probable that, like other molybdenum-containing hydroxylases, quinoline binds at the molybdenum center, too. The absence of the rapid type Q signals in the cyanide-inactivated (desulfo) form of the enzyme after the substrate is added also supports this assumption. Only a minor portion of the preparation consists of nonfunctional enzyme giving rise to the Mo(V) resting signal also observed in xanthine dehydrogenases from *V. alcalescens* (Dalton et al., 1976) and turkey liver (Barber et al., 1976), aldehyde oxidase from *Desulfovibrio gigas* (Turner et al., 1987), and carbon monoxide oxidases from pseudomonads (Bray et al., 1983). In preparations of milk xanthine oxidase another Mo(V) species is present, responsible for the slow signal and ascribed to the inactive desulfo form of this enzyme (Bray, 1975, 1989; Gutteridge et al., 1978b; Massey & Edmondson, 1970). This desulfo xanthine oxidase is considered a preparation or storage artifact (Bray, 1975, 1980). However, for quinoline oxidoreductase this signal was observed in neither the dithionite- nor the quinoline-reduced sample,

Table III: EPR Parameters of Fe-S Centers in Quinoline Oxidoreductase from *Pseudomonas putida* 86 and Related Molybdoenzymes

enzyme	center	g_1	g_2	g_3	g_{av}	reference
quinoline oxidoreductase (*P. putida* 86)	Fe-S I (dithionite)	2.027	1.948	1.899	1.958	this work
	Fe-S I (quinoline)	2.035	1.95[a]	1.898	1.961	
	Fe-S II (dithionite)	2.067	1.973	1.871	1.970	
	Fe-S II (quinoline)	2.072	1.97[a]	1.866	1.969	
carbon monoxide oxidase (*P. carboxydovorans*)	Fe-S I	2.023	1.946	1.899	1.956	Bray et al. (1983)
	Fe-S II	2.137	2.009	1.900	2.015	
aldehyde oxidoreductase (*Desulfovibrio gigas*)	Fe-S I	2.021	1.938	1.919	1.959	Bray et al. (1991)
	Fe-S II	2.057	1.970	1.900	1.976	
xanthine dehydrogenase (*Clostridium acidiurici*)	Fe-S I	2.034	1.945	1.918	1.966	Wagner et al. (1984)
	Fe-S II	2.075	1.924	1.871	1.957	
xanthine oxidase (bovine milk)	Fe-S I	2.022	1.935	1.899	1.952	Bray (1975)
	Fe-S II	2.12	2.007	1.91	2.01	

[a] Values cannot be determined precisely due to superposition with Mo(V) signals.

even when periods before freezing the sample were extended to 60 min. Obviously in preparations of quinoline oxidoreductase no detectable amounts of the desulfo form are present. In desulfo quinoline oxidoreductase, prepared by treatment with cyanide, a Mo(V) slow type Q signal appeared upon reduction with dithionite after prolonged reaction times. With the restrictions mentioned above, the parameters directly derived from the spectrum show an increased rhombicity of the *g*-values and a more pronounced anisotropy of only one resolved proton interaction, when compared to the simulation parameters of the slow signal of milk xanthine oxidase (Bray, 1980). This may be indicative of a considerably distorted arrangement of the Mo center in the desulfo species of quinoline oxidoreductase. Similar to milk xanthine oxidase, the rapid type Q signal of quinoline oxidoreductase exhibits a solvent-exchangeable proton coupling of comparable size (1.3 vs 1.18 and 1.54 mT). A smaller proton interaction of 0.46 mT present in milk xanthine oxidase with various substrates (Morpeth et al., 1984; Gutteridge et al., 1978a,b; George & Bray, 1988) and confirmed by ENDOR studies (Howes et al., 1990) could not be resolved in quinoline oxidoreductase. The rapid type Q signal has axial symmetry, whereas in milk xanthine oxidase a rhombic distortion is present. This may be indicative of a less distorted environment of the molybdenum center in quinoline oxidoreductase than in milk xanthine oxidase. For the axial spectrum of quinoline oxidoreductase the hyperfine interaction of the less abundant Mo isotopes with nonzero nuclear spin can be estimated from the experimental spectra, taking the average from the broad lines for both isotopes. For the sample in nondeuterated solvent the $A_{\parallel}$ and $A_{\perp}$ features exhibit the expected doublet splitting of the proton hyperfine interaction. The Mo coupling constants are close to the values obtained on isotopically enriched samples of milk xanthine oxidase (Table II).

In order to monitor the interaction of the substrate with this new type of reactive center (molybdopterin cytosine dinucleotide molybdenum cofactor), quinoline deuterated at the catalytically relevant position 2 was employed. A transfer of the deuteron at position 2 to the cyanolyzable sulfur ligand of the molybdenum center during catalysis could reveal spectral changes in comparison with the protonated substrate. However, the large hyperfine interaction in the rapid type Q signal persisted. One possible explanation requires that the hydrogen not be involved in the catalytic cycle and that the exchangeable coupling may originate from the protein moiety or solvent. Alternatively, a rapid-exchange process is probable in which the proton transferred from quinoline to the molybdenum center is rapidly substituted by the solvent. Such a mechanism has been proven to exist in milk xanthine oxidase by rapid-freeze EPR experiments (Gutteridge et al., 1978a,b) but remains to be demonstrated for quinoline oxidoreductase.

Reduction of quinoline oxidoreductase with dithionite and to a smaller extent also with quinoline leads to the formation of an organic radical. In dithionite-reduced samples it constitutes a major spectral component. In both cases the signal is easily saturated at high powers and at low temperatures, typical for a free radical species. The EPR line width of 1.91 mT is indicative of the presence of a neutral, blue flavin radical species ($FADH^{\bullet}$) similar to milk xanthine oxidase (Palmer et al., 1971). However, in the optical spectrum of the dithionite-reduced enzyme no absorbance band in the region of 600–700 nm, which would be expected for a blue semiquinone radical, is found. Therefore it cannot be ruled out at present that the organic radical signal arises from some other radical species, e.g., the pterin moiety. Unfortunately, extraction of the flavin is not feasible without irreversible denaturation of the enzyme, in order to determine the signal-producing species.

The rhombic signals of Fe-S centers observed at temperatures below 60 K are similar to some extent to the EPR spectra found in other molybdenum-containing hydroxylases, e.g., milk xanthine oxidase, and are therefore categorized in an analogous way (Bray, 1975; Bray et al., 1991). The *g*-factors of the Fe-S I center in quinoline oxidoreductase show only little variation from those of other enzymes with characteristic g_{av} values around 1.95 to 1.96. The g_{av} value is especially close to the value for carbon monoxide oxidases, the only other enzymes with a molybdenum molybdopterin cytosine dinucleotide cofactor. This implies similar structures of the signal-producing species (Fe-S I) in the various prokaryotic and eukaryotic enzymes.

On the other hand, there are significant differences in *g*-factors for the Fe-S II signals indicative of a more pronounced variability of this signal-producing species. The g_{av} value of quinoline oxidoreductase closely resembles that of the aldehyde oxidoreductase of *D. gigas*, but shows a significant discrepancy with that of carbon monoxide oxidase, with a g_{av} value of 2.014.

The small but reproducible differences in the *g*-factors of both Fe-S species in quinoline oxidoreductase reduced by its substrate quinoline or by the nonphysiological reagent dithionite have not been reported so far for other enzymes of this class. The presence of the substrate increases the *g*-anisotropy slightly for both Fe-S I and Fe-S II centers, which may indicate some structural changes occurring at the Fe-S centers upon substrate binding at the catalytic site. Small changes in the composition of the buffer upon addition of dithionite cannot be excluded as a source for the observed differences in *g*-anisotropy. In addition the variance in temperature and power dependence of the signals in the quinoline- and the dithionite-reduced enzyme also points to distinct effects of the substrate on the relaxation behavior. In

this context, it is noteworthy that in both dithionite-reduced native and desulfo quinoline oxidoreductase samples at 77 K broad features attributable to Fe-S centers are distinctly present. A detailed investigation of the saturation behavior of the various signals present in quinoline oxidoreductase, focusing on possible magnetic interactions, is under way in our laboratory.

Though neither a complete description of the complex saturation behavior nor a quantitative estimate of the Fe-S content of the enzyme can be presented at this early stage of the EPR work, it is suggested by the spectral analogy to, e.g., the aldehyde oxidoreductase of *D. gigas* (Bray et al., 1991) that quinoline oxidoreductase contains two nonidentical [2Fe-2S] clusters. This assumption is supported by the cofactor composition, determined as Mo:Fe:S:FAD, 1.6:6.9:8.5:1.6 (Bauder et al., 1990), and the UV/vis spectrum typical for a 1:4:4:1 ratio of Mo:Fe:S:flavin (Coughlan, 1980).

The results presented here demonstrate the efficiency of the preparation procedure to obtain an enzyme of highest homogeneity. The EPR data indicate a relationship of quinoline oxidoreductase to other molybdenum-containing hydroxylases of eukaryotic and bacterial origin, although some distinct differences exist. For the first time a clear correlation between the molybdopterin cytosine dinucleotide molybdenum cofactor, the reduction of the enzyme by its substrate quinoline, and the appearance of the Mo(V) rapid type Q EPR signal is revealed. To enlarge our knowledge of the poorly characterized enzymes possessing this derivative of molybdopterin in addition to a monoxo–monosulfido group at the molybdenum center, further work is required to elucidate mechanistic and structural details in the catalytic cycle of quinoline oxidoreductase, for which high-resolution EPR techniques (ENDOR, ESEEM) will also have to be applied.

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