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Sulfide-quinone reductase activity in membranes of the chemotrophic bacterium *Paracoccus denitrificans* GB17

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Abstract Reduction of exogenous ubiquinone and of cytochromes by sulfide in membranes of the chemotrophic bacterium *Paracoccus denitrificans* GB17 was studied. For sulfide-ubiquinone reductase activity, K_m values of 26 ± 4 and 3.1 ± 0.6 μM were determined from titrations with sulfide and decyl-ubiquinone, respectively. A maximal rate of up to 0.3 μmol decyl-ubiquinone reduced (mg protein)$^{-1}$ min^{-1} was estimated. The reaction was sensitive to quinone-analogous inhibitors, but insensitive to cyanide. Reduction of cytochromes by sulfide was monitored with an LED-array spectrophotometer. Under oxic conditions, reduction rates and extents of reduction were lower than those under anoxic conditions. Reoxidation of cytochromes was oxygen-dependent and cyanide-sensitive. The multiphasic behavior of transient reduction of cytochrome *b* with limiting amounts of sulfide reflects that sulfide, in addition to acting as an electron donor, is a slowly binding inhibitor of cytochrome *c* oxidase. The initial peak of cytochrome *b* reduction is dependent on electron flow to an oxidant, either oxygen or ferricyanide, and is stimulated by antimycin A. This oxidant-induced reduction of cytochrome *b* suggests that electron transport from sulfide in *P. denitrificans* GB17 employs the cytochrome bc_1 complex via the quinone pool.

Key words *Paracoccus denitrificans* · Sulfide oxidation · Sulfide-quinone reductase · Cytochrome *bc* complex · Flavocytochrome *c*

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Abbreviations *SQR* Sulfide-quinone reductase · *Decyl-UQ* Decyl-ubiquinone · *cyt* Cytochrome

Introduction

Inorganic reduced sulfur compounds serve as electron donors in many phototrophic and chemotrophic bacteria, mostly with sulfate as the major oxidation product [for reviews, see Friedrich (1998) and Kelly et al. (1997)]. The bacterium *Paracoccus denitrificans* GB17, formerly named *Thiosphaera pantotropha* GB17 (Ludwig et al. 1993), grows lithotrophically with sulfide and thiosulfate as electron donors aerobically and anaerobically under denitrifying conditions (Robertson and Kuenen 1983). In bacteria, mainly two enzymatic systems are considered to be involved in the oxidation of sulfide to sulfur: flavocytochrome *c* and sulfide-quinone reductase (SQR).

Flavocytochromes *c*, located in the periplasm of several bacteria, are soluble enzymes showing sulfide:cytochrome *c* oxidoreductase activity in vitro [for a review, see Brune (1995)]. For that reason it has been suggested that flavocytochrome *c* plays an essential role in sulfide oxidation in vivo. Indeed, the partial amino acid sequence deduced from the *soxF* gene of *P. denitrificans* GB17 exhibits significant similarity to the flavin subunit of flavocytochrome *c* of the photosynthetic bacterium *Chromatium vinosum* (Wodara et al. 1997). Therefore, flavocytochrome *c* might function in sulfide oxidation in *P. denitrificans* GB17 as well. However, flavocytochrome *c* does not occur in a variety of sulfide-oxidizing bacteria and seems to be confined to species additionally capable of thiosulfate oxidation.

During the last twenty years, evidence has accumulated for a second, membrane-bound sulfide-oxidizing system in phototrophic bacteria as reviewed by Shahak et al. (1997) and Friedrich (1998). This activity has been identified as SQR (E.C.1.8.5.′) activity and was first detected in thylakoids of the filamentous cyanobacterium *Oscillatoria limnetica* (Cohen et al. 1975). SQR activity has been attributed to an inducible, membrane-bound, 57-

kDa flavoprotein (Arieli et al. 1994). Sulfide oxidation by SQR and participation of the cytochrome *bc* complex in electron flow from sulfide to cytochrome *c* (see Fig. 4) has been established in membranes of *O. limnetica* (Shahak et al. 1987), Chlorobia (Klughammer et al. 1995), and the non-thiosulfate-utilizing sulfide oxidizer *Rhodobacter capsulatus* (Shahak et al. 1994). Recently, SQR of *Rba. capsulatus* was purified, and its gene was sequenced and functionally expressed in *Escherichia coli* (Schütz et al. 1997). *Rba. capsulatus* is a close relative to *P. denitrificans* within the α-subdivision of proteobacteria (Stackebrandt et al. 1996).

The wide distribution of SQR activity among prokaryotes and the close phylogenetic relatedness to *Rba. capsulatus* led to our investigation of SQR activity in the chemotrophic *P. denitrificans*. Here we describe sulfide-quinone oxidoreductase activity and the participation of the cytochrome bc_1 complex in sulfide oxidation in membranes of *P. denitrificans* GB17.

Materials and methods

Bacterial culture conditions

P. denitrificans (GB17) was cultivated at 30° C in mineral medium with 20 mM sodium thiosulfate as described (Chandra and Friedrich 1986) with the following modification: the concentration of phosphate was reduced to 3.5 mM, and the pH was kept constant by titration with 5 M sodium carbonate. Cells were cultivated in a 300-l fermenter (Bioengineering, Wald, Switzerland) with 220 l of working volume at an aeration rate of 100 l air min^{-1}

Preparation of membranes

Cells were harvested by cross-flow filtration (Sartorius, Göttingen, Germany) at 10° C, were collected by centrifugation, and were resuspended (100 g, wet wt.) in 150 ml 55 mM sodium-potassium phosphate buffer (pH 7.5). After addition of approximately 1 mg of DNase, cells were disrupted by French press treatment as described (Fischer et al. 1996).

Membranes were prepared by differential centrifugation. Whole cells and cell debris were removed by centrifugation at $10{,}000 \times g$ for 15 min. Membranes were collected after centrifugation at $200{,}000 \times g$ for 2 h, washed twice with 20 ml of the same buffer, and resuspended to give a concentration of 22.6 mg protein ml^{-1}.

Sulfide-quinone reductase assay

Sulfide-quinone reductase activity was measured by sulfide-dependent decyl-ubiquinone (decyl-UQ) reduction in an Aminco DW-2 spectrophotometer (American Instrument, Md., USA), recorded at 275–300 nm (Shahak et al. 1994) using a millimolar differential extinction coefficient of 15 cm^{-1} (Morton 1965). The reaction mixture routinely contained 50 mM Bis-Tris (pH 6.5), 20 mM glucose, 20 μM decyl-UQ, and membranes equivalent to 6 μg protein ml^{-1}. In the presence of the ionophores valinomycin or nigericin, the reaction mixture was supplemented with 20 mM potassium chloride. Anoxic conditions were established by flushing with N_2 and adding one unit of glucose oxidase ml^{-1} and approximately ten units of catalase ml^{-1}. The reaction was started by the addition of 100 μM Na_2S. The decyl-UQ reduction rate was determined by the initial slope of the decrease of the absorption at 275 nm minus the absorption of 300 nm. Rates were corrected for the non-enzymatic reaction. Inhibitor concentrations were determined spectroscopically by using extinction coefficients given by Von Jagow and Link (1986). For aurachin C (Oettmeier et al. 1990) dissolved in methanol, extinction coefficients of 32.2 mM cm^{-1} at 251 nm or 11.4 mM cm^{-1} at 346 nm were used (W. Oettmeier, Lehrstuhl Biochemie der Pflanzen, Ruhr-Universität Bochum, Germany, personal communication).

Measurement of reduction of cytochromes

Spectral changes of cytochromes were recorded with the LED-array spectrophotometer described previously (Klughammer et al. 1990). This instrument allows simultaneous recordings of 16 different wavelengths in the range of 508–590 nm with maximal time resolution of 1 ms/point. The reaction mixture contained 100 mM glycylglycine (pH 7.0). Anoxic conditions were established under nitrogen with glucose/glucose oxidase and catalase as described above. Membranes were added equivalent to 0.5 or 0.7 mg protein ml^{-1}. Decyl-UQ was reduced according to Rich (1978).

Spectral changes were deconvoluted into cytochrome *c* and *b* kinetics with model spectra (Fig. 1) obtained in the following way:

1. Membranes were first reduced with 1 mM ascorbate. A cytochrome c_{551} spectrum was obtained from the difference between 11 and 14 s during transient oxidation with 10 μM ferricyanide (not shown). This was a minor component and did not improve the curve-fitting substantially. Therefore, it was not considered further.

2. The cytochrome c_{552} spectrum (Fig. 1) was obtained by the difference of the spectrum of N_2-flushed membranes reduced by 60 μM Na_2S minus the spectrum recorded 58 s after the start of reoxidation.

3. After addition of 1 mM dithionite to membranes prereduced by 3 mM ascorbate, a fast cytochrome c_{552} reduction was followed by a slow component that peaked at 560 nm. The cytochrome b_{560} spectrum (Fig. 1) was derived from the difference of the spectra at 30 s minus that at 10 s after dithionite addition by subtracting the c_{552} component.

4. The cytochrome b_{566} spectrum with a split α-peak (Fig. 1; Berry and Trumpower 1985) was obtained by further addition of 0.1 μM

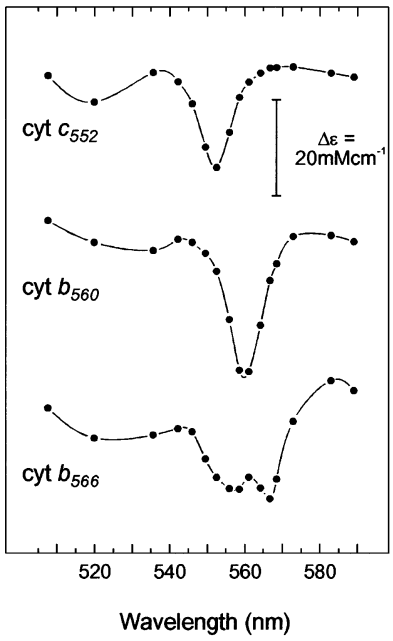

Fig. 1 Model spectra of cytochrome c_{552}, cytochrome b_{560} and cytochrome b_{566}. The oxidized *minus* reduced difference spectra used for the fitting procedure are shown. The spectra were obtained as described under Materials and methods

benzyl viologen to dithionite-reduced membranes after subtraction of the b_{560} component.

A curve-fitting routine (Klughammer et al. 1990) optimally fitted the components that peaked at 550.6, 552.3, and 560 nm and the split peak component with maxima at 558 and 566 nm to the observed changes together with a second-order parabola for the correction of nonspecific spectral changes. Differential millimolar extinction coefficients of 20 mM^{-1} cm^{-1} were assumed for the *c*-type cytochromes (c_{551}, 551–570 nm, c_{552}, 552–570 nm). For the *b*-type cytochromes, coefficients calculated by Berry and Trumpower (1985) were used: for cytochrome b_{560}, 29.7 mM^{-1} cm^{-1} at 560–575 nm and 10.8 mM^{-1} cm^{-1} at 566–575 nm; for cytochrome b_{566}, 13.9 mM^{-1} cm^{-1} at 560–575 nm and 18.2 mM^{-1} cm^{-1} at 566–575 nm. In the presence of antimycin A, it had to be assumed that the cytochrome b_{560} component was red-shifted (Kamensky et al. 1985) from 560 to 560.6 nm for satisfying curve-fitting.

Measurement of sulfide-dependent oxygen consumption

Oxygen uptake was measured polarographically with a Clark-type oxygen electrode (Hansatech, Great Britain) at 25°C in 3 ml of a buffer containing 100 mM Tris-HCl and 10 mM potassium phosphate (pH 8.0). Calculations were made on the basis of an oxygen concentration of 253 µM in air-saturated water at this temperature. Sulfide was determined in accordance with Trüper and Schlegel (1964). Membranes equivalent to 0.1 mg protein ml^{-1} were present. The reaction was started by the addition of 100 µM Na_2S. Rates were corrected for the non-enzymatic reaction.

Chemicals

Decyl-UQ, antimycin A, myothiazol, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone (FCCP), valinomycin, and glucose oxidase type II were obtained from Sigma (St. Louis, Mo., USA). Nigericin and catalase were obtained from Boehringer (Mannheim, Germany). PQ-1 was synthesized according to Rich et al. (1987). Aurachin C and stigmatellin were kindly provided by G. H. Höfle (Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Germany). All other chemicals were of reagent grade and were obtained from commercial sources.

Results and discussion

Reduction of decyl-UQ by sulfide

Externally added decyl-UQ was rapidly reduced by sulfide under oxic and anoxic conditions in the presence of membranes from *P. denitrificans* GB17 [data not shown; see Shahak et al. (1994) for comparison)]. The reaction in the absence of membranes or with heated membranes was negligible. Under anoxic conditions, a rate of 0.3 µmol decyl-UQ reduced (mg protein)$^{-1}$ min^{-1} was obtained. This rate was approximately threefold higher than that found in membranes of the phototrophic bacterium *Rba. capsulatus* (Schütz et al. 1997). Due to reoxidation of ubiquinol by oxygen, under oxic conditions the apparent rate and the extent of decyl-UQ reduction were reduced to approximately 70%. A similar behavior was documented earlier for membranes of *Rba. capsulatus* (Shahak et al. 1994). The addition of the uncoupling agent FCCP or of valinomycin or nigericin to final concentrations of 3 µM, or of combinations of valinomycin and nigericin did not stimulate the reduction rate in the absence or presence of oxygen. Furthermore, no stimulation of SQR activity by uncoupling agents was observed in membranes of *Rba. capsulatus* (Shahak et al. 1994). The optimal pH of the reaction was 7.3, with half-maximal rates at pH 6.0 and 9.5. While the pH optimum of the reaction was comparable to that of membranes from *Rba. capsulatus* (Shahak et al. 1994), the pH range of SQR activity was broader. At pH 6.5, 80% of the maximal activity was present still. K_m values for sulfide and for decyl-UQ were determined to be 26 ± 4 µM and 3.1 ± 0.6 µM, respectively. Thus, the K_m for sulfide was approximately three- to fourfold higher than that determined in membranes of *Rba. capsulatus* and *O. limnetica*, while the K_m values for the artificial quinone substrate in *Paracoccus* and *Rhodobacter* membranes were in the same range and approximately tenfold lower than in *Oscillatoria* membranes using plastoquinone as electron acceptor (Table 1).

Table 1 K_m values for sulfide-quinone reductase (*SQR*) and the efficiency of inhibitors in membranes of *Paracoccus denitrificans*GB17, *Rhodobacter capsulatus* (Shahak et al. 1994), *Oscillatoria limnetica* (Arieli et al. 1991, 1994), and *Chlorobium limicola* f. *thiosulfatophilum* (Shahak et al. 1992). The rates of reduction of decyl-ubiquinone were measured as described in Materials and methods. I_{50} values give the micromolar concentrations of the quionone-analogous inhibitors and potassium cyanide (*KCN*) for 50% inhibition (*none* no inhibition and *nd* not determined)

	P. denitrificans (GB17)	*Rba. capsulatus*	*O. limnetica*	*Chl. limicola* f. *thiosulfatophilum*
K_m (µM)				
Sulfide	26 ± 4	5	8	nd
Decyl-ubiquione	3.1 ± 0.6	2	nd	nd
Plastoquinone-1	nd	nd	32	< 20
I_{50} (µM)				
Antimycin A	15 ± 3	50	none	0.096
Stigmatellin	20 ± 2	2.5	6.8	0.005
Myxothiazol	22 ± 2	43	none	6
Aurachin C	28 ± 4	0.23	50	0.012
KCN	none	120	12	10

Reduction of decyl-UQ by sulfide in membranes of *P. denitrificans* GB17 was sensitive to quinone-analogous inhibitors (Table 1). These inhibitors compete with the quinone substrate at the quinone binding site of the SQR (see Q_S in Fig. 4). The different behavior of SQRs from different organisms towards quinone-analogous inhibitors reflects similar differences in quinone binding known for the quinol-oxidizing cytochrome *bc* complexes of these organisms. Aurachin C and stigmatellin, the most effective SQR inhibitors in membranes of *Rba. capsulatus*, *O. limnetica*, and *Chl. limicola* f. *thiosulfatophilum*, were less effective in membranes of *Paracoccus*. SQRs in membranes of the phylogenetically close relatives *P. denitrificans* GB17 and *Rba. capsulatus* were less sensitive than *Chlorobium* SQR to antimycin A and myxothiazol, which did not inhibit *Oscillatoria* SQR at all. Antimycin A and myxothiazol are inhibitors of the ubiquinol-oxidiz-

ing cytochrome bc_1 complex of mitochondria and bacteria, but do not inhibit the plastoquinol-oxidizing cytochrome b_6f complex present in thylakoids of plants and cyanobacteria [for a review, see Hauska et al. (1996)]. The menaquinol-oxidizing cytochrome *bc* complex of Chlorobiaceae combines properties of both (Schütz et al. 1994) and is antimycin-A-sensitive (Klughammer et al. 1995).

Cyanide is a potent inhibitor of many flavoproteins in micromolar concentrations (Massey and Ghisla 1983) and inhibits sulfide oxidation by flavocytochrome *c* both in phototrophic bacteria (Yamanaka and Kusai 1976) and in chemotrophic bacteria (Visser et al. 1997). Cyanide did not inhibit quinone reduction by sulfide in *P. denitrificans* GB17 membranes, in contrast to SQR in membranes of *Rba. capsulatus*, *O. limnetica*, and *Chl. limicola* f. *thiosulfatophilum* (Table 1).

Cytochromes in membranes of *P. denitrificans* GB17

Four different cytochrome components could be distinguished in membranes of *P. denitrificans* GB17. Reduction with 1 mM ascorbate revealed low amounts of a component peaking at 551 nm with a shoulder at 560 nm. Further addition of ascorbate did not result in further cytochrome reduction. Addition of 1 mM dithionite to ascorbate-reduced membranes resulted in fast reduction of a component absorbing at 552 nm (cyt c_{552}) and in a slow reduction of a 560-nm component (cyt b_{560}). Further reduction by dithionite resulted in a spectral change peaking at 558 nm with a shoulder at approximately 566 nm (cyt b_{560} and cyt b_{566}), and the addition of 0.1 µM benzyl viologen finally resulted in a split α-peak with maxima at 557 and 566 nm (cyt b_{566}; Berry and Trumpower 1985). Spectra of the cytochromes c_{552}, b_{560}, and cytochrome b_{566} are shown in Fig. 1.

A component peaking at 551 nm that was found by Kula et al. (1981) has been expounded as a mixture of membrane-bound cytochrome c_1 peaking at 553 nm and periplasmic cytochrome c_{550} (Berry and Trumpower 1985). In our measurements, the cytochrome c_{551}, a minor component, did not improve the fit substantially and therefore was omitted. Traces of cytochrome b_{560}, reducible by ascorbate, have also been reported before (Berry and Trumpower 1985). The dominant cytochrome c_{552}, probably the tightly bound *c*-type cytochrome functioning in electron transfer from cytochrome bc_1 to cytochrome oxidase, has been obtained only after addition of dithionite. Berry and Trumpower (1985) have found cytochrome c_{552} fully reducible by ascorbate in the isolated "supercomplex" of the cytochrome bc_1 complex and cytochrome *c* oxidase (see Fig. 4). The difference might be due to the absence of lipophilic mediators in our measurements. According to the findings of others (Kula et al. 1981; Berry and Trumpower 1985), the 560-nm component has been attributed to the high-potential heme *b* of the bc_1 complex. Further reduction has revealed the presence of two spectrally distinct *b*-type cytochromes in *Paracoccus* membranes, one with a peak again at 560 nm and one with a split α-peak representing the low-potential heme b_{566} of the cytochrome bc_1 complex (Berry and Trumpower 1985). Analyses of the spectra yielded cytochrome contents of 67, 658, 187, and 125 nmol (g protein)$^{-1}$ for cytochromes c_{551}, c_{552}, b_{560}, and b_{566}, respectively, corresponding to molar ratios of approximately 1 : 10 : 3 : 2. A molar ratio of 1 : 1 : 1 for the hemes b_{560} and b_{566} in cytochrome *b* and heme *c* in cytochrome c_1, with a maximum at 553 nm, should be expected for the cytochrome bc_1 complex (Berry and Trumpower 1985). However, in membranes of *P. denitrificans*, other additional dithionite-reduceable *b*- and *c*-type cytochromes are present (Stouthamer 1991; Ludwig 1992), which could not be resolved by the spectroscopic analysis applied.

Reduction of cytochromes by sulfide in membranes of *P. denitrificans*

Sulfide added to a final concentration of 60 µM under oxic conditions transiently reduced 80% of dithionite-reducible cytochrome c_{552} (Fig. 2 A). Of the b_{560} component, approximately 10% was transiently reduced (Fig. 2 A), while the absorption change of the b_{566} component was negligible (not shown). Addition of antimycin A decreased the rate and extent of the cytochrome c_{552} reduction (Fig. 2 B). At the same time, however, reduction of heme b_{560} was stimulated fivefold to approximately 50% (Fig. 2 B). Also heme b_{566} was reduced in this case (to approximately 30%). In presence of cyanide, 80% of cytochrome c_{552} was again transiently reduced (Fig. 2 C). The rate of reduction was stimulated, and the rate of reoxidation was diminished. Approximately 5% of heme b_{560} was transiently reduced (Fig. 2 C). Under microoxic conditions (Fig. 2 D), after flushing with nitrogen, cytochrome c_{552} was reduced to an extent similar to that under oxic conditions (Fig. 2 A). After oxygen had been consumed, cytochrome c_{552} was further reduced up to 90%. Initally, 8% of heme b_{560} was reduced. Again, absorption changes of heme b_{566} were negligible in Fig. 2 C and D. Estimated from the traces at a higher time resolution (not shown), cytochrome c_{552} reduction rates were 9.5 nmol (mg protein)$^{-1}$ min^{-1} under oxic conditions (Fig. 2 A), 14 nmol (mg protein)$^{-1}$ min^{-1} in the presence of cyanide (Fig. 2 C), and 11 nmol (mg protein)$^{-1}$ min^{-1} under microoxic conditions (Fig. 2 D). In the presence of antimycin A, after an initial rate of 1 nmol (mg protein)$^{-1}$ min^{-1}, the cytochrome c_{552} reduction rate was decreased to 0.6 nmol (mg protein)$^{-1}$ min^{-1} (Fig. 2 B).

The increased rate of reduction of cytochrome c_{552} by sulfide in the presence of cyanide indicates electron flow from sulfide to oxygen via SQR and quinol oxidase (see Fig. 4). The quinol oxidase in membranes of *Paracoccus* is a supercomplex formed by the cytochrome bc_1 complex and cytochrome *c* oxidase (Berry and Trumpower 1985). Cyanide blocks the reoxidation of cytochrome *c* by cytochrome *c* oxidase (Bolgiano et al. 1989). In contrast to the case of membranes of Chlorobia (Klughammer et al. 1995) and *Rba. capsulatus* (Shahak et al. 1994), cy-

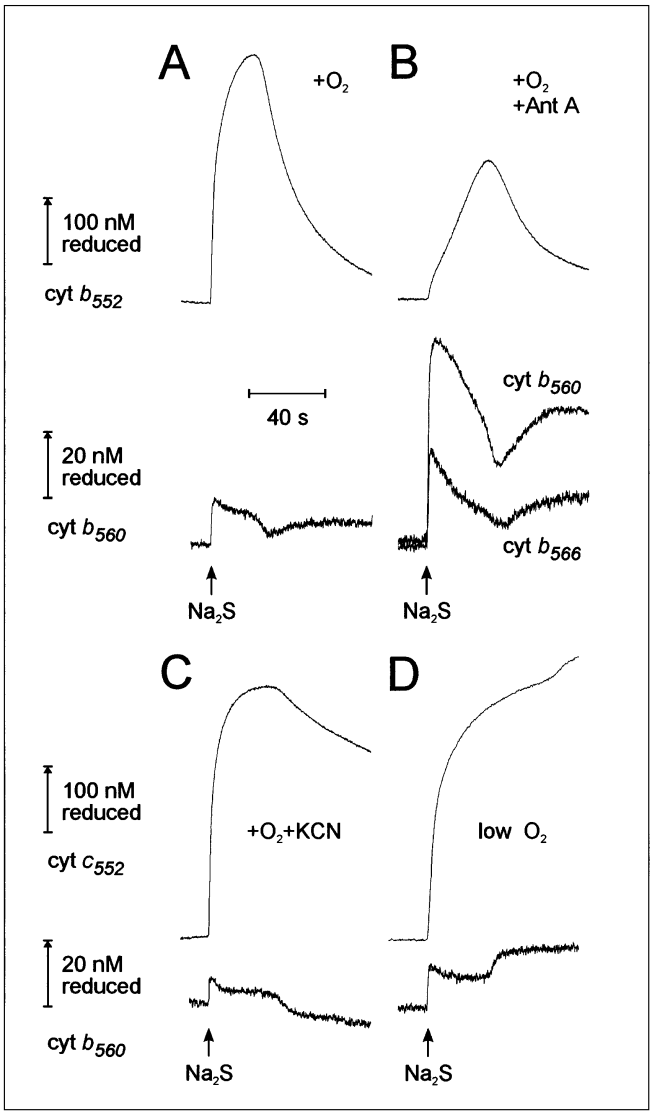

Fig. 2 A–D Reduction of cytochromes by sulfide in membranes of *Paracoccus denitrificans* GB17. Sulfide-dependent reduction of **A** cyt c_{552} and b_{560} under oxic conditions. **B** Sulfide-dependent reduction of cyt c_{552}, b_{560}, and b_{566} in the presence of 10 μM antimycin A. **C** Sulfide-dependent reduction of cyt c_{552} and b_{560}, in the presence of 2 mM potassium cyanide (*KCN*). **D** Sulfide-dependent reduction of cyt c_{552} and b_{560} under microoxic conditions after flushing with nitrogen. Reduction of cyt b_{566} was negligible in **A**, **C**, and **D**. Membranes present were equivalent to 0.7 mg ml^{-1}. *Arrows* indicate addition of 60 μM Na_2S

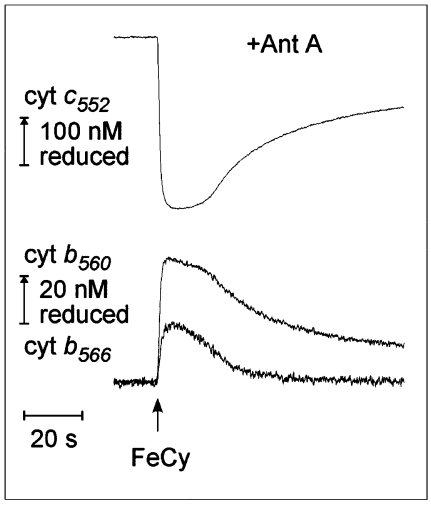

Fig. 3 Oxidant-induced reduction of cytochromes b_{560} and b_{566} in membranes of *Paracoccus denitrificans* GB17 with sulfide as electron donor. Cytochromes were reduced by 180 μM Na_2S. Antimycin A (*Ant A*) was present at 10 μM. Reaction was started by addition of ferricyanide (*FeCy*) to 50 μM as indicated by an *arrow*. Membranes present were equivalent to 0.7 mg ml^{-1}

tochrome *c* reduction rates were not decreased by cyanide in membranes of *P. denitrificans* GB17. This agrees with the insensitivity of SQR to cyanide in membranes of this chemotrophic bacterium (Table 1). With a rate of 600 nmol of electrons (mg protein)$^{-1}$ min^{-1} transferred from sulfide to decyl-ubiquinone, the turnover of SQR was much faster than the maximal reduction rate of cytochrome c_{552} [14 nmol (mg protein)$^{-1}$ min^{-1}; Fig. 2C]. Thus, SQR is not rate-limiting in sulfide oxidation. If the naturally occurring quinone in membranes of *P. denitrificans* GB17 is reduced as efficiently as is the artificial decyl-ubiquinone, then either the turnover of the cytochrome *bc* complex is rate-limiting or other quinol-oxidizing pathways exist. Probably, an alternative oxidase (Ludwig 1992; De Gier et al. 1996) competes for the quinol reduced by SQR (see Fig. 4).

The heme *b* kinetics were polyphasic, which can be explained by the fact that sulfide is a "slow-binding" inhibitor of the cytochrome *c* oxidase (Nicholls 1975) in addition to its donating electrons to the ubiquinol pool. After the initial peak of reduction of *b*-type cytochromes, reoxidation occurs, reflecting progressive inhibition of electron flow to oxygen. Consumption of sulfide results in the recovery of electron flow through cytochromes to oxygen with a second reduction phase of the hemes b_{560} and b_{566}. Accordingly, this second phase of reduction was sensitive to cyanide (Fig. 2C). Using decyl-ubiquinol as reductant, no second phase of reduction of *b*-type cytochromes was observed (data not shown). Under microoxic conditions, the second phase of heme b_{560} reduction started before a second phase of cytochrome c_{552} reduction indicated full consumption of oxygen (Fig. 2D). This may be taken as an indication that sulfide inhibition of cytochrome *c* oxidase is already released in the presence of residual sulfide at micromolar concentrations, showing that the oxidase in *Paracoccus* is somewhat less sensitive to sulfide than the oxidase in mitochondria (Nicholls 1975).

Under anoxic conditions, after cytochromes in membranes of *P. denitrificans* GB17 had been reduced in the presence of antimycin A but with a threefold concentration of sulfide, addition of 50 μM ferricyanide transiently oxidized all cytochrome c_{552} and transiently stimulated reduction of cytochromes b_{560} and b_{566} up to 50 and 30% (Fig. 3). Similar results were obtained after pulses of oxygen (data not shown). These experiments demonstrate oxidant-induced reduction of cytochrome *b* of the cytochro-

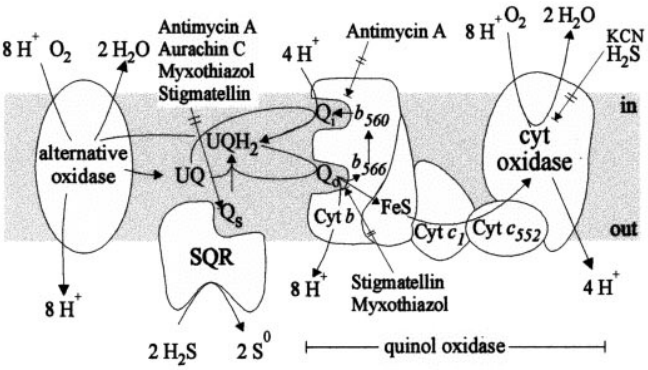

Fig. 4 Model of the electron transport from sulfide to oxygen in membranes of *Paracoccus denitrificans* GB17. Sites of inhibition by the inhibitors are indicated by *crossed-out arrows* [*QS* quinone binding site of sulfide-quinone reductase (*SQR*), Q_O quinol oxidation site of cytochrome bc_1 complex, Q_i quinone reduction site of cytochrome bc_1 complex, *Cyt b* cytochrome *b*, b_{560} and b_{566} high- and low-potential heme of cytochrome *b*, *FeS* Rieske iron-sulfur protein, *Cyt* c_1 cytochrome c_1 of the cytochrome *bc* complex, *Cyt* c_{522} cytochrome c_{522}, and *cyt oxidase* cytochrome oxidase]

me bc_1 complex with sulfide as reductant and indicate that oxidation of sulfide by SQR is coupled with the proton-motive Q-cycle mechanism in membranes of *P. denitrificans* GB17 [Fig. 4; for a review see Hauska et al. (1996)].

Sulfide-dependent uptake of oxygen

The initial rates for sulfide-dependent consumption of oxygen and for the oxidation of sulfide in membranes of *P. denitrificans* GB17 were 63 ± 6 nmol O_2 (mg protein)$^{-1}$ min^{-1} and 150 ± 16 nmol sulfide (mg protein)$^{-1}$ min^{-1}, respectively, determined from four experiments. The total ratio of oxygen consumed per sulfide oxidized was 2.0 ± 0.1. The reaction with heated membranes was negligible. The concentration of antimycin A for 50% inhibition of the oxygen uptake rate was 15 μM.

The rate of oxidation of sulfide, equivalent to 300 nmol electrons (mg protein)$^{-1}$ min^{-1}, and the rate of reduction of oxygen, equivalent to 246 nmol electrons (mg protein)$^{-1}$ min^{-1}, are only half the rate of 600 nmol electrons (mg protein)$^{-1}$ min^{-1} transferred from sulfide to externally added decyl-ubiquinone. The naturally occurring quinone in membranes of *P. denitrificans* GB17 might be limiting in the electron transfer from sulfide to oxygen. However, the rates are much higher than the rate of 14 nmol electrons (mg protein)$^{-1}$ min^{-1} transferred to cytochrome c_{552}. This confirms the assumption that most of the electrons from sulfide run through an alternative oxidase pathway to oxygen (Fig. 4), and this oxidase is not (or only somewhat) inhibited by sulfide under the sulfide concentration used. The agreement of the concentrations of antimycin A for 50% inhibition of the uptake rate of oxygen and for 50% inhibition of the reduction rate of decyl-ubiquinone by this quinone-analogous inhibitor (Table 1) indicates that all the electrons transferred from sulfide to oxygen pass through the quinone pool. The total ratio of 1 mol of oxygen consumed per 2 mol of sulfide oxidized suggests that all the electrons from sulfide are transferred to oxygen and that the product of the oxidation of sulfide may be elemental sulfur, higher polythionates, or polysulfides. Similar ratios were found with cells of *Thiobacillus ferrooxidans* (Hazeu et al. 1988) and *Thiobacillus caldus* (Hallberg et al. 1996) in the presence of uncouplers or N-ethylmaleimide. Those agents stopped the oxidation of sulfide at the redox level of S^0 in these cells.

For several chemotrophic bacteria it has been demonstrated that quinones and membrane-bound cytochromes are involved in the oxidation of thiosulfate and elemental sulfur (Moriarty and Nicholas 1970; Saxena and Aleem 1973; Beffa et al. 1992; Hallberg et al. 1996) as well as in the oxidation of sulfide. The product of the oxidation of sulfide in membranes of *P. denitrificans* GB17 is unknown. Therefore, it cannot be excluded that some of the reduction of decyl-ubiquinone, cytochromes, and oxygen is due to intermediary products of the oxidation of sulfide. However, the insensitivity of the sulfide-dependent reduction of decyl-ubiquinone to uncouplers, the good correspondence between the rate of oxidation of sulfide and the rate of consumption of oxygen, and the sulfide-to-oxygen ratio of 2 for the total oxidation of sulfide make this unlikely. Nevertheless, the products of the oxidation of sulfide in membranes of *P. denitrificans* GB17 need to be determined in order to clear up this question.

The nature of SQR activity in membranes of *P. denitrificans* GB17

From our results we conclude that in membranes of *P. denitrificans* GB17, sulfide is oxidized by the membrane-bound SQR and quinone becomes reduced (Fig. 4). Subsequent reoxidation of quinol by the proton-motive Q-cycle mechanism of the cytochrome bc_1 complex leads to reduction of cytochrome *c*, as has been shown earlier for phototrophic bacteria (Shahak et al. 1994, 1997; Klughammer et al. 1995). Cytochrome *c* is reoxidized by cytochrome *c* oxidase, and the electrons are transferred to oxygen. Additionally, quinol is reoxidized by an alternative oxidase [De Gier et al. 1996; for a review, see Stouthamer (1991)]. With sulfide concentrations that block the cytochrome *c* oxidase, only the alternative pathway is active (Fig. 4).

The protein responsible for SQR activity in this chemotroph remains unknown. Using antibodies raised against native SQR of *Rba. capsulatus*, SQR antigens from *P. denitrificans* GB17 could not be detected. Also, DNA-DNA hybridization was not successful. Similar negative results were obtained with DNA from Chlorobiaceae and from membranes of Chlorobiaceae and *Chromatium vinosum* (M. Bronstein, R. Zimmermann, M. Schütz, unpublished work). With the exception of *Rba. capsulatus*, these strains oxidize thiosulfate and contain flavocytochrome *c* [for a review, see Friedrich (1998)]. Therefore, either flavocytochrome *c* bound to membranes

or SQR might be responsible for hydrogen sulfide oxidation. However, flavocytochrome *c* of *P. denitrificans* GB17 is a soluble enzyme [located in the periplasm, as is evident from the signal peptide deduced from the *soxF* gene coding for the flavoprotein (Wodara et al. 1997)], and should be lost during membrane preparation. Furthermore, flavocytochrome *c* sulfide dehydrogenase from *Thiobacillus* sp. W5, isolated by Visser et al. (1997), did not show sulfide-quinone reductase activity, but sulfide-dependent reduction of ubiquinone-2 was observed in crude extracts of this chemotroph. On the other hand, SQRs of phototrophic bacteria were significantly less sensitive to cyanide (Shahak et al. 1997) as compared to flavocytochrome *c* (Yamanaka and Kusai 1976), and SQR activity in membranes of *P. denitrificans* GB17 was insensitive to cyanide. This suggests that both are different enzymatic systems and that both might function in the oxidation of sulfide in vivo. The question could be settled by the deletion of the *soxF* gene and by the deletion of the *sqr* gene in *P. denitrificans* GB17, a bacterium which has been studied well genetically.

Recently, SQR activity has also been reported in the chemotrophs *Aquifex aeolicus* (Shahak et al. 1997), *Thiobacillus* sp. W5 (Visser et al. 1997), and even in mitochondria of the lugworm *Arenicola marina* (M. Klein, M. Schütz, G. Hauska and M. K. Grieshaber, unpublished work). The occurrence of SQR activity in these phylogenetically widely distant sulfide-oxidizing organisms might reflect a role of SQR in early evolution.

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