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Reduction of cytochromes with menaquinol and sulfide in membranes from green sulfur bacteria *

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Abstract

Reduction of cytochromes in chlorosome-free membranes of *Chlorobia* was studied anaerobically, with an LED array spectrophotometer. For *Chlorobium tepidum* these membranes contained 0.2 moles cytochrome per mole of bacteriochlorophyll *a*. The observed change upon complete reduction of oxidized membranes with dithionite could be satisfactorily fitted with three cytochrome components having absorption peaks at 553 (cyt *c*), 558 and 563 nm (cyt *b*), in relative amounts of 5:1:2. About 20% of total cytochrome 553 were reducible by ascorbate. Menaquinol reduced all of the 553-component, and this reduction was sensitive to stigmatellin, NQNO and antimycin A. The reduction was insensitive to KCN. However, it was transient at low concentrations of menaquinol in the absence of KCN, but permanent in its presence, demonstrating that electron transport into an oxidation pool was blocked. The 563-component was only slightly reduced by menaquinol unless NQNO or antimycin were present. The stimulation of cytochrome 563-reduction by these inhibitors was more pronounced in the presence of ferricyanide. This phenomenon reflects 'oxidant-induced reduction' of cytochrome *b* and demonstrates that a Q-cycle is operative in *Chlorobia*. Also, sulfide fully reduced cytochrome 553, but more slowly than menaquinol. KCN inhibited in this case, as did stigmatellin, NQNO and antimycin A. NQNO was a better inhibitor than antimycin A. Cytochrome 563 again was hardly reduced unless antimycin A was added. The effect was more difficult to observe with NQNO. This supports the conclusion that sulfide oxidation proceeds via the quinone pool and the cytochrome *bc*-complex in green sulfur bacteria.

Abbreviations: BChl – bacteriochlorophyll; cyt – cytochrome; NQNO – 2-n-nonyl-4-hydroxyquinoline-N-oxide; SQR – sulfide-quinone reductase

Introduction

Sulfide serves as electron donor in green sulfur bacteria, and in several other phototrophic organisms (Brune 1989), but the path of sulfide oxidation is still ambiguous. In contrast to the older view of a flavocy-

* Dedicated to Prof. Dr. Aloys Wild on occasion of his 65th birthday.

tochrome *c* as the sulfide oxidant (Kusai and Yamanaka 1973), evidence accumulates that sulfide is oxidized by the quinone pool, via sulfide-quinone oxido-reductase (SQR). This enzyme is inducible in the cyanobacterium *Oscillatoria limnetica* from which it has recently been purified to homogeneity in an active form (Arieli et al. 1994). It has been characterized as a 57 kDa flavo-protein. Its N-terminus has been found to be largely identical to that of a similar enzyme from *Rhodobacter capsulatus* (M. Schütz et al., in preparation).

Recently, SQR activity with externally added ubiquinone, and its inhibitor sensitivity, has been compared to the reduction of cytochromes by sulfide in membranes from *Rb. capsulatus* (Shahak et al. 1994). This led us to conclude that the major path of electrons from sulfide involves the ubiquinone pool plus the cytochrome bc_1-complex. Previously, we have demonstrated SQR-activity with externally added quinone also for the strictly anaerobic green sulfur bacterium *Chlorobium limicola* (Shahak et al. 1992), and here we provide a comparison of cytochrome reduction with menaquinol and sulfide in this organism. The results substantiate the pathway of electrons from sulfide via SQR to the menaquinone pool and the cytochrome *bc*-complex. In addition, as expected from the recent characterization of a transcription unit for a Rieske FeS-protein and cytochrome *b* (Schütz et al. 1994), the operation of the Q-cycle in *Chlorobium* is demonstrated by the observation of oxidant-induced reduction of cytochrome *b*.

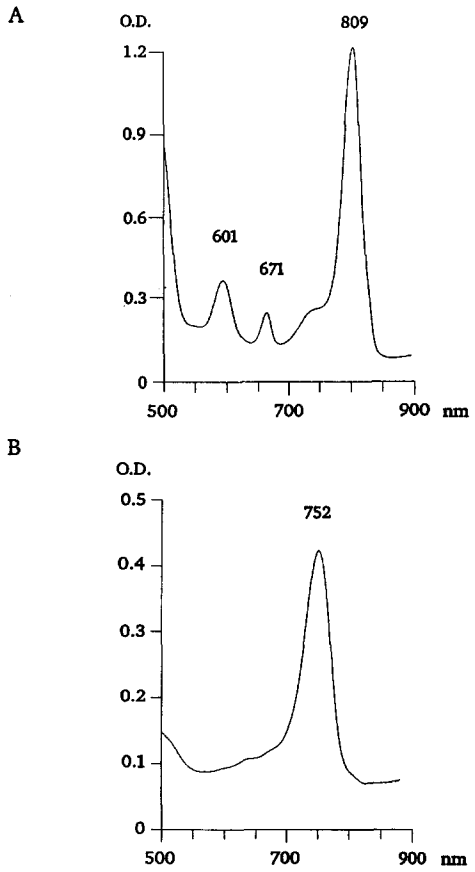

Fig. 1. Spectra of two membrane fractions from *Chlorobium tepidum*. The fractions were obtained as described under Materials and methods. (A) Loosely packed fraction, collected in about 1,5 ml/20 l culture and diluted 1:20; (B) Tightly packed pellet, resuspended in about 150 ml/20 l culture and diluted 1:500.

Materials and methods

Chlorobium tepidum was kindly donated by Dr M.T. Madigan/Carbondale, USA, as well as by Dr M. Miller/Odense, Denmark. It was grown as described by Wahlund et al. (1991) in 20 l-bottles. Cells were harvested at the end of the log-phase of growth, at an OD_{750nm} of the culture of 3.5 to 4.0. From a 20 l-culture 17–21 g wet weight of cells were obtained. They were frozen in liquid nitrogen and stored at −70 °C until use.

All the following steps were carried out at about 4° C. The cells from a 20 l culture were resuspended in 150 ml of 20 mM Tricine-Tris, pH 7.25, 20 μM FMN, which had been first flushed with nitrogen, followed by illumination with strong white light (250 W projector lamp) for at least 10 min to remove oxygen (Nelson et al. 1972). The suspension was passed three times through a French press (Aminco) at 18–20 000

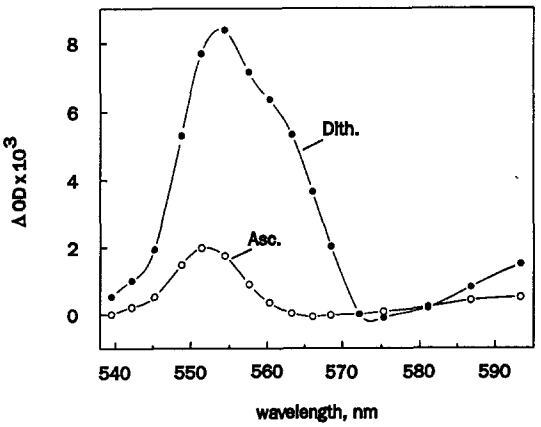

Fig. 2. Cytochrome difference spectra of membranes from *Chlorobium tepidum*. The stirred cuvette contained chlorosome deficient membranes equivalent to 4 μM BChl *a* per ml Tricine-Tris, 20 mM, pH 7.25. Ferricyanide was added to 0.1 mM, and difference spectra were recorded after further additions of 1 mM ascorbate and 30 μM dithionite.

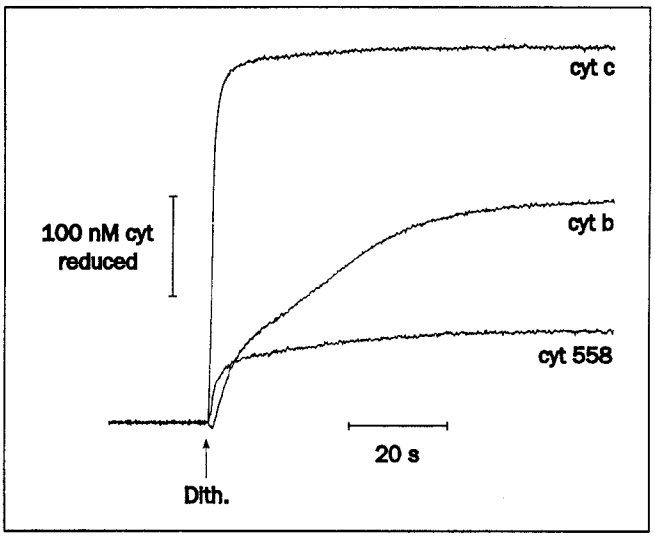

Fig. 3. Reduction kinetics with dithionite. The time course of the spectral change after the addition of dithionite in the experiment of Fig. 2 is deconvoluted into 3 components, cytochrome *c*, cytochrome *b* and a third component peaking at 558 nm.

psi, which resulted in 80% breakage. Residual cells and large fragments were removed by centrifugation at 23 500 g for 20 min, which was followed by sedimenting the membranes in the ultracentrifuge at 120 000 *g* for 1 h. A loosely packed sediment was obtained, on top of a tightly packed pellet which contained all the chlorosomes (Fig. 1). The loosely packed fraction (about 1.5 ml) was collected with a Pasteur-pipette, and was stored frozen at −70 °C until use. It contained about 0.2 mg BChl *a* per ml. BChl *a* was measured after Olson et al. (1976).

Cytochrome changes were recorded under nitrogen with the LED-array spectrophotometer previously described in detail (Klughammer et al. 1990), and as employed for *Rb. capsulatus* before (Shahak et al. 1994). The simultaneous recordings at 16 different wavelengths in the range from 530 to 600 nm were deconvoluted to cytochrome *c*- and *b*-kinetics in the following way: A cytochrome *c*-spectrum peaking at 553 nm was obtained from the difference of the sulfide-reduced and the ferricyanide-oxidized sample in Fig. 7A. It differed only slightly from the one obtained by reduction with ascorbate in Fig. 2, and thus cytochrome *c* is treated as only one spectral component in this analysis. A pure cytochrome *b*-spectrum peaking at 563 nm was obtained after addition of 4 μM NQNO, 50 s after reduction of the sample with 120 μM sulfide (oxidant-induced reduction). Under these conditions cytochrome *c*-changes were negligible. For a good fit of complete reduction by dithionite a third, minor component peaking at 558 nm had to be assumed (Fig. 3). This component was not further analysed. The fitting routine was described before (Shahak et al. 1994), again assuming differential millimolar extinction coefficients of 20 as a satisfactory approximation. Differentiation between cytochrome *c*-components peaking at 553 and 552 nm did not improve the fit.

Decyl-menaquinone was a kind gift of Drs P. Rich and J. Moody/Bodmin, UK. It was reduced to decyl-menaquinol following Rich (1978), and was stored in EtOH/ 10 mM HCl in the freezer.

Results and discussion

Measurements of absorption changes in green sulfur bacteria are hampered by the heavy pigmentation of their membranes with BChl *c* or *d* of the chlorosomes. To overcome this difficulty cultures have been developed in which synthesis of BChl *c* was suppressed by ethylene (Miller et al. 1992).

In our efforts to purify the P840-reaction center from *Chlorobium tepidum* (C. Hager et al., in preparation), we ran across a chlorosome-free membrane fraction (Fig. 1) without the use of ethylene. This fraction represents only about 2% of total BChl a of the homogenate. Nevertheless, we assume that it is representative of the membrane, from which the chlorosomes have been physically detached. The preparation predominantly shows sealed vesicles in electronmicro-

graphs, and is able to build up a pH-gradient in the light (Shuldiner et al. 1972) when ascorbate and phenazine methosulfate are present (C. Hager and L. Englmeier, unpuplished).

The cytochrome content in these membranes is high (Figs. 2 and 3). They contain about 0.125 moles of cytochrome *c*-553, 0.05 moles of cytochrome *b*-563, and about 0.025 moles of cytochrome-558 per mole of BChl *a* (Fig. 3). About one fifth of cytochrome *c*-553, which actually peaks at 552 nm, is reduced by ascorbate (Fig. 2). Cytochrome b reveals two kinetic components of about equal amplitude, one rapidly and one slowly reduced by dithionite (Fig. 3), characteristic for the two hemes of cytochrome *b* in *bc*-complexes (Hauska et al., in press). Even the faster cytochrome *b* component is reduced more slowly than cytochrome *c*-553 by dithionite.

Menaquinol as reductant

Figure 4A shows that 4 μM decyl-menaquinol reduced about 80% of cytochrome *c* in a transient which leveled off to about 30%. This transient was also observed upon a second addition of menaquinol, and eventually was titrated out after repeated additions. A total of about 16 μM decyl-menaquinol was required, which corresponds to an oxidation pool of 4 equivalents per molecule of BChl *a*. Interestingly, the phenomenon was sensitive to KCN (Fig. 4D), suggesting that electrons are fed from cytochrome *c* into the oxidation pool via a cyanide-sensitive redox center. Cytochrome *b* was reduced by menaquinol to a very small extent only, unless NQNO (Fig. 4B), or antimycin A (10 μM; not shown) were present. In their presence more than half of the cytochrome *b*-complement went reduced. Both inhibitors decreased the rate of cytochrome *c*-reduction, as did stigmatellin (Fig. 4C). The later did not likewise stimulate the reduction of cytochrome *b*, in accordance with the different sites of inhibitor action. According to the Q-cycle mechanism of *bc*-complexes stigmatellin inhibits at the quinol oxidation site Q_o, before cytochrome *b*, while antimycin A and NQNO act on the quinone reduction site Q_i, after cytochrome *b* reduction (Link et al. 1993; Shahak et al. 1994). These inhibitors lock the electrons in cytochrome *b*, and thus stimulate 'oxidant-induced reduction' of cytochrome *b*, a key feature of the Q-cycle. When decyl-menaquinol was added in the presence of excess ferricyanide, cytochrome reduction was largely suppressed (Fig. 5A), but when the Q_i-inhibitors NQNO and antimycin were additionally present the strong oxidant ferricyanide caused a pronounced oxidant-induced reduction of cytochrome *b* which exceeded the cytochrome *c* change (Fig. 5B, C). The extents of stimulated cytochrome *b* reduction were again more than 50%, resembling the change with NQNO in the absence of ferricyanide (Fig. 4B).

Inhibition of electron flow and stimulation of cytochrome *b*-reduction by antimycin A has been reported before (Knaff and Buchanan, 1975).

Inhibitor effects on the spectrum of cytochrome b

The spectral changes depicted in Fig. 6 reflect the binding of the inhibitors antimycin A, stigmatellin and NQNO, in vicinity of the cytochrome *b* hemes. Antimycin A causes a redshift (Fig. 6A), as is also found for mitochondrial (von Jagow and Ohnishi 1985). It can be attributed to a 561 nm-component in *Chlorobium*. It is not clear to which kinetic cytochrome *b*-component of Fig. 3 it is related. Stigmatellin causes a more complex change (Fig. 6B), mainly a blueshift of a 563 nm-component. In contrast, a change looking like a redshift of cytochrome *b* by stigmatellin is observed with the mitochondrial bc_1-complex (von Jagow and Ohnishi 1985), and also with the chloroplast b_6f-complex. In both cases both hemes-*b* contribute to the change (Hauska et al. 1989). As with the mitochondrial bc_1-complex (von Jagow and Ohnishi 1985), the shifts caused by antimycin A and stigmatellin are additive (not shown). At first sight these observations suggest again that antimycin shifts the absorption of the high-potential heme at the Q_i-site, with an absorption peak at 561 nm in *Chlorobium*, while stigmatellin affects the low potential heme at the Q_o-site, with an absorption peak at 563 nm. Unexpectedly, NQNO, a Q_i-site inhibitor like antimycin, affects the longer wavelength, 563-component (Fig. 6C), pointing out, that the observed spectral changes need not necessarily reflect the heme proximal to the binding site. In the chloroplast b_6f-complex stigmatellin predominantly affects the spectrum of the high-potential heme, which is distal to the Q_o-site, where stigmatellin binds (Hauska et al. 1989). NQNO yields a blueshift of the 563-component, which is more pronounced than the one with stigmatellin (Fig. 6C). The blueshift is again in contrast to a reported redshift for mitochondrial bc_1-complex (Kamensky et al. 1985). Valinomycin/K^+, which leads to a redshift of the cytochrome b_6 spectrum in chloroplasts (Klughammer and Schreiber 1993), had no significant effect in *Chlorobium*.

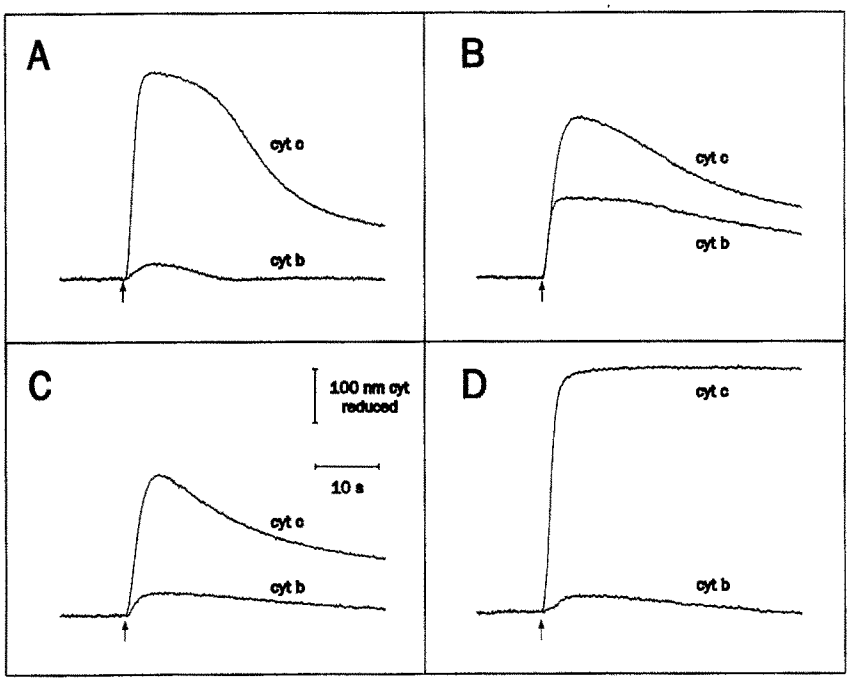

Fig. 4. Cytochrome reduction with decyl-menaquinol and inhibitor effects. The cuvette contained the same membrane suspension as in Fig. 2. The reaction was started by addition of decyl-menaquinol to 4 μM (arrow). (A) without further addition. (B) plus 4 μM NQNO, (C) plus 2 μM stigmatellin, (D) plus 1 mM KCN.

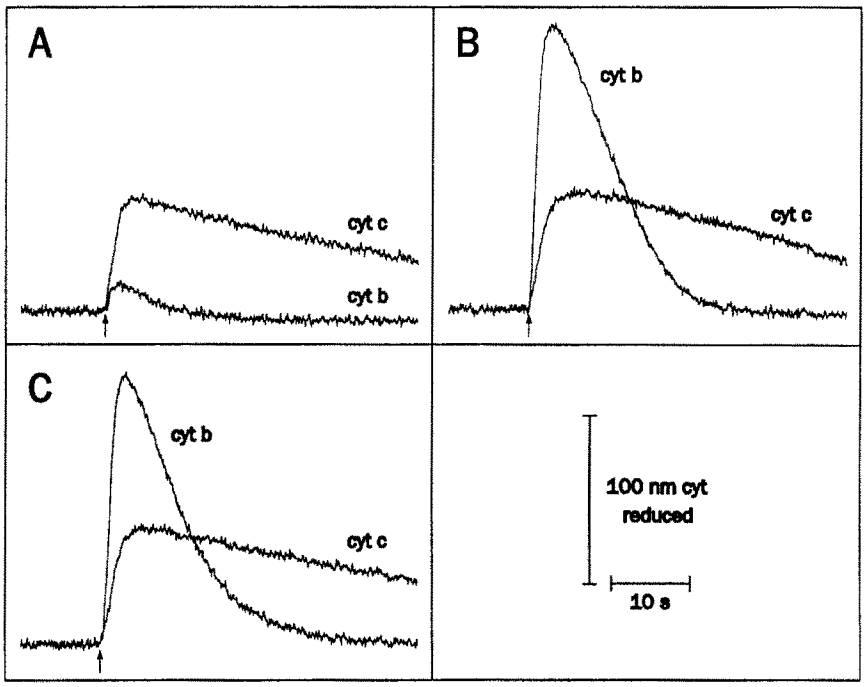

Fig. 5. Oxidant-induced reduction of cytochrome *b* with decyl-menaquinol. The reaction mixture additionally contained 0.1 mM ferricyanide. The arrow indicates the start of the reaction with 4 μM decyl-menaquinol. (A) without further addition, (B) plus 4 μM NQNO, (C) plus 10 μM antimycin A.

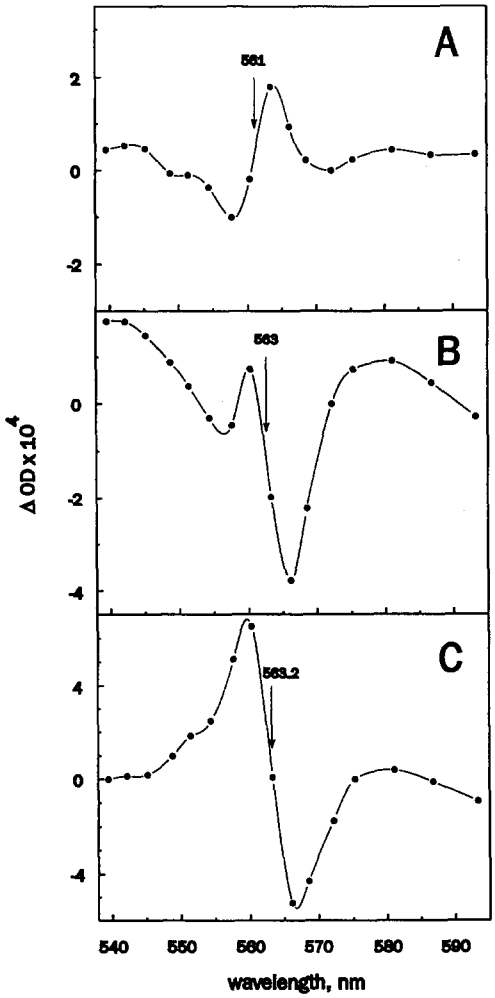

Fig. 6. Spectral shifts of cytochrome *b* by inhibitors. Membranes were present equivalent to 12 μM BChl *a*. The spectral changes after the addition of the inhibitors were recorded. (A) 10 μM antimycin A, (B) 4 μM stigmatellin, (C) 4 μM NQNO.

From the spectral changes in Fig. 6 it can be concluded that the cytochrome *b*-complement in *Chlorobium* comprises at least two forms, as is already indicated by the two kinetic components in Fig. 3. Redox titrations of cytochrome *b* in *Chlorobium limicola* can indeed be interpreted as reflecting two hemes with rather close redox potentials (Hurt and Hauska 1984). The two components could not be spectrally distinguished, both peaking at 562 nm. The best fit was obtained with two components of –70 and –140 mV (leCoutre 1991).

Sulfide as reductant

Figure 7A shows that sulfide efficiently reduces cytochrome *c* with practically no reduction of cytochrome *b*. NQNO inhibited cytochrome *c*-reduction substantially more than can be accounted for by its action at the Q_i-site of the bc-complex (Fig. 4B). NQNO thus is a potent inhibitor of the *Chlorobium* SQR itself (Shahak et al. 1992), probably binding at the quinone reduction site Q_s as discussed before (Shahak et al. 1994). Nevertheless, a stimulation of cytochrome *b*-reduction can be observed in the presence of NQNO, which is better seen in the presence of antimycin A (Fig. 7C), which seems not to inhibit it significantly beyond its action at the Q_i-site. SQR proper thus is not very sensitive to antimycin, which is in contrast to our earlier observation with *C. limicola* that SQR measured with externally added decylubiquinone was about equally sensitive to NQNO and antimycin A (Shahak et al. 1992).

Cytochrome reduction by sulfide was inhibited efficiently by stigmatellin (not shown), as expected from its effect on SQR (Shahak et al. 1992). KCN inhibited with sigmoidal kinetics (Fig. 7D), as has been observed before with *Rb. capsulatus*, and has been interpreted as competition between sulfide and cyanide (Shahak et al. 1994), probably at the flavine (Arieli et al. 1994).

In the presence of ferricyanide cytochrome *c* reduction with sulfide is largely suppressed (Fig. 8A). Reduction of cytochrome *b* by sulfide is stimulated, however, in the presence of antimycin A. This 'oxidant-induced reduction' demonstrates that electrons flow from sulfide involves the cytochrome *bc*-complex in *Chlorobium*. It remains to be established, whether this is the main route of electron transport, or whether part of the electrons in vivo flow via flavocytochrome c (Kusai and Yamanaka 1973; Brune 1989).

With a similar membrane fraction from *C. limicola* similar observations have been made with menaquinol and sulfide. In both *Chlorobium* systems saturating red light counteracted the reduction of cytochromes completely. Thiosulfate, up to 1 mM, did not reduce any cytochrome in either system (not shown).

Acknowledgement

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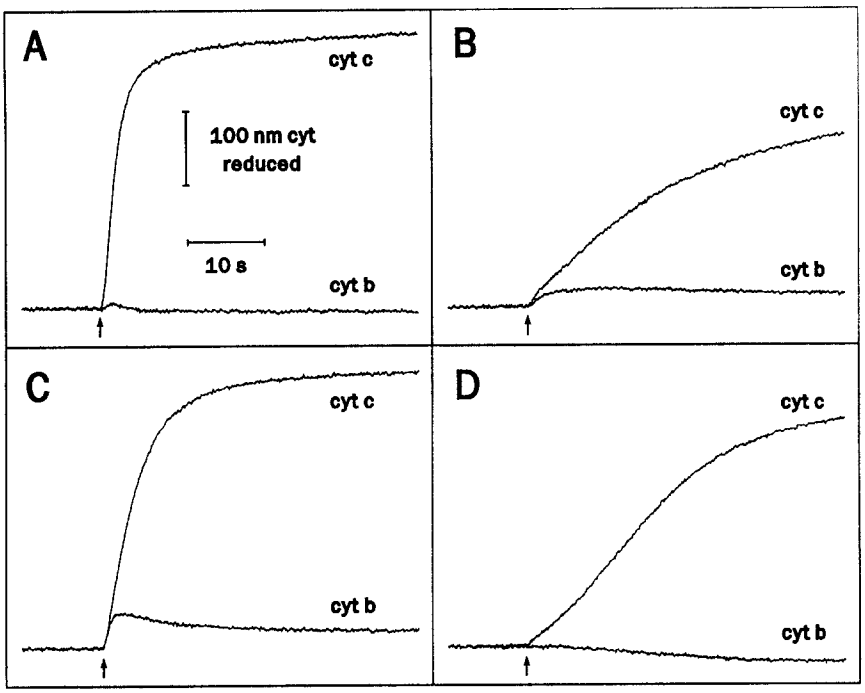

Fig. 7. Cytochrome reduction with sulfide. The reaction was measured as for Fig. 4, but was started by 24 μM sulfide. (A) without further addition, (B) 5 μM NQNO, (C) 10 μM antimycin A, (D) 1 mM KCN.

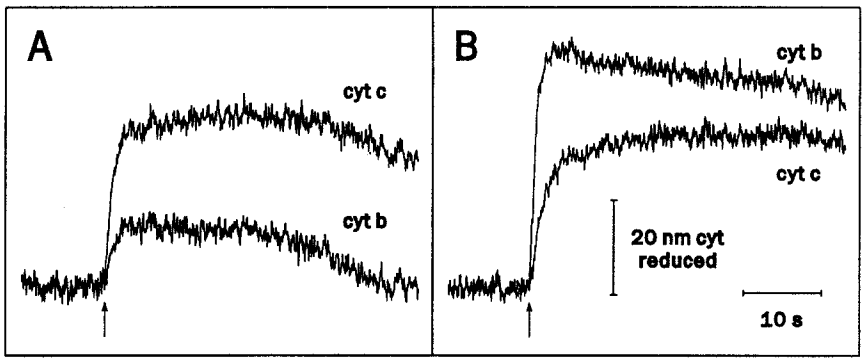

Fig. 8. Oxidant-induced reduction of cytochrome *b* with sulfide. The conditions were as for Fig. 6, but the reaction was started with 24 μM sulfide. (A) without further addition, (B) 10 μM antimycin A

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