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Utilization of sulfide and elemental sulfur by *Ectothiorhodospira halochloris**

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Abstract. *Ectothiorhodospira halochloris* grows photoheterotrophically with a variety of sulfur sources. During sulfide oxidation to elemental sulfur considerable amounts of polysulfides may be accumulated transiently. When grown on elemental sulfur no sulfate was produced by oxidation, but sulfide and polysulfide were formed by reduction. Only one soluble cytochrome *c*-551 was isolated and purified. It was a small acidic hemeprotein with a molecular weight of 6,300, an isoelectric point of 3.1 and a redox potential of −11 mV at pH 7.0. It showed three absorption maxima in the reduced state (α = 551 nm; β = 523 nm; γ = 417 nm). The addition of various *c*-type cytochromes to a suspension of spheroplasts stimulated the velocity of sulfide oxidation. This stimulation was best with the small acidic cytochromes from *E. halochloris* or *Ectothiorhodospira abdelmalekii*. Sulfide oxidation was stopped by several uncoupling agents, ionophores and electron transport inhibitors. Antimycin A, rotenone and cyanide had no effect on sulfide oxidation.

Key words: *Ectothiorhodospira halochloris* – Cytochrome *c*-551 – Sulfide oxidation – Elemental sulfur reduction – Polysulfide – Sulfur metabolism

Members of the genus *Ectothiorhodospira* may be isolated from extremely saline, marine or freshwater habitats (Trüper 1968; Raymond and Sistrom 1967, 1969). *Ectothiorhodospira halochloris* was isolated from a hypersaline lake of the Wadi Natrun, Egypt. In these lakes pH values as high as 11 and salt concentrations up to saturation have been measured (Imhoff and Trüper 1977, 1981). The striking feature of the genus *Ectothiorhodospira* is the ability to deposit elemental sulfur outside the cells, while the other members of the family Chromatiaceae deposit it inside the cells. This difference makes it easier to investigate sulfide and elemental sulfur oxidation in members of the genus *Ectothiorhodospira* than in members of the genus *Chromatium*. There are comparatively little data concerning the enzymatic mechanism of sulfide oxidation in phototrophic bacteria. In the initial studies of Kusai and Yamanaka (1973 a, b) who were using *Chlorobium limicola f. thiosulfatophilum*, it was suggested that a flavocytochrome *c*-553 accepted electrons from sulfide before they were further transferred to oxidized reaction center bacteriochlorophyll. Later the same was proposed for members of the genus *Chromatium* (Petushkova and Ivanovsky 1976; Fischer and Trüper 1977; Fukumori and Yamanaka 1979).

In a preceding publication we reported that sulfide was quantitatively oxidized to elemental sulfur by *Ectothiorhodospira abdelmalekii* with an intermediary accumulation of polysulfides. This reaction was stimulated by the addition of native cytochrome *c*, supporting the idea that *c*-type cytochromes play a catalytical role in sulfide oxidation (Then and Trüper 1983).

The present paper summarizes experiments on the anaerobic oxidation of elemental sulfur in whole cells of *E. halochloris* and presents some molecular properties of cytochrome *c*-551 from this organism. The experiments were carried out to elucidate the role of cytochromes in sulfide and elemental sulfur metabolism and compare these results with those from *E. abdelmalekii*.

Materials and methods

Ectothiorhodospira halochloris (DSM 1059) was grown photoheterotrophically in the medium described by Then and Trüper (1983). All standard methods were carried out as previously described (Then and Trüper 1983). Sulfate was determined as described by Dodgson (1961). The sulfur content of the cells was determined as sulfate after complete digestion in $HClO_3/H_2O_2$ according to Novozamsky and van Eck (1977). A hydrophilic sulfur sol was prepared according to Roy and Trudinger (1970). Alkaline protease was obtained from Sigma, München, FRG. All other chemicals were purchased as described by Then and Trüper (1983).

Results

Growth of Ectothiorhodospira halochloris on various sulfur compounds

Good growth occurred under photoheterotrophic conditions with sulfide (initial concentration 0.8 mM), elemental sulfur added as a hydrophilic sulfur sol (10 mM), sulfite (1.5 mM), sulfate (1 mM), thiosulfate (4 mM), tetrathionate (0.5 mM), cysteine (2 mM) and methionine (0.1 mM). None of the sulfur sources mentioned above supported growth when acetate was omitted from the medium described in "Materials and methods". During growth with sulfide a substantial amount of polysulfides were transiently

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* Dedicated to Prof. Dr. H. G. Schlegel on the occasion of his 60th birthday

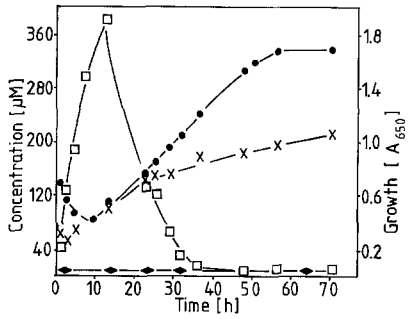

Fig. 1. Oxidation of elemental sulfur by a growing culture of *Ectothiorhodospira halochloris*. 10 mM elemental sulfur as a sterile hydrophilic sol was added to the culture. The cells were covered with a 1 cm thick layer of sterile liquid paraffin and stirred magnetically. Samples were taken through the paraffin layer. As most of the elemental sulfur precipitated and the amount of the elemental sulfur remained almost constant the absorbance at 650 nm remains a relative value for growth of the culture. (□) Sulfide; (×) polysulfide sulfur; (◆) sulfate; (●) absorbance (A) of growing culture at 650 nm

accumulated. At the end of sulfide oxidation elemental sulfur droplets appeared in the medium.

Anaerobic oxidation of elemental sulfur

The kinetics of anaerobic utilization of elemental sulfur was studied with a growing cell culture using a hydrophilic sulfur sol. In the first 10 h the hydrophilic sulfur completely precipitated in the saline medium (Fig. 1), causing the optical density to drop during this time. Due to the inhomogeneity of the sulfur distribution it was not possible to determine the level of the elemental sulfur. Initially a large amount of sulfide was produced, which we concluded was formed by reduction of elemental sulfur with acetate as electron donor.

As is commonly known, free elemental sulfur dissolves in an alkaline solution of sulfide to form polysulfides (Fehér and Laue 1956). Thus the reason for the polysulfide formation is a purely chemical reaction between sulfide and elemental sulfur. No sulfate production could be detected during the experiment. As it is not easy to determine small amounts of sulfate in a hypersaline medium, we could not be certain that no sulfate was formed. So we tried to strike a balance sheet over all sulfur compounds. A major problem is an accurate quantitative determination of elemental sulfur. To overcome this problem the cultures were fed with elemental sulfur placed in a dialysis bag. Good growth occurred under these conditions, but due to a longer lag phase growth stopped after 10 days. Tha analytical balance sheet over all sulfur compounds showed that 97% of the elemental sulfur (Table 1) introduced into the medium could be found in the specified fractions. So there is little evidence of sulfate formation. Thus under photoheterotrophic conditions *E. halochloris* does not appear to be able to further oxidize elemental sulfur.

Purification and properties of cytochrome c-551

We found only one soluble cytochrome *c*-551 in *E. halochloris*. The cytochrome was eluted from the DEAE-column in the oxidized form. In this form it showed absorption

Table 1. Balance sheet of sulfur compounds. *Ectothiorhodospira halochloris* was grown on elemental sulfur. The medium contained 2 g Na-acetate/l and the protein content at the end of growth was 180 mg/l. The sulfur within the dialysis bag and in the cells was determined chemically. Results shown are the average of duplicate samples

Sulfur compounds	Concentration (mM)
Sulfur in dialysis bag	5.775
Sulfur in the cells	0.937
Sulfide in the medium	0.031
Polysulfide sulfur in the medium	0.720
Total	7.463
Control	7.663

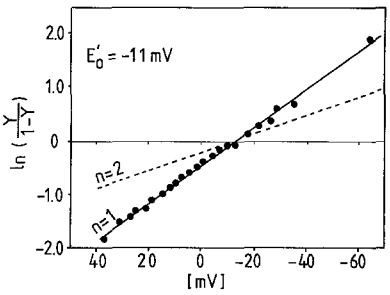

Fig. 2. Redox titration of *Ectothiorhodospira halochloris* cytochrome *c*-551. The absorbance changes of cytochrome *c*-551 at 551 nm were plotted against redox potential. The midpoint oxidation-reduction potential at pH 7.0 and 2 M NaCl was −11 mV. *Y*, A_{551}/A_{max}; *A*, absorbance; *n*, number of electrons; (——) titration curve measured; (– – –) theoretical curve according to the Nernst equation for $n = 2$

maxima at 280, 359, 411 and at 530 nm. After the addition of dithionite three typical absorbtion maxima could be detected in the reduced state: $\alpha = 551$ nm; $\beta = 523$ nm; $\gamma = 417$ nm. The apparent relative molecular mass was determined by comparative gel filtration on Sephadex G-75 with three marker proteins and calculated to be 6,300. The Sephadex G-75 column was equilibrated in 50 mM Tris/HCl, pH 7.8, containing 2 M NaCl. The high concentration of salt in the elution buffer was necessary because the cytochrome showed three times higher molecular weights without NaCl in the elution buffer. Lower salt concentrations might be responsible for the formation of aggregates. Similar effects were described for acidic rubredoxins by Steinmetz and Fischer (1982; Steinmetz et al. 1983).

Flat bed electrofocussing on Ampholine gel pH 2.5–5.0 showed only a single band with an isoelectric point of pH 3.1 (±0.2). The midpoint oxidation-reduction potential at pH 7.0 of cytochrome *c*-551 was −11 mV (Fig. 2). The reaction mixture contained 2 M NaCl. Without additional salt an even lower potential of −59 mV was determined. The intercept on the abscissa in Fig. 2 gives the midpoint oxidation-reduction potential, while the slope is identical with the theoretical value of the Nernst equation for $n = 1$. Intercept and slope were determined by a correlation analysis with a correlation coefficient of $R = 0.986$. As the slope of the titration curve was identical with the theoretical slope of the Nernst equation for $n = 1$, we concluded that only

Table 2. Influence of various *c*-type cytochromes on sulfide oxidation by spheroplasts of *Ectothiorhodospira abdelmalekii*. Spheroplasts (2.6 mg protein/ml) were suspended in saline 10 mM carbonate buffer, pH 8.2, and incubated anaerobically at 40° C and 4,000 lx. One part of the spheroplasts were previously incubated with an alkaline protease (0.2 mg/ml) in saline carbonate buffer, pH 8.1, for 3.5 h. Then the cells were centrifuged (6,000 *g*, 10 min), washed and resuspended in carbonate buffer. The experiment was started with 3 mM sulfide. 140 nmol horse heart cytochrome *c*, 65 nmol *Ectothiorhodospira halochloris* cytochrome *c*-551 and 36 nmol *E. abdelmalekii* cytochrome *c*-551 were used. Results shown are the average of duplicate samples

Conditions	Polysulfide sulfur (mM)				
	0 h	1 h	2 h	3 h	4 h
Spheroplasts					
Spheroplasts	0.43	0.50	0.59	0.70	0.71
Horse heart cyt. *c*	0.51	0.67	0.72	0.98	1.06
E. halochloris cyt. *c*	0.53	0.79	0.82	1.18	1.23
E. abdelmalekii cyt. *c*	0.56	0.79	0.82	1.14	1.16
Spheroplasts incubated with a protease					
Spheroplasts	0.54	0.83	1.10	1.16	1.42
Horse heart cyt. *c*	0.58	0.93	0.99	1.52	1.45
E. halochloris cyt. *c*	0.65	1.18	1.45	1.70	1.73
E. abdelmalekii cyt. *c*	0.69	1.09	1.45	1.79	1.81

one electron was transferred in the heme part of the cytochrome.

Effects of external c-type cytochromes on sulfide oxidation

It could be shown that additional native cytochrome *c*-551 from *Ectothiorhodospira abdelmalekii* had a stimulating effect on sulfide oxidation velocity only with spheroplasts of this organism (Then and Trüper 1983). Table 2 shows the results with different cytochromes on sulfide oxidation of spheroplasts from *E. abdelmalekii*. All cytochromes tested had a stimulating effect on sulfide oxidation as compared with the control. The acidic cytochromes from *E. halochloris* and *E. abdelmalekii* produced a stronger effect on sulfide oxidation than horse heart cytochrome *c*. The polysulfide production was best with cytochrome *c*-551 from *E. halochloris* (73% higher compared with control), followed by cytochrome *c*-551 from *E. abdelmalekii* (62%) and horse heart cytochrome *c* (49%).

Spheroplasts incubated with an alkaline protease were also investigated. The remaining cytochrome *c* content of these cells was 75% compared with that of non-digested cells. The protein content of these was reduced to 1.4 mg protein/ml (Table 2). The spheroplasts were still able to oxidize sulfide and the sulfide oxidation velocity was even higher than with non-digested cells, but the stimulation was not as high as with non-digested spheroplasts. Polysulfide production was best with native cytochrome *c*-551 (26% higher than control), followed by cytochrome *c*-551 from *E. halochloris* (21%) and horse heart cytochrome *c* (1.8%).

Influence of various inhibitors on sulfide oxidation

Table 3 shows the effects of several uncouplers, ionophores and electron transport inhibitors on sulfide oxidation. All

Table 3. Influence of various inhibitors on sulfide oxidation. Concentrated cell suspensions of *Ectothiorhodospira halochloris* (4.5 mg protein/ml) were incubated anaerobically in a cylindrical 10 ml glass vessel with a water jacket. The experiment was started with 3 mM sulfide. Cells were incubated for 10 min in the dark at pH 8.2 and 40° C. The light (4,000 lx) was turned on and after 10 min the inhibitor was added. Sulfide was determined in the medium. (+) immediate inhibition of sulfide oxidation; (−) no inhibition

Inhibitor	
Antimycin A (4 μM)	−
Valinomycin (2 μM)	+
Dichlorophenyl-dimethylurea (400 μM)	+
Cyanide (200 μM)	−
Amytal (200 μM)	+
Rotenone (20 μM)	−
Dicyclohexylcarbodiimide (2 μM)	−
Heptylhydroxyquinolin-N-oxide (2 μM)	+
Chlorocarbonylcyanidephenylhydrazone (12 μM)	+
Carbonylcyanide-p-trifluorophenyl-hydrazone (2 μM)	+

uncouplers used prevented sulfide oxidation. Antimycin A did not block sulfide oxidation. This support the idea that electrons from sulfide were accepted after the blocking site of Antimycin A. Cyanide which blocks the oxidation of cytochrome aa_3 in mitochondria (Jones 1976) had no effect on sulfide oxidation. Rotenone, which was found to block electron transport in the span between NADH and ubiqinone in mitochondria, also had no effect on sulfide oxidation.

Discussion

The present work has shown that *Ectothiorhodospira halochloris* is only able to grow photoheterotrophically on a wide variety of sulfur sources. Under these conditions the organism was not able to further oxidize elemental sulfur to sulfate. Instead of oxidizing elemental sulfur the cells reduced it to sulfide. Similar results were obtained for several members of the genus *Chromatium* during dark-anaerobic metabolism (Trüper 1964; van Gemerden 1967, 1968) and in the methanogenic bacteria (Stetter and Gaag 1983). The sulfur reduction occurred in the first 15 h of growth and then declined. The reducing equivalents probably come from acetate.

The formation of polysulfides in the alkaline medium is a purely chemical reaction driven by the production of sulfide by the organism. A quantitative balance sheet of sulfur showed no evidence that sulfate was produced, but it proved an assimilatory sulfur reduction to sulfide. Thus we conclude that elemental sulfur is only used as an assimilatory sulfur source while acetate acts as a source for reducing equivalents and carbon.

E. halochloris as well as *E. abdelmalekii* seem to possess only one cytochrome *c*-551 (Then and Trüper 1983). These cytochromes have many properties in common and differ only slightly in their molecular weights, isoelectric points and oxidation-reduction potentials.

The stimulation of sulfide oxidation by the addition of *c*-type cytochromes to spheroplasts of *E. abdelmalekii* showed that the cytochromes were the limiting factor in sulfide oxidation by the spheroplasts. A comparison of the different

cytochromes showed that the cytochromes *c*-551 from *E. halochloris* and *E. abdelmalekii* had an almost identical effect on sulfide oxidation. This was not surprising as they differ only slightly in their molecular properties. Horse heart cytochrome *c* did not stimulate sulfide oxidation to the same extent. One reason for this may be its redox potential. The oxidation-reduction potential of the cytochromes from *E. halochloris* and *E. abdelmalekii* is near 0 mV, while the potential of horse heart cytochrome is +260 mV (Dickerson and Timkovich 1975). Therefore it is not as easy to oxidize horse heart cytochrome as the cytochromes from the green *Ectothiorhodospira* species. This is supported by the fact that horse heart cytochrome *c* had no effect on spheroplasts that were previously incubated with a protease. Perhaps due to structural changes in the cell membrane of the spheroplasts sulfide oxidation was only stimulated by the cytochromes of the green *Ectothiorhodospira* species. We believe that the additional cytochromes were first reduced by sulfide in the medium and subsequently oxidized by the natural electron acceptor in the cell membrane. Intact cells were not able to oxidize the external cytochromes (Then and Trüper 1983). The physiological electron acceptor of the cytochrome could be a so far unknown component of the photosynthetic electron transport chain, as sulfide oxidation of the cytochromes occurred only in the light. As too little is presently known about the composition of the photosynthetic reaction center of the two bacteriochlorophyll *b*-containing *Ectothiorhodospira* species, we can only propose that the single cytochrome (*c*-551) found in these species may also have the function of the electron donor for the photosynthetic reaction center. Also in the phototrophic green sulfur bacterium, *Chlorobium vibrioforme*, only one single cytochrome *c* has been found (Steinmetz et al. 1983).

Little is known about sulfide: cytochrome *c* reductase (Trüper and Fischer 1982), but results from Chlorobiaceae (Kusai and Yamanaka 1973 a, b; Steinmetz and Fischer 1982) show that flavocytochrome *c*-553 was a likely candidate for sulfide oxidation. Two abilities were thought to be relevant for these cytochromes: They should be quickly reduced by sulfide and this catalytic ability should be inhibited by cyanide.

Sulfide oxidation by *E. halochloris* and *E. abdelmalekii* (results not shown) was not inhibited by cyanide under physiological conditions. This is a remarkable result and casts doubt upon the value of isolated cytochrome *c* inhibition by cyanide. We have not studied other possible cyanide effects upon *E. halochloris*. The effects of the other inhibitors studied are not clear, because on one hand very little knowledge exists about inhibitor effects in sulfur and especially sulfide metabolism; on the other hand so little is known about the components of the electron transport chain in the extreme haloalkaliphilic *Ectothiorhodospira* species that one cannot a priori assume that all species blocking sites of the inhibitors studied are there at all. Lacking inhibition by antimycin and rotenone versus inhibition by amytal and heptylhydroxyquinolin-N-oxide may therefore appear contradictory, but cannot be – at least presently – explained. The inhibitory effects of ionophore and uncoupling agents show that sulfide oxidation depends on an energized state of the cell membrane and an intact electron flow.

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