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Cytochromes of the Green Sulfur Bacterium *Chlorobium vibrioforme* f. *thiosulfatophilum*. Purification, Characterization and Sulfur Metabolism

Manfred A. Steinmetz and Ulrich Fischer

Institut für Mikrobiologie der Rheinischen Friedrich-Wilhelms-Universität, Meckenheimer Allee 168, D-5300 Bonn 1, Federal Republic of Germany

Abstract. Three cytochromes of the thiosulfate-utilizing green sulfur bacterium *Chlorobium vibrioforme* f. *thiosulfatophilum* were highly purified by ion exchange column chromatography and ammonium sulfate fractionation. All three cytochromes are located in the soluble fraction. Cytochrome c-551 (highest purity index obtained: $A_{280}/A_{416} = 0.39$) shows maxima at 551 nm (α-band), 521 nm (β-band), and 416 nm (γ-band) for the reduced form. This cytochrome is an acidic protein with a molecular weight of 32,000, a redox potential of 150 mV, and an isoelectric point at pH 6.0. Cytochrome c-553 (highest purity index obtained: $A_{280}/A_{417} = 0.8$) is also an acidic protein with maxima at 553,5 nm, 523,5 nm and 417 nm for the reduced form, a molecular weight of 63,000, a redox potential of 90 mV, an isoelectric point at pH 6.3, and it contains FAD as flavin component. It is autoxidizable and participates in sulfide oxidation, but cannot catalyze the reverse reaction. The cytochrome c-555 (highest purity index obtained: $A_{280}/A_{418} = 0.16$) is a small basic protein with maxima at 555 nm, 523 nm and 418 nm (reduced form), a molecular weight of 12,500, an isoelectric point between pH 10 and 10.5, and a redox potential of 155 mV. The ratio of the cytochrome contents to each other is constant and does not change when the organism has only thiosulfate or sulfide as the main electron donor in the medium.

The soluble fraction further contains the non-heme iron-containing proteins rubredoxin and ferredoxin. The anaerobic sulfide oxidation in a growing culture of *Chlorobium vibrioforme* f. *thiosulfatophilum* is accompanied by a rapid formation of thiosulfate, which is only utilized when sulfide is no longer available, while the elemental sulfur concentration increases constantly until thiosulfate is consumed.

Key words: *Chlorobium vibrioforme* f. *thiosulfatophilum* – Cytochrome c-551 – Flavocytochrome c-553 – Cytochrome c-555 – Sulfur metabolism

In a previous paper we reported about the cytochromes and other iron-sulfur proteins (ferredoxin and rubredoxin) of the non-thiosulfate-utilizing green sulfur bacterium *Chlorobium limicola* strain 6330 (Steinmetz and Fischer 1981). With the only exception that this organism lacked cytochrome c-551, we were able to isolate the same electron transfer proteins already found in the thiosulfate-utilizing *C. limicola* f. *thiosulfatophilum* (Meyer et al. 1968, 1971). From the studies of Kusai and Yamanaka (1973a, b) it was known that cytochrome c-551 of *C. limicola* f. *thiosulfatophilum* serves as the first endogenous electron acceptor of thiosulfate oxidation from which the electrons are transferred via cytochrome c-555 to oxidized bacteriochlorophyll. That special cytochromes or non-heme iron proteins participate in thiosulfate metabolism has been reported by Trudinger (1961a, b) for cytochrome c-553,5 of *Thiobacillus neapolitanus* and by Hatchikian et al. (1972) for cytochrome cc_3' of *Desulfovibrio gigas*. Appelt et al. (1979) could show that a soluble cytochrome c accepts the electrons from a thiosulfate:cytochrome c oxidoreductase in *Rhodopseudomonas palustris*, while in *Chromatium vinosum* two possible electron acceptors are present during thiosulfate oxidation: a membrane-bound cytochrome c (Knobloch et al. 1981) and the soluble high-potential-iron-sulfur-protein (HIPIP) (Fukumori and Yamanaka 1979b).

So far known, the flavocytochromes of the phototrophic sulfur bacteria are involved in sulfide or sulfite oxidation. Flavocytochrome c-550 of *Thiocapsa roseopersicina* has adenylylsulfate reductase activity (Trüper and Rogers 1971), while the flavocytochromes of *C. limicola* f. *thiosulfatophilum* (c-553) (Kusai and Yamanaka 1973c), *C. limicola* strain 6330 (c-553) (Steinmetz and Fischer 1981) and *Chromatium vinosum* (c-552) (Yamanaka and Kusai 1976; Fischer and Trüper 1979) are sulfide:cytochrome c reductases catalyzing the oxidation of sulfide. In addition to this enzymatic activity, the flavocytochrome c-552 of the latter organism catalyzes also the reverse reaction from elemental sulfur to sulfide (Fukumori and Yamanaka 1979a). Another interesting aspect is the sulfide oxidation by whole cells of the green sulfur bacteria. During anaerobic sulfide oxidation by a growing culture of *C. limicola* f. *thiosulfatophilum* thiosulfate appeared as the first main product in the medium before elemental sulfur was formed (Schedel 1978). In contrast to Schedel's findings (1978) we could show that cells of the non-thiosulfate-utilizing *C. limicola* strain 6330 oxidized sulfide directly to elemental sulfur, which was only utilized, when sulfide was no longer available (Steinmetz and Fischer 1981).

Here we present some properties of the soluble cytochromes and some quantitative measurements of anaerobic sulfide oxidation of *C. vibrioforme* f. *thiosulfatophilum*. It was the aim of this study to compare the results with those of *C. limicola* f. *thiosulfatophilum* and *C. limicola* strain 6330.

Material and Methods

Growth and Harvest of Cells

Chlorobium vibrioforme f. *thiosulfatophilum* strain NCIB 8327 (obtained from the Deutsche Sammlung von Mikro-

Non-common abbreviations: C, Chlorobium; SDS, sodium dodecylsulfate; HIPIP, high-potential-iron-sulfur-protein

Offprint requests to: U. Fischer

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organismen, D-3400 Göttingen, DSM Number 263) was grown photolithoautotrophically at 30° C and 1,000 lux in 20 l carboys. The medium (Pfennig 1965) contained in 1 l: 0.52 g $CaCl_2 \times 2\,H_2O$; 1.2 ml trace element solution (Pfennig and Lippert 1966); 1.2 ml of a 0.002% vitamin B_{12} solution; 0.4 g KH_2PO_4; 0.4 g NH_4Cl; 0.4 g $MgCl_2 \times 6\,H_2O$; 0.4 g KCl; 5.2 g $NaHCO_3$; 0.75 g $Na_2S \times 9\,H_2O$ and 4.4 g $Na_2S_2O_3$. To get higher cell yields, the cultures (grown only with sulfide) were fed three times with 1 l of a 0.075% $Na_2S \times 9\,H_2O$ solution. The pH of the medium was controlled after feeding and regulated with some ml of a sterilized 2 M H_2SO_4 solution to a pH of 6.9–7.1. Cells were harvested with a Christ Cryofuge (10,000 rpm) 4 days after inoculation.

Purification of the Cytochromes

The cytochromes were prepared by the method according to Meyer et al. (1968), modified by Fischer (1977), and described in full details recently by Steinmetz and Fischer (1981), with the only exception that cytochrome c-551 and flavocytochrome c-553 were chromatographed on DE-52 instead of CM-52 cellulose after ammonium fractionation (50–75% saturation) and desalting on Sephadex G-25.

Spectrophotometric Determination

Absorption spectra were determined in a Beckman Acta M VI double beam spectrophotometer using 1 cm quartz cells. The reduced spectra were obtained by the addition of some crystals of dithionite to the sample.

Elemental Sulfur Reductase Assay

Colloidal sulfur was obtained from thiosulfate acidification with concentrated H_2SO_4 according to the method described by Roy and Trudinger (1970). Elemental sulfur reductase activity was determined as an anaerobic oxidation of reduced benzylviologen with sodium dithionite as reductant at pH 8.5, described by Fukumori and Yamanaka (1979a), and manometrically in Warburg vessels in a hydrogenase dependent system according to the method of Fauque et al. (1979). Hydrogenase of *Desulfovibrio gigas* was prepared according to Schedel and Trüper (1979).

Protein Estimation

Protein was determined according to Lowry et al. (1951).

Analytical Determinations

Sulfide was determined as described by Pachmayr (1960) and modified after Trüper and Schlegel (1964).

Elemental sulfur was determined according to Bartlett and Skoog (1954) modified after Steinmetz and Fischer (1981).

Thiosulfate was determined by the method according to Urban (1961) modified after Steinmetz and Fischer (1981).

Identification of Flavin

Flavin was identified by paper chromatography according to Kilgour et al. (1957). The organic phase of the following solvent system was used after the mixture was allowed to stand for 3 h: n-butanol-acetic acid-water (40:10:50, by vol.). The flavocytochrome was denatured by heat treatment (10 min at 80° C in a water bath), chromatographed and the R_f value of the released flavin component was compared with that for FAD and FMN. Flavins were detected by their fluorescence at 254 nm (ultraviolet lamp Desaga Min UVIS).

Molecular Weight

Molecular weight of the cytochromes was measured by electrophoresis on SDS acrylamid gel by the method of Weber and Osborne (1969) following the instruction manuals of the Boehringer Combithek No. 101365 and No. 236292.

Redox Potential

Redoxtitrations were performed anaerobically at pH 7.0 and 25° C by the stepwise addition of a 25 mM $K_3[Fe(CN)_6]$ solution and a 100 mM $Na_2S_2O_4$ solution. Redox potential was measured with a PHM 64 Research pH Meter, Radiometer Kopenhagen and a platinum and a calomel electrode unter stirring in a 1 cm cell and the relative absorption changes at the α-maxima of the different cytochromes were followed with time in a Beckman Acta M VI recording double beam spectrophotometer until equilibrium conditions were reached. Cytochromes were suspended in 40 mM 3(N-morpholino) propane sulfonate; 0.15 M mannitol; 50 mM sucrose; 10 mM EDTA; 10 mM oxalic acid, pH 7.0. The following redox mediators were added: 1 mM $FeCl_3$; 1.2 mM durochinone; 0.4 mM menadione; 20 mM 2-hydroxy-1,4-naphthochinone; 0.1 mM anthrachinone sulfonate; 0.1 mM diaminodurene; 50 µM phenazine ethosulfate; 50 µM phenazine methosulfate.

Isoelectric Point

Isoelectric point was determined by flat bed electrofocusing in a LKB Multiphor 2117 on "Ampholine PAG-plates, pH range 3.5–9.5" after the method given in the LKB instruction manual for electrofocusing. After electrofocusing the proteins were stained with Coomassie blue. To carry out flat bed electrofocusing the cytochrome samples had to be desalted by gel-filtration on a Sephadex G-25 column.

Chemicals

Standard chemicals (analytical grade) were obtained from Merck, Darmstadt, benzylviologen from Serva, Heidelberg, Biochemicals and Combitheks from Boehringer, Mannheim, DEAE- and CM-Cellulose from Whatman Biochemicals Ltd, Maidstone, England, Sephadex G-25 and G-75 from Pharmacia, Uppsala, Sweden, Ampholine PAG-plate from LKB, Stockholm, Sweden, phenazine ethosulfate from ICN Pharmaceuticals, Inc., Plainview, New York, USA and all other redox mediators from EGA Chemie, Steinheim, West-Germany.

Results

Purity and Spectral Properties of Cytochrome c-551

Figure 1 shows the spectrum of oxidized and reduced cytochrome c-551 of *Chlorobium vibrioforme* f. *thiosulfatophilum*. The oxidized form had a γ-band at 410 nm which shifted in the reduced form – after the addition of dithionite – towards 416 nm and an α-band at 551 nm and a β-band at 521 nm

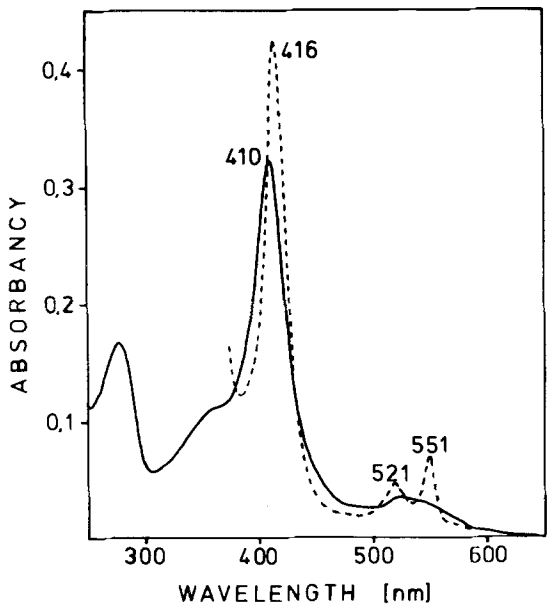

Fig. 1. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) cytochrome c-551 of *Chlorobium vibrioforme* f. *thiosulfatophilum*. 1 cm cells contained 3.7 nmol of cytochrome c-551 (calculated using E_{mM} of 114; Bartsch 1971) in 1 ml 0.1 M potassium phosphate buffer, pH 7.0. (——) Oxidized; (– – – –) reduced

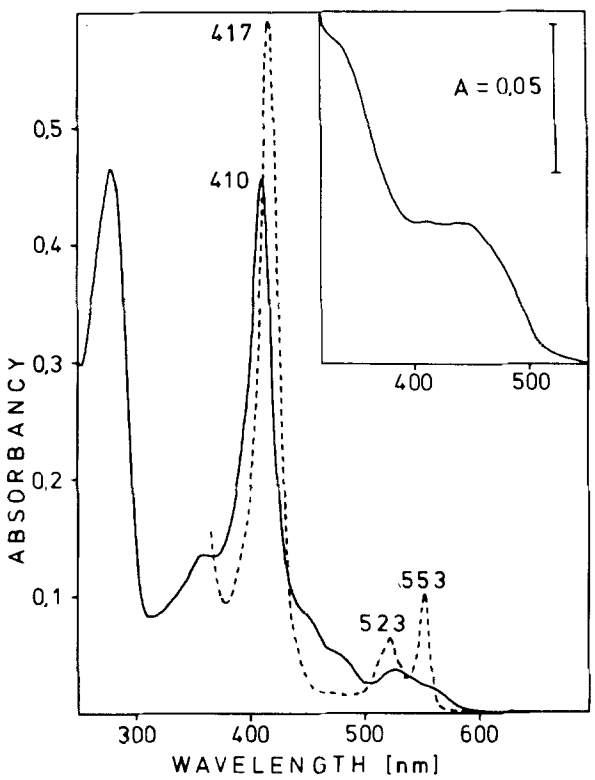

Fig. 2. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) flavocytochrome c-553 of *C. vibrioforme* f. *thiosulfatophilum*. 1 cm cells contained 3.8 nmol of flavocytochrome c-553 (calculated using E_{mM} of 156; Bartsch 1971) in 1 ml 0.1 M potassium phosphate buffer, pH 7.0. Insert shows the absorption spectrum of the separated oxidized flavin containing subunit of flavocytochrome c-553. In the reduced form (plus a few crystals of sodium dithionite) the visible spectrum is completely bleached out. 1 cm cells contained 5 nmol of the flavin subunit (calculated using E_{mM} of 9.8 for FAD, Appaji et al. 1967) in 1 ml 0.1 M Tris-HCl, pH 11. (——) Oxidized; (– – – –) reduced

appeared additionally. The best absorbance ratio (A_{280}/A_{416}) obtained for this cytochrome was 0.39. Cytochrome c-551 was an acidic protein and we could isolate 1.18 µmol out of 100 g wet cell material.

Purity and Spectral Properties of Flavocytochrome c-553

Flavocytochrome c-553 was an acidic protein and was isolated in a partially reduced form but became oxidized after standing for some hours at the open air. The spectrum of the oxidized form of the flavocytochrome c-553 showed two shoulders at 440 nm and 480 nm, indicating the flavin component, and a γ-band at 410 nm. The dithionite reduced spectrum revealed the characteristic maxima of a flavocytochrome c: $\alpha = 553$ nm, $\beta = 523$ nm, and $\gamma = 417$ nm, while the flavin shoulders were bleached out (Fig. 2). The best absorbance ratio (A_{280}/A_{417}) obtained for this flavocytochrome was 0.8. We could isolate 0.34 µmol of flavocytochrome c-553 out of 100 g wet cell paste. The insert of Fig. 2 shows the separated oxidized flavin-containing subunit of flavocytochrome c-553. For the separating procedure of the flavin-containing subunit, a flavocytochrome c-553 solution was adjusted to pH 5.5 with 0.1 N HCl, chromatographed on a small CM-52 cellulose column (equilibrated in 1 mM phosphate buffer, pH 6.0) and eluted with an increasing pH gradient (50 mM Tris-HCl, pH 7.0 – 11). The flavin-containing subunit was eluted at pH 11. By paper chromatography (Schleicher and Schüll No. 2043 b) and its fluorescence at 254 nm, flavin adenine dinucleotide (FAD) was identified as the flavin component (R_f-value: 0.07). The flavocomponent of the recently described flavocytochrome c-553 of *C. limicola* strain 6330 (Steinmetz and Fischer 1981) was also FAD, as examined by the above described procedure.

Purity and Spectral Properties of Cytochrome c-555

Cytochrome c-555 was a small, basic protein with an absorption maximum at 412 nm in the oxidized form. The reduced cytochrome – after adding dithionite – showed maxima at 555 nm (α-band), 521 nm (β-band) and 418 nm (γ-band) and a little shoulder in the α-band at 551 nm (Insert of Fig. 3). This shoulder was found for all cytochromes c-555 of the Chlorobiaceae and was typical for f-type cytochromes. The best absorbance ratio (A_{280}/A_{418}) obtained for this cytochrome was 0.16 (Fig. 3). Out of 100 g wet cell material we could isolate 0.93 µmol of cytochrome c-555.

Molecular Weight Estimation of Cytochrome c-551, Flavocytochrome c-553 and Cytochrome c-555

The molecular weight of the three cytochromes were estimated by polyacryl amide gel electrophoresis (5 % gel or 7.5 % gel) in the presence of 0.1 % SDS by comparing the migration path of the cytochromes with that of several marker proteins with known molecular weight. By this procedure we determined a molecular weight of 32,000 for cytochrome c-551, 63,000 for flavocytochrome c-553, and 12,500 for cytochrome c-555 (Fig. 4).

Isoelectric Point of Cytochrome c-551, Flavocytochrome c-553 and Cytochrome c-555

The isoelectric point of the three cytochromes of *C. vibrioforme* f. *thiosulfatophilum* was found at pH 6.0 for cytochrome c-551, at pH 6.3 for flavocytochrome c-553, and between

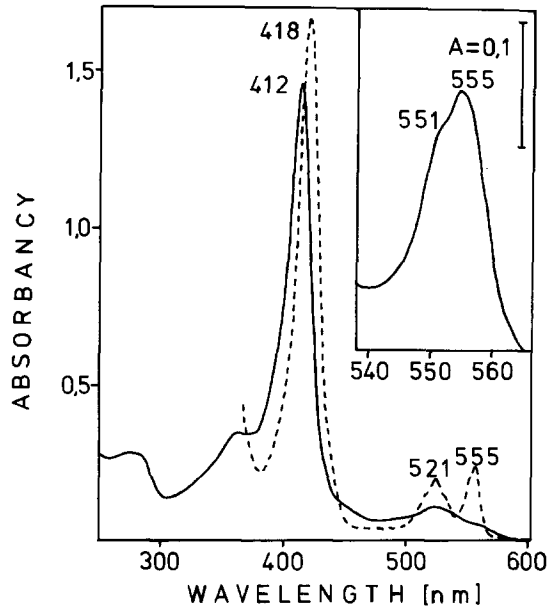

Fig. 3. Absorption spectra of oxidized and reduced (plus a few crystals of sodium dithionite) cytochrome c-555 of *C. vibrioforme* f. *thiosulfatophilum*. 1 cm cells contained 9.1 nmol of cytochrome c-555 (calculated using E_{mM} of 184; Bartsch 1971) in 1 ml 0.1 M potassium phosphate buffer, pH 7.0. Insert shows enlarged α-peak of cytochrome c-555 with the typical shoulder of f-type cytochromes at 551 nm. (——) Oxidized; (– – – –) reduced

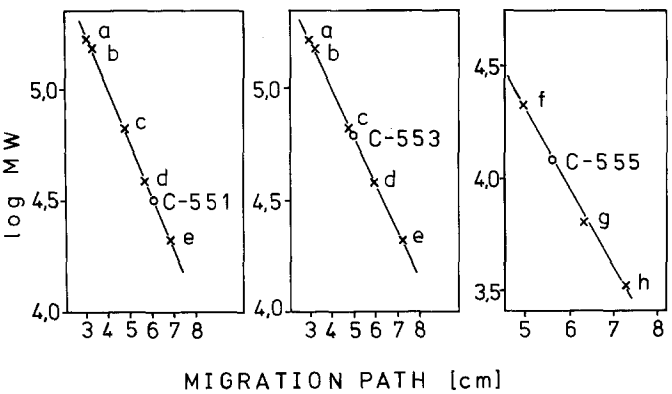

Fig. 4. Estimation of the molecular weight of *C. vibrioforme* f. *thiosulfatophilum* cytochromes c-551, c-553 and c-555 by SDS-electrophoresis. The migration path of the cytochromes was compared with that of several calibration proteins from Boehringer Combithek No. 161365: *a* = β'-subunit (MW: 165,000), *b* = β-subunit (MW: 155,000), *d* = α-subunit (MW: 39,000) of RNA polymerase from *Escherichia coli*; *c* = albumin from bovine serum (MW: 68,000), *e* = trypsin inhibitor from soy bean (MW: 21,500) and from Boehringer Combithek No. 236292: *f* = trypsin inhibitor from soy bean (MW: 21,500), *g* = apoprotinin (MW: 6,500), *h* = insulin chain B (MW: 3,400)

pH 10 and 10.5 for the basic cytochrome c-555 by flat bed electrofocusing.

Redox Potential of Cytochrome c-551, Flavocytochrome c-553 and Cytochrome c-555

The midpoint oxidation-reduction potentials at pH 7.0 were +150 mV for cytochrome c-551, +90 mV for flavocytochrome c-553, and +155 mV for cytochrome c-555 (Fig. 5). The amount of transferred electrons for each cytochrome was

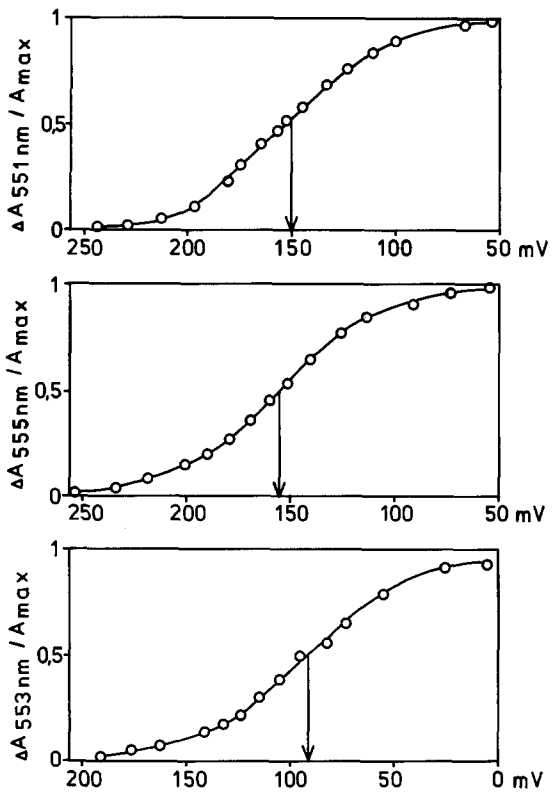

Fig. 5. Ferricyanide titration curves of *C. vibrioforme* f. *thiosulfatophilum* cytochromes c-551, c-553 and c-555. The absorbance changes of the cytochromes were recorded at their α-maxima (cytochrome c-551 at 551 nm, flavocytochrome c-553 at 553 nm, cytochrome c-555 at 555 nm). The redox titration was performed with 14 nmol of cytochrome c-551, 2 nmol of flavocytochrome c-553, and 13 nmol of cytochrome c-555. For detailed information see "Material and Methods"

determined by comparing the ferricyanide titration curves with the theoretical values – calculated by the equation of Nernst – for n = 1 and n = 2 and a redox potential (E_0') of +150 mV (cytochrome c-551), +90 mV (flavocytochrome c-553), and +155 mV (cytochrome c-555). We found that the values of each ferricyanide titrated redox potential were identical with the theoretical calculation for n = 1, indicating that only one electron was transferred in the heme part of each cytochrome. Therefore, we concluded that each cytochrome possessed only one heme group per protein molecule.

A midpoint redox potential of +140 mV at pH 7.0 was found for the recently mentioned small cytochrome c-555 of *C. limicola* strain 6330 (Steinmetz and Fischer 1981) by the method described in this paper.

Influence of Thiosulfate and Sulfide on the Cytochrome Pattern

Cells of *C. vibrioforme* f. *thiosulfatophilum*, grown on sulfide without thiosulfate in the medium, contained the same three soluble cytochromes as they were found in cells which grew on thiosulfate as the main electron donor. We could not find a significant increase or decrease of the cytochrome concentration when sulfide was replaced by thiosulfate or vice versa, and the cytochromes (c-551 : c-553 : c-555) remained nearly in a constant ratio to each other (3.5 : 1 : 2.7 with thiosulfate and 4 : 1 : 1.7 with sulfide). Therefore, we concluded that in this organism the cytochrome formation was independent of the utilized electron donor.

Catalytic Activity of Flavocytochrome c-553

Both sulfur reductase assays (see "Methods") were used to find out whether flavocytochrome c-553 of *C. vibrioforme* f. *thiosulfatophilum* catalyzed the reduction of elemental sulfur to sulfide. When the method of Fukumori and Yamanaka (1979a) with reduced benzylviologen and dithionite at alkaline pH under anaerobic conditions was used, we found that benzylviologen was oxidized and that even sulfide was formed before flavocytochrome c-553 was added. Lyons and Nickless (1968) reported that under alkaline conditions dithionite was hydrolyzed into sulfide and sulfite. Therefore, it seemed that the formation of sulfide in this assay was of a purely chemical nature and that this assay was unsuitable for the measurement of an elemental sulfur reductase. Using the hydrogenase dependent assay of Fauque et al. (1979) we measured a consumption of 7 nmol H_2/min in the presence of 15 nmol flavocytochrome c-553 and 8.7 nmol H_2/min in the control, while Fauque et al. (1979) had a 250 – 300 fold higher activity using cytochrome c_3 of *Desulfovibrio desulfuricans* Norway 4 to catalyze the reduction of elemental sulfur. A comparison of these results showed that flavocytochrome c-553 of *C. vibrioforme* f. *thiosulfatophilum* was not an elemental sulfur reductase. On the other hand the flavocytochrome c-553 was immediately reduced after the addition of sulfide (40 µmol), and we have some indication that flavocytochrome c-553 may be a sulfide:cytochrome c reductase.

Rubredoxin and Ferredoxin

Besides the described three soluble cytochromes we also found the two non-heme iron proteins rubredoxin and ferredoxin in the soluble fraction. Rubredoxin showed nearly the same maxima at 370 nm, 485 nm and 570 nm in the oxidized form as they were recently described for rubredoxin of *C. limicola* strain 6330 (Steinmetz and Fischer 1981).

Anaerobic Sulfide and Thiosulfate Oxidation in a Growing Culture of C. vibrioforme f. *thiosulfatophilum*

For this experiment, *C. vibrioforme* f. *thiosulfatophilum* was cultivated at 28° C and 1,000 lux in a 5 l carboy in the medium described in "Methods", inoculated with 1 l cell suspension (0.5 mg protein/ml) and stirred magnetically. Samples of 10 – 15 ml were removed from the growing culture at different intervals of time for determination of sulfur compounds. Figure 6 shows that thiosulfate concentration increased until sulfide was completely consumed and that thiosulfate was the first main product of sulfide oxidation. The relatively high level of thiosulfate and sulfide in the beginning was dependent on the culture medium. The following decrease of thiosulfate was accompanied by a transiently accumulation of elemental sulfur, which was oxidized after all thiosulfate had been converted, indicating that *C. vibrioforme* f. *thiosulfatophilum* metabolized thiosulfate via elemental sulfur.

Discussion

In the thiosulfate-utilizing green sulfur bacterium *Chlorobium vibrioforme* f. *thiosulfatophilum* three soluble c-type cytochromes – cytochrome c-551, flavocytochrome c-553 and cytochrome c-555 – as well as two non-heme iron electron transfer proteins – bacterial ferredoxin and rubredoxin –

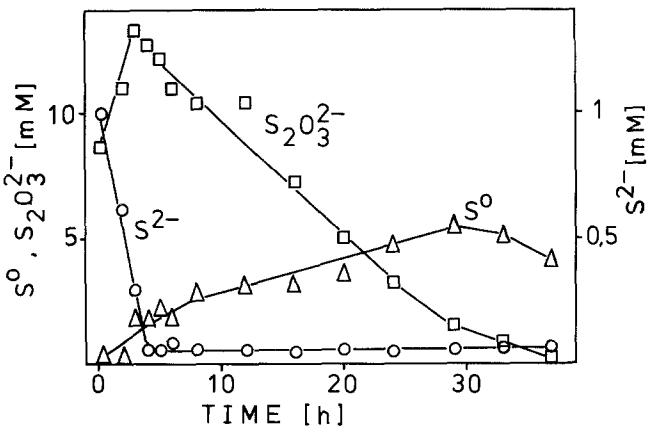

Fig. 6. Anaerobic sulfide and thiosulfate oxidation by *C. vibrioforme* f. *thiosulfatophilum* in a 5 l carboy (protein concentration = 0.1 mg/ml). For analytical methods see "Material and Methods". (□——□) Thiosulfate; (○——○) sulfide; (△——△) elemental sulfur

have been found. Table 1 summarizes some molecular properties of these three cytochromes and compares them with the corresponding cytochromes of *C. limicola* f. *thiosulfatophilum* and *C. limicola* strain 6330. In addition to the recently described two cytochromes (c-553 and c-555) of the non-thiosulfate-utilizing *C. limicola* strain 6330 (Steinmetz and Fischer 1981) *C. vibrioforme* f. *thiosulfatophilum* contains also cytochrome c-551. With the only exception that cytochrome c-551 of *C. vibrioforme* f. *thiosulfatophilum* possessed only one heme group per protein molecule instead of two, found for *C. limicola* f. *thiosulfatophilum* cytochrome c-551 (Meyer et al. 1968), all other properties were very similar. The discrepancy in molecular weights might depend on different estimation methods. In *C. limicola* f. *thiosulfatophilum* cytochrome c-551 served as the first endogenous electron acceptor in thiosulfate oxidation before the electrons were transported via cytochrome c-555 to bacteriochlorophyll (Kusai and Yamanaka 1973a, b). The occurrence of cytochrome c-551 only in the above mentioned thiosulfate-utilizing Chlorobiaceae indicates that indeed this cytochrome has an important function in electron transfer during thiosulfate metabolism. This finding is in good accordance with the result obtained from the non-thiosulfate-utilizing *C. limicola* strain 6330 where cytochrome c-551 is absent (Steinmetz and Fischer 1981). That special cytochromes or even electron transport chains are necessary for the oxidation of reduced sulfur compounds has been reported for thiobacilli as well as for phototrophic bacteria. Moriarty and Nicholas (1970) could show that the cytochromes b, c and d were involved in sulfide oxidation while the cytochromes b, c and a functioned in sulfite oxidation in *Thiobacillus concretivorus*. Appelt et al. (1979) described a soluble c-type cytochrome as electron acceptor in *Rhodopseudomonas palustris* participating in thiosulfate oxidation, while Knobloch et al. (1981) found a membrane-bound cytochrome c involved in this process in *Chromatium vinosum*. The latter report was in contrast to the result of Fukumori and Yamanaka (1979b), who found that soluble HIPIP of *Chromatium vinosum* was the most effective electron acceptor for the thiosulfate-oxidizing enzyme. These contrary findings could imply that i) two different independent electron transfer pathways for thiosulfate oxidation exist in *Chromatium vinosum* or ii) HIPIP and the membrane-bound cytochrome are parts of a single electron transport chain.

Table 1. Comparison of molecular properties of the soluble cytochromes of *Chlorobium vibrioforme* f. *thiosulfatophilum* with those of *Chlorobium limicola* f. *thiosulfatophilum* and *Chlorobium limicola* strain 6330

Organism and cytochromes	*Chlorobium limicola* f. *thiosulfatophilum*[a]			*Chlorobium vibrioforme* f. *thiosulfatophilum*[b]			*Chlorobium limicola* strain 6330[c] (c-551 not present)	
Molecular properties	c-551	c-553	c-555	c-551	c-553	c-555	c-553	c-555
Molecular weight	45,000	50,000	10,000	32,000	63,000	12,500	56,000	10,000
Redox potential	+135	+98	+145	+150	+90	+155	+65	+140[b]
Heme groups	2	1	1	1	1	1	1	1
Flavin group	–	FAD	–	–	FAD	–	FAD[b]	–
Isoelectric point	6.0	6.7	10.5	6.0	6.3	10–10.5	9.0	9.5–10
Purity index (A_{280}/γ-band) (ox = oxidized form)	0.39	0.94_{ox}	0.16	0.39	0.8	0.16	0.96	0.13_{ox}
Maxima (nm)								
Oxidized (γ-band)	410.5	410	412.5	410	410	412	410	412
Reduced (α- and γ-band)	551	553.5	555 (551)	551	553.5	555 (551)	553.5	555 (551)
	416	416.7	418.5	416	417	418	417	417.5

[a] Data from Meyer et al. (1968) and Bartsch (1978)
[b] Own results
[c] Data from Steinmetz and Fischer (1981)

Further studies must clarify which of the proposed ways is realized.

From the redox potential of +90 mV and the isoelectric point at pH 6.3 we think that there is a closer relationship between the two acidic flavocytochromes c-553 of the thiosulfate-utilizing strains of the Chlorobiaceae than to the basic flavocytochrome c-553 of *C. limicola* strain 6330. The flavocytochromes c-553 of the so far examined three strains of Chlorobiaceae were slowly autoxidized (Bartsch et al. 1968; Steinmetz and Fischer 1981) and showed great similarity with regard to the other properties listed in Table 1, while there were some differences to flavocytochrome c-552 of *Chromatium vinosum*, discussed in a previous paper by Steinmetz and Fischer (1981).

It seems that there is no unique function for flavocytochromes concerning sulfur metabolism in phototrophic bacteria. The flavocytochrome c-553 of *C. limicola* f. *thiosulfatophilum* (Kusai and Yamanaka 1973c) and *C. limicola* strain 6330 (Steinmetz and Fischer 1981) and *Chromatium vinosum* flavocytochrome c-552 (Yamanaka and Kusai 1976) are sulfide:cytochrome c reductases, while flavocytochrome c-550 of *Thiocapsa roseopersicina* shows adenylylsulfate reductase activity (Trüper and Rogers 1971), and sulfide oxidation is catalyzed by a thermostable cytochrome c-550 with elemental sulfur as reaction product (Fischer and Trüper 1977). Elemental sulfur reductase activity was also described for flavocytochrome c-552 of *Chromatium vinosum* by Fukumori and Yamanaka (1979a) indicating that this hemoprotein was able to catalyze also the reverse reaction of sulfide oxidation. To clarify the question whether flavocytochrome c-553 of *C. vibrioforme* f. *thiosulfatophilum* possessed elemental sulfur reductase activity or not, we used the method of Fukumori and Yamanaka (1979a) and found that sulfide was detectable even before flavocytochrome c-553 was added to the reaction mixture and that the formation of sulfide was a purely chemical reaction, because dithionite was hydrolyzed into sulfide and sulfite at alkaline pH (Lyons and Nickless 1968). Concerning the redox potentials of −240 mV for the reaction of elemental sulfur to sulfide and of +90 mV for flavocytochrome c-553 one cannot expect that this reaction is realized in vivo by this hemoprotein without being coupled to an energy producing process. This assumption is even supported by the fact that sulfide formation from elemental sulfur in *C. limicola* f. *thiosulfatophilum* is a light-dependent process (Paschinger et al. 1974). Regarding the redox potential, it is understandable that cytochromes c_3 of the sulfate-reducing bacteria are more qualified to catalyze elemental sulfur reduction on the basis of their extreme negative redox potentials of around −250 mV (Fauque et al. 1979). On the other hand, flavocytochrome c-553 of *C. vibrioforme* f. *thiosulfatophilum* is rapidly reduced by sulfide, so this hemoprotein may act as a sulfide:cytochrome c reductase.

Cytochrome c-555 of *C. vibrioforme* f. *thiosulfatophilum* is a small, basic and soluble cytochrome. From the molecular properties (see Table 1) it resembles very much the already described small cytochromes c-555 of *C. limicola* f. *thiosulfatophilum* (Gibson 1961; Meyer et al. 1968) and *C. limicola* strain 6330 (Steinmetz and Fischer 1981) and may have the same function in sulfur metabolism and electron transport, as it is proposed by Kusai and Yamanaka (1973 a, b, c). The authors could show that cytochrome c-555 of *C. limicola* f. *thiosulfatophilum* stimulated the reduction rate of cytochrome c-551 during thiosulfate oxidation and accepted the electrons from flavocytochrome c-553 (catalyzing sulfide oxidation) and cytochrome c-551 (endogenous acceptor of the thiosulfate oxidizing enzyme) before they were transferred to bacteriochlorophyll. Further studies must help to clear whether cytochrome c-555 of *C. vibrioforme* f. *thiosulfatophilum* has a similar stimulation effect and whether this organism possesses a thiosulfate oxidizing enzyme.

The same authors also reported that cells of *C. limicola* f. *thiosulfatophilum* grown only with sulfide in the medium,

contained more flavocytochrome c-553 than those cultivated with thiosulfate as the main electron source. This is in contrast to our results. When we did the same experiment with *C. vibrioforme* f. *thiosulfatophilum* we found that the ratio of cytochromes to each other was nearly the same, independent of the sulfur compound. On the basis of these contrary findings we suppose that perhaps different regulation mechanisms may be developed concerning the influence of sulfur compounds on cytochrome concentration and pattern in both thiosulfate-utilizing species of the Chlorobiaceae.

Bacterial ferredoxin and rubredoxin, two non-heme iron containing proteins, are also present in *C. vibrioforme* f. *thiosulfatophilum*. Oxidized rubredoxin had the same spectral properties as they were already mentioned for this protein from *C. limicola* f. *thiosulfatophilum* (Meyer et al. 1971) and *C. limicola* strain 6330 (Steinmetz and Fischer 1981), and it seems that rubredoxin is really a characteristic protein of the Chlorobiaceae among the phototrophic bacteria, as it has been proposed by Meyer et al. (1971). Its physiological function in sulfur or carbon metabolism is still unclear. The occurrence of cytochrome c-551, flavocytochrome c-553, and cytochrome c-555 in *C. vibrioforme* f. *thiosulfatophilum* coincides with the results of Meyer et al. (1968) for *C. limicola* f. *thiosulfatophilum*. Therefore it is possible that the proposed electron transport pathways during sulfide and thiosulfate oxidation in the latter organism (Kusai and Yamanaka 1973a, b, c) are also realized in the former. In connection with the functions of cytochromes in the oxidation of sulfide and thiosulfate one should also consider the anaerobic sulfur turnover by whole cells. In a preceding paper we reported that whole cells of *C. limicola* strain 6330 oxidized sulfide directly via elemental sulfur, but did not utilize the thiosulfate formed by a non-enzymatic reaction (Steinmetz and Fischer 1981). During anaerobic sulfide oxidation by *C. limicola* f. *thiosulfatophilum*, thiosulfate was the first main product, which appeared in the medium, while elemental sulfur concentration increased a short time later. Only when all sulfide had been consumed, elemental sulfur was further oxidized by the cells (Schedel 1978). We have also studied the anaerobic sulfide oxidation by a growing culture of *C. vibrioforme* f. *thiosulfatophilum* and agree with Schedel's findings (1978) that the first product formed is thiosulfate and that the thiosulfate concentration increases until sulfide is no longer available in the medium. The decrease of thiosulfate is accompanied by a constant increase of elemental sulfur, which is only oxidized, when all thiosulfate is consumed. This indicates that thiosulfate is converted via elemental sulfur by *C. vibrioforme* f. *thiosulfatophilum*. Further studies will help to elucidate, which enzymatic reactions are involved in elemental sulfur oxidation in green sulfur bacteria.

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