Arch Microbiol (1981) 130:334–338

Archives of
Microbiology

The Enzymatic System Thiosulfate: Cytochrome *c* Oxidoreductase from Photolithoautotrophically Grown *Chromatium vinosum*

Wolfgang Schmitt, Guido Schleifer, and Karl Knobloch

Institut für Botanik und Pharmazeutische Biologie der Universität Erlangen-Nürnberg, Schlossgarten 4, D-8520 Erlangen, Federal Republic of Germany

Abstract. *Chromatium vinosum* cells form a vesicular type intracytoplasmic membrane system during phototrophic growth on thiosulfate. – An enzyme protein transferring electrons from thiosulfate to cytochromes of type *c* was enriched from S-144. The colorless thiosulfate:cytochrome *c* oxidoreductase was characterized by a molecular weight of 36,000 (after dodecylsulfate treatment) and 35,000 (by gel filtration). Isoelectric focusing revealed a pI range of 4.4 to 4.7. Apparent K_m values for the cytochromes tested were in the μM range. – The endogenous electron acceptor compound, isolated from the chromatophore fraction P-144, was found to be a membrane-bound cytochrome *c*-552. The homogeneous cytochrome protein had an average pI value of 4.65 and a molecular weight of 71,500 determined by gel filtration. By dodecylsulfate electrophoresis it was cleaved into two proteins representing particle weights of 45,000 and 20,000.

Key words: Photosynthesis – *Chromatium vinosum* Thiosulfate – Cytochrome *c* – Oxidoreductase

Light and inorganic reduced sulfur are sufficient to provide the purple sulfur bacterium *Chromatium vinosum* as far as energy and electron sources are concerned. Metabolic products of thiosulfate oxidation have been investigated (Smith 1966; Smith and Lascelles 1966), and the in vivo reduction of a cytochrome *c*-552 by electron-donating substrates, like thiosulfate, was reported (cf. Sybesma 1970). The existence of an enzymatic system thiosulfate:cytochrome *c* oxidoreductase has been assumed (van Grondelle et al. 1977). A flavocytochrome *c*-552 acting as the enzymatic system which transferred electrons from the inorganic sulfur source, sulfide, to cytochromes of type *c* (from different origin) applied in in vitro experiments has been investigated (Fukumori and Yamanaka 1979a). An enzymatic system using thiosulfate has been described to be operative in the "non-sulfur purple bacterium" *Rhodopseudomonas palustris* (Schleifer et al. 1981).

Offprint requests to: K. Knobloch

Abbreviations: HiPIP, high potential nonheme iron protein; IEF, isoelectric focusing; SDS, dodecylsulfate, sodium salt; Temed, N,N,N′, N′-tetramethylethylenediamine

0302-8933/81/0130/0334/$01.00

In this paper, we report on a thiosulfate:cytochrome *c* oxidoreductase system isolated from the "sulfur purple bacterium" *C. vinosum*.

Materials and Methods

Chromatium vinosum ("strain D", ATCC 17899) was grown photolithoautotrophically on thiosulfate in 10 l carboys at 10,000 lux and 25° C in an inorganic medium according to Fuller (cf. Bose 1963). The cells were harvested at 4° C after 2 days of growth. About 58 g of cells (wet weight) were obtained from 40 l culture medium.

Protein Isolation and Purification

The preparation of the cell-free supernatant fraction S-144 containing the enzyme protein was as described previously (Schleifer et al. 1981). The different purification steps applied for further enrichment of the enzyme are given in Fig. 1.

The chromatophore fraction P-144 was resuspended and homogenized in 30 ml Tris-HCl buffer (0.05 M, pH 8.0). 150 ml cold acetone (−18° C) were poured quickly into the suspension followed by continuous stirring for 30 min at −18° C. Then, the chromatophore suspension was centrifuged for 15 min at 15,000 × *g* at −10° C (modified, cf. Bartsch 1971). The resulting "pellet 15" was flushed with nitrogen at −18° C to remove all traces of acetone, resuspended in Tris-HCl buffer (0.05 M, pH 8.0) and treated by ultrasonic oscillation (400 W) at 4° C in intervalls of 30 s (adding up to a total sonication time of 3 min). The protein solution then was centrifuged for 90 min at 144,000 × *g*. The resulting supernatant ("sup. 144") contained the acceptor cytochrome *c*. Further purification steps are shown in Fig. 2.

All steps of isolation and purification were carried out at 4° C, unless indicated specifically.

SDS Polyacrylamide Disc Gel Electrophoresis was performed according to the standard procedure described by Weber and Osborn (1969). In order to prepare an incubation at different temperature ranges, all samples were dissolved in sodium phosphate buffer (0.05 M, pH 7.1) containing 2% SDS and 2% mercaptoethanol; the incubation time for the different cytochrome samples was either (1) 12–16 h at room temperature or (2) 1 min at 60° C followed by 12–16 h at room temperature, or (3) 10 min at 60° C followed by 12–16 h at room temperature, and (4) 10 min at boiling temperature

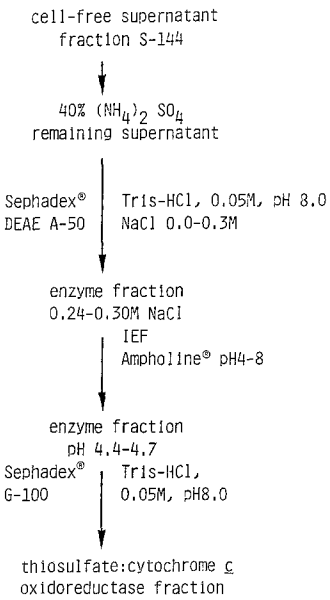

Fig. 1. Steps of enrichment and partial purification of the thiosulfate:cytochrome *c* oxidoreductase protein from *C. vinosum*

followed by 30 min at 60° C. Gel concentration was 12%, and 1.5% crosslinker was applied.

Other methods applied and chemicals used were described by Schleifer et al. (1981).

Results

The enzyme thiosulfate:cytochrome *c* oxidoreductase was part of the cell-free supernatant fraction S-144, whereas the electron acceptor protein, a cytochrome of type *c*, had to be isolated from the chromatophore membrane fraction P-144.

Oxidoreductase Protein

The enzyme fraction obtained after the purification steps given in Fig. 1 showed three main protein bands when tested by polyacrylamide disc gel electrophoresis. Comparative investigations by varying the purification procedure (Fig. 1) enabled us to identify one of these bands to be the enzyme.

For this enzyme protein a particle weight of 35,000 (gel filtration) and 36,000 (SDS electrophoresis) was obtained.

Preparative and analytical IEF revealed a pI value in the range of 4.4 to 4.7 for the enzymatic active protein.

Apparent K_m values obtained were in the μM range (Table 1).

The oxidoreductase protein was unstable with regard to increasing temperature. A treatment at 40° C for 10 min led to a decrease in activity of about 20%; treatment at 60° C for 10 min resulted in a loss of about 50% activity, and incubation at 80° C resulted in protein denaturation. – The addition of CN^-, SCN^-, SO_3^{2-} or urea at 0.1 to 0.5 mM concentrations did not inhibit the activity of the oxidoreductase protein.

Acceptor Cytochrome c

The homogeneity of cytochrome *c* was demonstrated by polyacrylamide disc gel electrophoresis which after staining with Coomassie blue revealed one single band (Fig. 3, I, A).

Table 1. Michaelis-Menten constants (K_m values) determined for the enzymatic system thiosulfate:cytochrome *c* oxidoreductase isolated from *C. vinosum*. – The data were obtained spectrophotometrically at 550 nm (horse heart cytochrome *c*) and at 552 nm (endogenous acceptor cytochrome *c* from *C. vinosum*) in 1 cm-cuvettes at room temperature. The sample and reference cuvettes in a total volume of 2.0 ml contained Tris-HCl buffer, 0.05 M, pH 8.0, various μM conc. of thiosulfate, mammalian cytochrome *c* (horse heart) in different μM conc. or corresponding amounts of *C. vinosum* acceptor cytochrome *c*, respectively. The reactions were started by adding the oxidoreductase protein containing fraction (protein amount ca. 0.5 mg)

Electron acceptor compound applied	Substrates (range of conc.s applied)	Calculated apparent K_m [μM] (average values from 3–4 experiments), method of calculation after	
		Woolf	Lineweaver-Burk
Horse heart cytochrome *c*	$S_2O_3^{2-}$ (40–400 μM)	6	26
	horse heart cyt. *c* (2.5–8.0 μM)	2	40
Endogenous *C. vinosum* cytochrome *c*	$S_2O_3^{2-}$ (0.25–1.0 μM)	0.4	0.2
	endogenous *C. vinosum* cyt. *c* (1–6 μM)	1.2	0.8

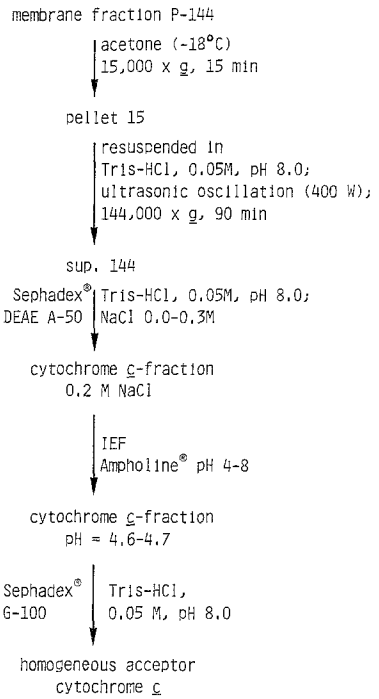

Fig. 2. Isolation and purification steps of the endogenous acceptor cytochrome *c* from *C. vinosum*

The "purity index" (A_{280}/A_γ; Bartsch 1971, 1978) resulted in a value of 0.6. Preparative and analytical IEF exhibited an average pI value of 4.65. Molecular weight determination on Sephadex G-100 led to an average value of 71,500. SDS

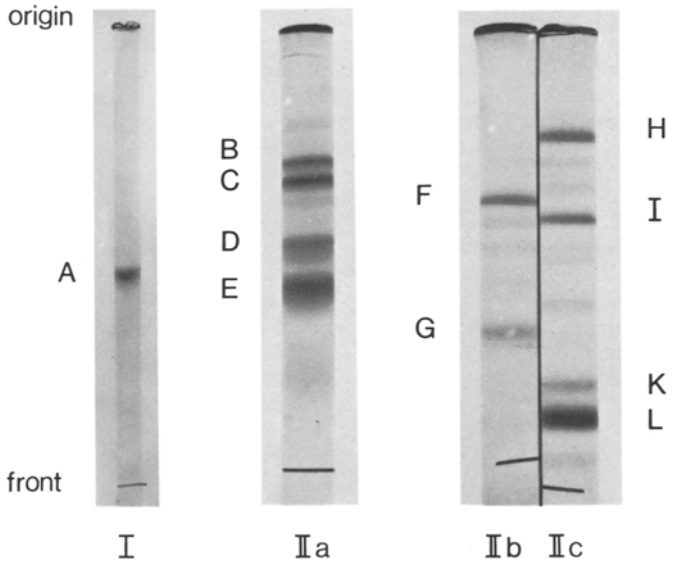

Fig. 3. I: Polyacrylamide (A) (conc.: gel 10%, crosslinker 1.5%, pH 8.9) and **IIa, b:** polyacrylamide SDS disc gel (B–G) (conc.: 12% gel, 1.5% crosslinker) electrophoresis of the purified acceptor cytochrome *c* from *C. vinosum*, **IIc:** protein standards in SDS (H: bovine serum albumin; I: aldolase; K: myoglobin; L: cytochrome *c*, horse heart). – The *C. vinosum* cytochrome's homogeneity is shown in I. After mild SDS treatment the cytochrome *c* reveals four (IIa) bands (B–E), whereas a longer treatment at higher temperatures results in two bands only (IIb) (F, G)

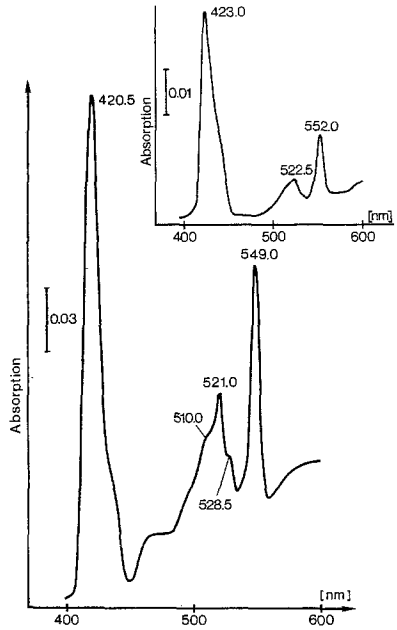

Fig. 4. Difference spectra of the purified cytochrome *c* from *C. vinosum*. The cytochrome was reduced with a few grains of dithionite and oxidized with air. Spectra were monitored with the two wavelength double beam spectrophotometer Perkin Elmer 356. The spectrum was recorded at 77 K, the insert at 293 K

electrophoresis after mild treatment resulted in four bands (Fig. 3, IIa). Their molecular weights were calculated to be close to 45,000 (B), 40,000 (C), 27,000 (D) and 20,000 (E). Treatment at higher temperature resulted in two protein bands only (Fig. 3, IIb), with average molecular weights of 45,000 (F) and 20,000 (G).

The dithionite-reduced minus oxidized difference absorption spectra are given in Fig. 4. The spectrum recorded at room temperature showed absorption maxima at 552.0, 522.5 and 423.0 nm. The absorption maxima obtained in liquid nitrogen at 77 K were seen at 549.0, 521.0 and 420.5 nm; in addition, the β-absorption was split into shoulders at 528.5 and 510.0 nm.

The absorption spectrum of the oxidized acceptor cytochrome *c* showed a γ-absorption at 408.0 nm; after reduction with dithionite, absorption maxima at 552.0, 521.0 and 415.5 nm were visible (Fig. 5).

The quantitative determination of the iron content of the acceptor cytochrome *c* by atomic absorption spectrophotometry resulted in 1.74 ppm iron when determined in a solution of the homogeneous acceptor cytochrome *c* which contained 1.35 mg protein per milliliter indicating the presence of 2 atoms of iron per cytochrome *c* molecule (71,500).

In search for a flavin component the reddish-yellow solution of the homogeneous acceptor cytochrome *c* was saturated with crystalline urea and kept at 4° C for 3 days. Elution of this solution with 8 M urea in Tris-HCl buffer (0.05 M, pH 8.0) from a Sephadex G-75 gel column, equilibrated in the identical buffer system (cf. Fukumori and Yamanaka 1979a), yielded two separated fractions. The first fraction obtained during elution showed a spectrum typical for a heme protein, in the reduced state revealing a γ-absorption peak at 408 nm. The second fraction exhibited an absorption maximum at 450 nm in the oxidized state; this

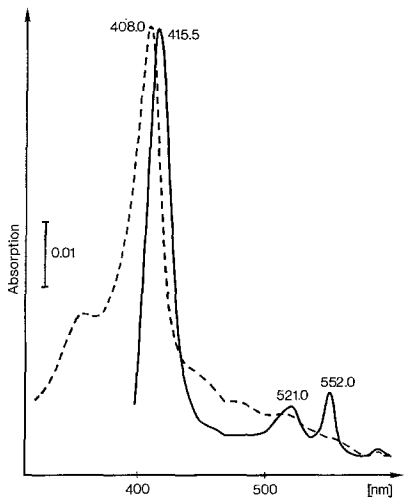

Fig. 5. Absorption spectra of the oxidized (*dashed line*) and reduced (*solid line*) acceptor cytochrome *c* from *C. vinosum*. The cytochrome in Tris-HCl buffer (0.05 M, pH 8.0) was oxidized with ammonium persulfate and reduced with sodium dithionite, and the spectra were recorded against Tris-HCl buffer as reagent blank

450 nm absorption disappeared after reduction by dithionite. – The spectrum of the homogeneous cytochrome *c* in the oxidized state revealed shoulders at 448 and 481 nm (Fig. 5). – The homogeneous cytochrome *c* (about 200 µg) when subjected to SDS electrophoresis was separated into two bands (see above) which remained visible in the unstained gel. One of the protein bands ("protein I", molecular weight about 20,000) was of reddish-brown color, the other ("protein II", molecular weight about 45,000) appeared al-

most colorless or slightly yellow. The protein I band, after elution from the gel, revealed a γ-absorption peak of a heme protein; the protein II band could not be characterized spectrophotometrically, a fact which could be explained by the SDS treatment (cf. Kennel and Kamen 1971 b).

Altogether nearly 10 mg of the enriched enzyme protein and about 6 mg of the homogeneous acceptor cytochrome *c* were isolated from 58 g washed cells harvested from 40 l suspension of photolithoautotrophically grown cells.

Discussion

Similar to the enzyme protein isolated from *Rhodopseudomonas palustris* (Schleifer et al. 1981) the thiosulfate:cytochrome *c* oxidoreductase from *Chromatium vinosum* could be enriched from S-144 (Fig. 1). The cytochrome of type *c* was isolated from P-144 (Fig. 2).

The different steps in protein isolation (Fig. 1) implied the solubility of the enzyme protein in spite of the fact that the presence of phospholipids had been proven in S-144 (Knobloch and Gemeinhardt 1979).

The 35,000 and 36,000 numbers for the molecular weight of the enzyme indicated that the protein resembled a single protein unit. The thiosulfate:cytochrome *c* oxidoreductase isolated from *R. palustris* consisted of two similar subunits (48,000 each) (Schleifer et al. 1981). – The enzyme in *Thiobacillus thioparus* was reported to represent 115,000 (Lyric and Suzuki 1970) and the corresponding protein from *Chlorobium thiosulfatophilum* (treated with SDS) yielded 80,000 (Kusai and Yamanaka 1973a, b).

We determined a pI range for the *C. vinosum* enzyme fraction of 4.4 to 4.7. The oxidoreductase protein from *R. palustris* (pI 5.45; Schleifer et al. 1981) and the one from *C. thiosulfatophilum* (pI 5.23) (Kusai and Yamanaka 1973a) exhibited similar pI values.

The K_m values for the *C. vinosum* enzyme (μM range) (Table 1) were at a similar level as reported for the *R. palustris* enzyme (Schleifer et al. 1981). Similar values were reported for the enzyme obtained from *T. thioparus* (Lyric and Suzuki 1970). For a HiPIP-reducing, thiosulfate-oxidizing enzyme from *C. vinosum* a K_m value of 130 μM was given, a K_m of 3.3 mM was obtained for ferricyanide, and horse heart cytochrome *c* was described to be a poor electron acceptor (Fukumori and Yamanaka 1979b).

The enzyme from *C. vinosum* was of a similar stability compared to the protein isolated from *R. palustris* (Schleifer et al. 1981).

We obtained some protein fractions which did reduce *c*-type cytochromes even in the absence of thiosulfate; however, just the enriched enzyme was able to reduce cytochrome *c* in vitro under both the following experimental conditions: (1) the reaction mixture contained thiosulfate plus cytochrome *c* and the reaction was started by adding the enzyme-containing fraction, (2) the reaction mixture contained cytochrome *c* plus the enzymatically active fraction and the reaction was started by adding thiosulfate.

The *C. vinosum* acceptor cytochrome *c* seems to be firmly attached to or embedded into the chromatophore membrane. We isolated the cytochrome from the chromatophores using cold acetone as a chemical solvent. A comparable method, using treatments with acetone plus cholate in order to extract a cytochrome *c*-556/552-complex from the chromatophore membranes of *C. vinosum* was reported, with no data given on its function (Kennel and Kamen 1971a).

A concentration of 0.04% β-mercaptoethanol reduced only the cytochrome *c*-556 of the above mentioned *c*-556/552-complex while the cytochrome *c*-552 remained in the oxidized state (Kennel and Kamen 1971b). If the same treatment was applied to our acceptor cytochrome *c*, no α-absorption at 556 nm was observed. The existence of a *c*-556 part accompanying the acceptor cytochrome *c* is unlikely.

The *C. vinosum* acceptor cytochrome, in a preparative as well as in an analytical IEF yielded two bands which were focused very close to each other. We interprete this fact by the assumption that the two bands represented different redox states of the same single protein.

The acceptor cytochrome *c* was characterized by a pI of 4.65 which is rather close to the pI value of 4.5 reported for a *C. vinosum* flavocytochrome *c*-552 (Bartsch 1978).

In our earlier experiments, mild treatment of the pure cytochrome by SDS in phosphate buffer led to the formation of four bands (Schmitt et al. 1980). We explain this as a partly incomplete separation of the protein since stronger treatment resulted in the formation of only two protein bands. Kennel and Kamen (1971b) describe three bands (MW: 45,000, 29,000, 23,000) for their cytochrome *c*-556/552-complex assuming the formation of aggregates by the smaller subunits; a particle weight of the same *c*-556/552-complex obtained under non-denaturating conditions is not reported.

Concerning the flavocytochrome *c*-552 from *C. vinosum* (Bartsch 1978), a flavoprotein subunit (particle weight 45,000) plus a cytochrome *c* subunit (particle weight 21,000) were described. Our experiments undertaken in order to dissociate the acceptor protein by treatment in 8 M urea resulted in two fractions with different spectra, one showing the typical γ-absorption of a heme component. The *C. vinosum* flavoprotein *c*-552 revealed an absorption maximum at 453 nm in the oxidized state; this absorption disappeared after reduction (Yamanaka et al. 1979; Fukumori and Yamanaka 1979a). We have observed a similar behavior for the second fraction obtained after the urea treatment. Specific absorption bands at 480 and 450 nm were described concerning the spectrum of an oxidized flavocytochrome *c*-552 from *C. vinosum* (Bartsch et al. 1968). The endogenous acceptor cytochrome *c*, purified during our experiments from *C. vinosum*, exhibited comparable spectral behavior at similar wavelengths (Fig. 5). Our data in comparison with those available from others seem to indicate that the acceptor cytochrome isolated from the *C. vinosum* chromatophores represents a flavocytochrome.

Bartsch (1978) described the *C. vinosum* *c*-552 flavocytochrome as a "soluble" protein which is trapped in vesicles formed during the disruption process and which could be released using acetone treatment. We report on a similar behavior of the acceptor cytochrome *c* in so far as its isolation requires an acetone treatment of the chromatophores. We have to state, however, that we are not able to isolate the cytochrome from the supernatant fraction S-144 which, besides the contents of both the cell compartments, holds membrane subunits plus the soluble proteins (cf. Knobloch and Gemeinhardt 1979).

It has been assumed for *C. vinosum* whole cells that electrons from thiosulfate are transferred to a mobile electron carrier represented by a cytochrome *c*-551 which couples with the oxidized chromatophore-bound *c*-type cytochromes (van Grondelle et al. 1977).

In our experiments in vitro we have described a soluble nonheme enzymatic system which transfers electrons to an

acceptor cytochrome *c*-552 isolated from the chromatophores from *C. vinosum*. Similar to the system from *R. palustris* (Schleifer et al. 1981) we were able to reconstitute the thiosulfate:cytochrome *c* oxidoreductase system in vitro using the purified components from *Chromatium*.

Acknowledgement. This work has been supported by the Deutsche Forschungsgemeinschaft.

References

Bartsch RG, Meyer TE, Robinson AB (1968) Complex c-type cytochromes with bound flavin. In: K Okunuki, MD Kamen, Y Sekuzu (eds) Structure and function of cytochromes. University of Tokyo Press, Tokyo, pp 443–451

Bartsch RG (1971) Cytochromes: Bacterial. In: A San Pietro (ed) Methods of enzymology, XXIII, A. Academic Press, New York, pp 344–363

Bartsch RG (1978) Cytochromes. In: RK Clayton, WR Sistrom (eds) The photosynthetic bacteria. Plenum Press, New York, pp 249–279

Bose SK (1963) Media for anaerobic growth of photosynthetic bacteria. In: H Gest, A San Pietro, LP Vernon (eds) Bacterial photosynthesis. The Antioch Press, Yellow Springs, pp 501–510

Fukumori Y, Yamanaka T (1979a) Flavocytochrome *c* of *Chromatium vinosum*. J Biochem 85:1405–1414

Fukumori Y, Yamanaka T (1979b) A high-potential nonheme iron protein (HiPIP)-linked, thiosulfate-oxidizing enzyme derived from *Chromatium vinosum*. Curr Microbiol 3:117–120

Grondelle R van, Duysens LNM, Wel JA van der, Wal HN van der (1977) Function and properties of a soluble *c*-type cytochrome *c*-551 in secondary photosynthetic electron transport in whole cells of *Chromatium vinosum* as studied with flash spectroscopy. Biochim Biophys Acta 461:188–201

Kennel SJ, Kamen MD (1971a) Iron-containing proteins in *Chromatium*. I. Solubilization of membrane-bound cytochrome. Biochim Biophys Acta 234:458–467

Kennel SJ, Kamen MD (1971b) Iron-containing proteins in *Chromatium*. II. Purification and properties of cholate-solubilized cytochrome complex. Biochim Biophys Acta 253:153–166

Knobloch K, Gemeinhardt F (1979) On the phospholipids and fatty acids in cell-free fractions from the Rhodospirillaceae *Rhodopseudomonas palustris* and *Rps. spheroides*. In: LA Appelquist, C Liljenberg (eds) Advances in the biochemistry and physiology of plant lipids. Elsevier/North-Holland Biomedical Press, Amsterdam

Kusai K, Yamanaka T (1973a) The oxidation mechanisms of thiosulphate and sulfide in *Chlorobium thiosulphatophilum:* Roles of cytochrome *c*-551 and cytochrome *c*-553. Biochim Biophys Acta 325:304–314

Kusai K, Yamanaka T (1973b) A novel function of cytochrome *c* (555, *Chlorobium thiosulfatophilum*) in oxidation of thiosulfate. Biochem Biophys Res Commun 51:107–112

Lyric RM, Suzuki I (1970) Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. III. Properties of thiosulfate-oxidizing enzyme and proposed pathway of thiosulfate oxidation. Can J Biochem 48:355–363

Schleifer G, Schmitt W, Knobloch K (1981) The enzymatic system thiosulfate : cytochrome *c* oxidoreductase from photolithoautotrophically grown *Rhodopseudomonas palustris*. Arch Microbiol 130:328–333

Schmitt W, Schleifer G, Knobloch K (1980) On the enzymatic system thiosulfate-cytochrome *c*-oxidoreductase from *Chromatium vinosum*. Isolation and purification of the acceptor cytochrome *c*. Hoppe-Seyler's Z Physiol Chem 361:1338

Smith AJ, Lascelles J (1966) Thiosulphate metabolism and rhodanese in *Chromatium* sp. strain D. J Gen Microbiol 42:357–370

Smith AJ (1966) The role of tetrathionate in the oxidation of thiosulphate by *Chromatium* sp. strain D. J Gen Microbiol 42:371–380

Sybesma C (1970) Photosynthetic bacteria. In: P Halldal (ed) Photobiology of microorganisms. Wiley-Interscience, London, pp 57–93

Weber K, Osborn M (1969) The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412

Yamanaka T, Fukumori Y, Okunuki K (1979) Production of subunits of flavocytochromes *c* derived from *Chlorobium limicola* f. *thiosulphatofilum* and *Chromatium vinosum*. Anal Biochem 95:209–213

Received January 2, 1981/Accepted September 8, 1981