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THE OXIDATION MECHANISMS OF THIOSULPHATE AND SULPHIDE IN *CHLOROBIUM THIOSULPHATOPHILUM*: ROLES OF CYTOCHROME *c*-551 AND CYTOCHROME *c*-553

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SUMMARY

A thiosulphate-cytochrome *c* reductase was highly purified from *Chlorobium thiosulphatophilum* and its properties were studied. The enzyme catalyses reduction with $Na_2S_2O_3$ of *c* cytochromes, including cytochrome *c*-551 of the bacterium. Cytochrome *c* (555, *C. thiosulphatophilum*) does not react directly with the enzyme at an appreciable rate but stimulates greatly the reduction by the enzyme of cytochrome *c*-551 with $Na_2S_2O_3$. The reduction of *c* cytochromes catalysed by the enzyme is strongly inhibited by cyanide and sulphite.

Cytochrome *c* (553, *C. thiosulphatophilum*), a *c*-type cytochrome with covalently bound flavin, was found to catalyse reduction with sulphide of *c* cytochromes, including cytochrome *c*-555. The reaction is strongly inhibited by cyanide. Cyanide seems to combine strongly with cytochrome *c*-553 probably at the flavin moiety. Thus, the absorption spectrum attributable to flavin of the haemoprotein is changed on addition of cyanide, and neither the original spectrum nor the activity reappears even after the cyanide-treated cytochrome has been subjected to gel filtration with a Sephadex G-25 column or to isoelectric focusing.

INTRODUCTION

Although *Chlorobium thiosulphatophilum* acquires energy and the reducing power necessary for its life processes by photosynthetic oxidation of $Na_2S_2O_3$ or Na_2S (ref. 1), very little is known about the enzymatic mechanisms in the oxidation of the sulphur compounds in the bacterium.

The bacterium possesses three kinds of *c*-type cytochromes, cytochromes *c*-551, *c*-553 and *c*-555 (ref. 2). As cytochrome *c*-555 resembles the *f*-type cytochrome in many respects[3], it seems to function in the organism in a similar way as the *f*-type cytochrome does in algae and higher plants. The functions of the other two kinds of cytochromes were obscure, although Mathewson *et al.*[4] have claimed that cytochrome *c*-551 is related to the oxidation of thiosulphate, as it is easily reduced with a crude extract of the organism on addition of thiosulphate while the other two *c*-type cytochromes are not. In the present investigation, we have succeeded in purifi-

cation of a thiosulphate-cytochrome *c* reductase and found that cytochrome *c*-551 functions as the electron acceptor for the enzyme.

Cytochrome *c*-553 has been highly purified by Meyer *et al.*[2] and found to have the covalently-bound flavin[5], but its function was unknown. We have found that cytochrome *c*-553 is a sulphide-cytochrome *c* reductase and that cytochrome *c*-555 functions as the electron acceptor for the cytochrome or enzyme. On the basis of these facts, the mechanisms in the photosynthetic electron transfer from $Na_2S_2O_3$ and Na_2S to $NAD(P)^+$ in the green sulphur bacterium are discussed. Some parts of the present investigation have been briefly reported[6].

MATERIALS AND METHODS

Materials

Cytochrome *c* (555, *C. thiosulphatophilum*)[3], cytochrome *c* (551, *Pseudomonas aeruginosa*)[7], and cytochrome *c* (550, *Thiobacillus novellus*)[8] were prepared by the methods established in our laboratory, and cytochromes *c*-551 and *c*-553 by the method of Meyer *et al.*[2]. Cytochrome *c* (550, *Saccharomyces oviformis*)[9] and cytochrome *c* (550, *Rhodospirillum rubrum*) (cytochrome c_2)[10] were kindly supplied by Sankyo Co., Ltd. (Tokyo, Japan), and by Dr T. Horio (Institute for Protein Research, Osaka University, Japan), respectively. Horse cytochrome *c* (Type VI), FMN and FAD were purchased from Sigma Chem. Co. (U.S.A.), and DEAE-cellulose from Serva Entwicklungslabor (Germany).

C. thiosulphatophilum

C. thiosulphatophilum (strain NCIB 8346), kindly supplied by Dr R. G. Bartsch (University of California, San Diego, U.S.A.) was cultivated in Larsen's medium for 5 days as described previously[3]. The harvested cells were stored at -20 °C until use.

Physical and chemical measurements

Determinations of spectral properties were performed at 20 °C in a Cary recording spectrophotometer, Model 14, 15 or 16, using cuvettes with a 1-cm light path. Protein contents were determined according to Lowry *et al.*[11]. Polyacrylamide gel (10%) electrophoresis in the presence of 1% sodium dodecyl sulphate and 0.5% mercaptoethanol was performed according to Wada and Snell[12], and the isoelectric point was determined by the isoelectric focusing method[13].

Purification of thiosulphate-cytochrome c reductase

Cells (50 g, wet wt) of *C. thiosulphatophilum* were suspended in 400 ml of 10 mM Tris–HCl buffer, pH 8.5, and the resulting suspension was poured into 2 l acetone which had been chilled at -5 °C. The mixture thus obtained was gently stirred for 10 min, and the cells collected by filtration and dried in air at room temperature. The acetone-dried cells (about 10 g) were suspended in 400 ml of 10 mM Tris–HCl buffer, pH 8.5 which was 10% saturated with $(NH_4)_2SO_4$. The resulting suspension was centrifuged at $12000 \times g$ for 20 min after standing overnight with continuous stirring. The supernatant thus obtained was fractionated with

$(NH_4)_2SO_4$; the precipitate formed between 45 and 65% saturation was collected by centrifugation and dissolved in 0.1 M Tris–HCl buffer, pH 8.5. The resulting solution was loaded on the DEAE-cellulose column (4 cm × 8 cm) which had been equilibrated with 0.1 M Tris–HCl, pH 8.5, after overnight dialysis against the same

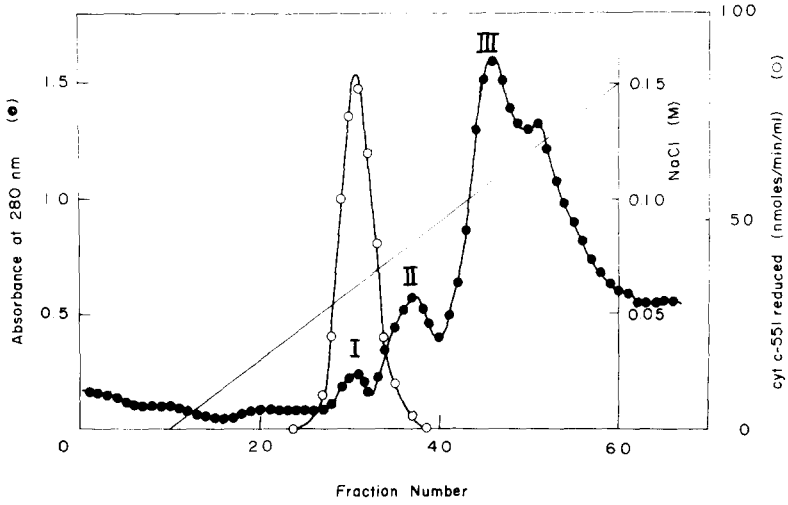


Fig. 1. Elution pattern of thiosulphate-cytochrome *c* reductase during chromatography on a DEAE-cellulose column. The eluate of Peaks I, II and III seen in the curve based on the absorbance at 280 nm contained the enzyme, cytochrome *c*-553 and cytochrome *c*-551, respectively.

buffer as used for the chromatography. After the column on which the enzyme was adsorbed was washed with 200 ml of the same buffer, the enzyme was eluted by the linear gradient solution which was produced from 200 ml each of 0.1 M Tris–HCl buffer, pH 8.5 and 0.1 M Tris–HCl buffer, pH 8.5 containing 0.2 M NaCl. Fractions of 4 ml were collected. The enzyme eluted at the NaCl concentration of 50–70 mM (Fig. 1). This chromatography step was repeated once and the main fraction thus obtained was used as the enzyme preparation.

RESULTS

Oxidation of thiosulphate

General properties of thiosulphate-cytochrome c reductase. The absorption spectrum of the enzyme preparation had only one peak at 280 nm. Therefore, it seems that the enzyme does not possess flavin or haem. Its molecular weight was determined to be 80000 by polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulphate and 0.5% mercaptoethanol. When the enzyme preparation was subjected to isoelectric focusing, a single peak was obtained in each of the elution curves determined both on the basis of the protein content and the activity. The two peaks obtained above were located at the same elution volume. The isoelectric point was determined to be at pH 5.23.

Reduction of cytochrome c-551 by thiosulphate-cytochrome c reductase. The enzyme reduced cytochrome *c* (551, *C. thiosulphatophilum*) at an appreciable rate

in the presence of $Na_2S_2O_3$. The reduction of cytochrome *c*-551 was greatly accelerated on addition of a small amount of cytochrome *c* (555, *C. thiosulphatophilum*), while cytochrome *c*-555 was hardly reduced with the enzyme unless cytochrome *c*-551 was added.

TABLE I

Effect of cytochrome *c*-555 on the reduction of cytochrome *c*-551 by thiosulphate-cytochrome *c* reductase with $Na_2S_2O_3$. The complete reaction mixture contained 0.1 M phosphate buffer, pH 6.0, 10 mM $Na_2S_2O_3$, 40 μM cytochrome *c*-551, 2.0 μM cytochrome *c*-555, and 1.1 μM enzyme in a total volume of 1.0 ml. The reactions were started by adding the enzyme and performed at 20 °C. The increase in the absorbance at 551 nm was followed spectrophotometrically with time.

Compounds omitted	*Cytochrome c-551 reduced (nmoles/min)*
None	16.7
Cytochrome *c*-555	4.1
Enzyme	1.1
$Na_2S_2O_3$	0.0
Cytochrome *c*-551	0.0

The reduction rate of cytochrome *c*-551 by the enzyme increased proportionally with the amount of cytochrome *c*-555 added while the molar ratio of the latter cytochrome to the enzyme was less than unity (Fig. 2). The acceleration effect of cytochrome *c*-555 was observed regardless of its redox states, while the cytochrome

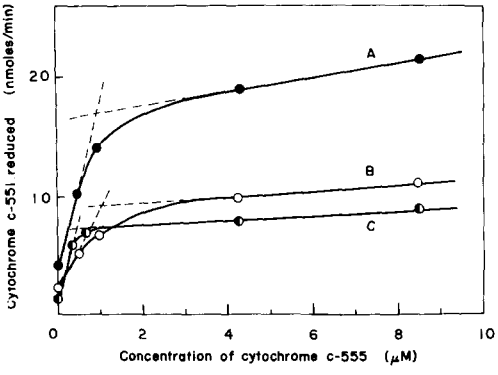


Fig. 2. Effect of cytochrome *c*-555 on the reduction of cytochrome *c*-551 with $Na_2S_2O_3$ catalysed by thiosulphate-cytochrome *c* reductase. The reaction mixture contained 0.1 M phosphate buffer, pH 6.0, 10 mM $Na_2S_2O_3$, 40 μM (A, C) or 20 μM (B) cytochrome *c* (551, *C. thiosulphatophilum*), and 1.1 μM (A, B) or 0.55 μM (C) enzyme in a total volume of 1.0 ml. The reactions were performed at 20 °C.

which had been treated with 5.0 M guanidine–HCl for 2 days at 20 °C was half as effective as the intact cytochrome. As shown in Table II, several *c*-type cytochromes were examined to test if they showed the acceleration effect on the reduction of cyto-

chrome *c*-551 by the enzyme. Cytochrome *c* (550, *R. rubrum*), cytochrome *c* (551, *P. aeruginosa*), cytochrome *c* (550, *T. novellus*), cytochrome *c* (550, *S. oviformis*) and horse cytochrome *c* showed the acceleration effect when each cytochrome *c* was used in place of cytochrome *c* (555, *C. thiosulphatophilum*), but none of them were so effective as cytochrome *c*-555. Cytochromes *c* (553, *C. thiosulphatophilum*) was not reduced by the enzyme with thiosulphate even in the presence of cytochrome *c*-555, nor did the cytochrome affect the thiosulphate-cytochrome *c*-551 reduction catalysed by the enzyme.

So far as tested, only thiosulphate was utilized by the enzyme; the enzyme did not act on sulphite, tetrathionate or dithionate with cytochromes *c*-551 and *c*-555 as the electron acceptor system. The thiosulphate-cytochrome *c*-551 reaction catalysed by the enzyme showed the highest activity at pH 6.0 in 2-(*N*-morpholino)ethanesulphonate buffer. The K_m value of the enzyme for thiosulphate was 1.7 mM. The enzyme did not show a rhodanese activity. The thiosulphate-cytochrome *c*-551 reduction catalysed by the enzyme was strongly inhibited by sulphite and cyanide (Table III). Addition of FAD or FMN (final concn., 1 μM), or metal ions such as Cu^{2+}, Zn^{2+}, Co^{2+} or Mn^{2+} (final concn., 1 μM) did not affect the reaction rate.

TABLE II

Acceleration effect of several kinds of *c* cytochromes on thiosulphate-cytochrome *c* reductase. The reaction mixture contained 0.1 M phosphate buffer, pH 6.0, 10 mM $Na_2S_2O_3$, 40 μM cytochrome *c*-551, 1.1 μM enzyme, and 2.0 μM each of various kinds of *c* cytochromes in a total volume of 1.0 ml. The reactions were performed at 20 °C and the increase in the absorbance at 551 nm was followed spectrophotometrically with time.

Source of cytochrome c	*α-Peak (nm)*	*Cytochrome c-551 reduced (nmoles/min)*
C. thiosulphatophilum	555	16.7
R. rubrum	550	8.0
P. aeruginosa	551	4.5
T. novellus	550	8.0
S. oviformis	550	6.0
Horse	550	6.5

TABLE III

Effects of sulphite and cyanide on the reduction of cytochrome *c*-551 catalysed by thiosulphate-cytochrome *c* reductase with $Na_2S_2O_3$. The conditions for activity determination were the same as for Table I except that sulphite or cyanide was added at the concentration indicated.

Compounds added	*Concn (mM)*	*Cytochrome c-551 reduced*	*Inhibition (%)*
None		16.7	0
Na_2SO_3	0.1	3.3	80
Na_2SO_3	0.05	6.7	60
KCN	0.1	8.9	47

TABLE IV

Reduction of cytochrome *c*-555 by thiosulphate-cytochrome *c* reductase with $Na_2S_2O_3$ in the presence of cytochrome *c*-551. The complete reaction mixture contained 0.1 M phosphate buffer pH 6.0, 10 mM $Na_2S_2O_3$, 40 μM cytochrome *c*-555, 4.5 μM cytochrome *c*-551 and 1.1 μM enzyme in a total volume of 1.0 ml. The reaction was performed at 20 °C and the increase in the absorbance at 555 nm was followed spectrophotometrically with time.

Compounds omitted	*Cytochrome c-555 reduced (nmoles/min)*
None	4.7
None*	2.4
Cytochrome *c*-551	0.35
Enzyme	0.35
$Na_2S_2O_3$	0.00
Cytochrome *c*-555	0.00

* Concentration of cytochrome *c*-551 was 2.3 μM.

TABLE V

Reduction of several kinds of *c*-type cytochromes by Na_2S and effect on their reduction rates of added cytochrome *c*-553. The reaction mixture contained 0.1 M Tris–HCl buffer, pH 7.4, 50 μM Na_2S and 50 μM each *c*-type cytochrome with (+) or without (−) 34 nM cytochrome *c*-553 in a total volume of 1.0 ml. The reaction was started by adding Na_2S and performed at 20 °C. The increase in the absorbance at the α-peak of each cytochrome was spectrophotometrically followed with time.

Cytochromes	*Additon of cytochrome c-553*	$\Delta A_\alpha/min$
Cytochrome *c*-551	−	0.18
	+	0.18
Cytochrome *c*-553		>4.0
Cytochrome *c*-555	−	0.02
	+	0.20
Yeast cytochrome *c*	−	0.15
	+	0.45

Reduction of cytochrome c-555. As already mentioned, cytochrome *c*-555 was hardly reduced directly by thiosulphate-cytochrome *c* reductase. However, on addition of a small amount of cytochrome *c*-551 to the reaction mixture, cytochrome *c*-555 was rapidly reduced by the enzyme with thiosulphate (Table IV). Cytochrome *c* (550, *S. oviformis*) was also fairly rapidly reduced by the enzyme in the presence of cytochrome *c*-551.

Oxidation product of thiosulphate. After the thiosulphate oxidation reaction by the enzyme had been performed with yeast cytochrome *c* (480 μM) as the electron acceptor, the reaction mixture was examined by paper chromatography to determine if polythionates were produced[14]. Polythionate such as dithionate, trithionate or tetrathionate was not detected.

Oxidation of sulphide

Reaction of cytochrome c-553 with sulphide. Cytochrome *c* (553, *C. thiosulphatophilum*) has the covalently-bound flavin (probably, FMN)[5]. As Table V shows, cytochrome *c*-553 was reduced by sodium sulphide quite more rapidly than cytochrome *c*-551, cytochrome *c*-555 and yeast cytochrome *c*. Cytochrome *c*-553 was reduced both at the flavin and haem moieties as judged from the change in the absorption spectrum. The reduction rates of cytochrome *c*-555 and yeast cytochrome *c* by sulphide were greatly accelerated on the addition of a small amount of cytochrome *c*-553. The reduction of cytochrome *c*-551 with sulphide was not affected on addition of cytochrome *c*-553, even at the concentration of 7 μM.

The reduction rate of yeast cytochrome *c* with sulphide catalysed by cytochrome *c*-553 was proportional to the concentration of cytochrome *c*-553 when the concentration of this haemoprotein was less than about 50 μM. When cytochrome *c*-553 was heated at 80 °C for 2 min, it completely lost its catalytic activity. The sulphide-cytochrome *c* reduction catalysed by cytochrome *c*-553 was strongly inhibited by cyanide. Atebrin was also an inhibitor of the reaction but much less effective than cyanide. Neither FAD nor FMN activated the reaction. When yeast cytochrome *c* was used as the electron acceptor for cytochrome *c*-553 with a limited amount of sulphide, 2 moles of cytochrome *c* were reduced per mole of sulphide added after a prolonged reaction, suggesting that the oxidation product of sulphide catalysed by cytochrome *c*-553 was elementary sulphur.

Effect of cyanide on absorption spectrum of cytochrome c-553. It has already been indicated by Bartsch *et al.*[5] that cytochrome *c*-553 does not react with CO. As the sulphide-cytochrome *c* reduction catalysed by cytochrome *c*-553 was strongly inhibited by the presence of cyanide at very low concentration, the effect of cyanide on the absorption spectrum of the haemoprotein was examined. When cyanide was

TABLE VI

Effect of inhibitors on the reduction of yeast cytochrome *c* with Na_2S catalysed by cytochrome *c*-553. The reaction mixture contained 0.1 M Tris–HCl buffer, pH 7.4, 50 μM yeast cytochrome *c*, 50 μM Na_2S, 35 nM cytochrome *c*-553 and various compounds at the concentration indicated in a total volume of 1.0 ml. The reaction was performed at 20 °C and the increase in the absorbance at 550 nm was spectrophotometrically followed with time.

Compounds added	*Concn* (μM)	$\Delta A_{550\ nm}/min$*	*Inhibition* (%)
None		0.300	0
Enzyme heated (80 °C, 2 min)		0.000	100
Atebrin	100	0.120	60
Cyanide	1.0	0.060	80
Cyanide	0.1	0.150	50
EDTA	100	0.300	0
o-Phenanthroline	100	0.300	0
NaN_3	10^4	0.280	7
CO	(1 atm)	0.300	0

* The figures in this column are the net increase in the absorbance obtained by subtracting the increase in the absence of the enzyme from each direct reading.

added to the solution of ferricytochrome *c*-553, its colour changed rapidly from brownish red to dark brown and its absorption spectrum was evidently affected; the absorbance at the peaks or shoulders at 480 and 450 nm which are thought to be attributable to the bound flavin[5] decreased, while that between 600 and 700 nm increased (Fig. 3). This spectral change was very rapid and remained even after

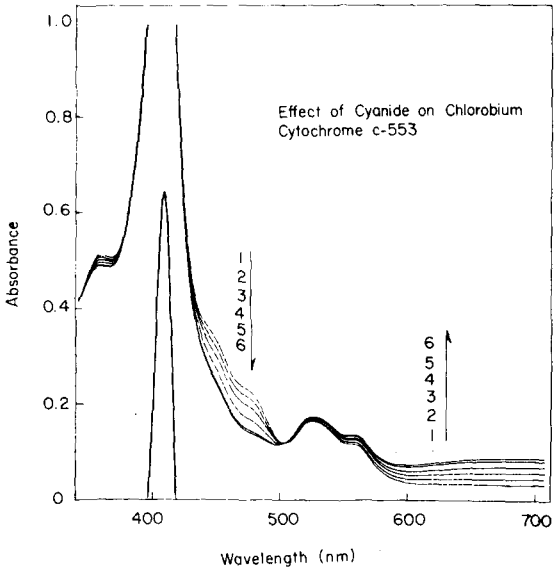


Fig. 3. Effect of cyanide on the absorption spectrum of ferricytochrome *c*-553. The cytochrome was dissolved in 0.1 M Tris–HCl, pH 7.4, at the concentration of 13 μM and the concentrations of cyanide added were; (1) 0 M, (2) 3 μM, (3) 6 μM, (4) 9 μM, (5) 12 μM, and (6) 15 μM.

addition of $K_3Fe(CN)_6$ to the solution of the cyanide-treated cytochrome, overnight dialysis against a large amount of a buffer, passage of the cyanide-treated cytochrome through a Sephadex G-25 column, or isoelectric focusing. The ferricytochrome *c*-553 preparation which had been treated with cyanide and then passed through a Sephadex G-25 column showed the same spectrum as the cyanide-treated ferricytochrome *c*-553, and it was one-third as active as the intact cytochrome *c*-553 in the sulphide-cytochrome *c* reduction. When 1 mM $HgCl_2$ was added to the cyanide-treated ferricytochrome *c*-553 (34 μM) and the mixture passed through a Sephadex G-25 column, the cytochrome *c*-553 preparation thus obtained showed the same absorption spectrum and enzymatic activity as the intact haemoprotein.

As already mentioned, ferricytochrome *c*-553 was quite rapidly reduced with sulphide. In the presence of cyanide, the reduction rate of cytochrome *c*-553 with sulphide greatly decreased. When 20 μM Na_2S was added to 7 μM cytochrome *c*-553 in the presence of 10 μM KCN, the reduction rate was about one-fifth of that in the absence of cyanide. When cytochrome *c*-553 was titrated with cyanide, the absorbance at 450 nm decreased in parallel with the sulphide-cytochrome *c* reductase activity.

The absorption spectrum of the cyanide-treated ferricytochrome *c*-553 was not changed after it was subjected to isoelectric focusing and its isoelectric point was at

pH 7.1; the isoelectric point of ferricytochrome *c*-553 did not change after treatment with cyanide, although Bartsch *et al.*[5] have reported that the isoelectric point of the cytochrome is at pH 6.7.

Reaction of cytochrome c-553 with cyanide and the redox states of the cytochrome. When a small amount of sulphide was added to ferricytochrome *c*-553 in the presence of cyanide, the cytochrome was reduced slowly and finally completely. The resulting reduced cytochrome became oxidized by autoxidation during prolonged standing. The ferricytochrome *c*-553 thus obtained showed the same absorption spectrum as the starting cyanide-treated ferricytochrome *c*-553. When the cyanide-treated ferricytochrome *c*-553 was reduced on addition of sulphide and then treated with the Sephadex G-25 column, the cytochrome preparation thus obtained, which was oxidized by the autoxidation, showed the same absorption spectrum and enzymatic activity as those of the intact cytochrome *c*-553.

DISCUSSION

Thiosulphate is oxidized by the action of a thiosulphate-cytochrome *c* reductase and electrons are transferred to cytochrome *c*-551. The overall reaction is greatly accelerated by participation of cytochrome *c*-555 which is not directly reduced by the thiosulphate-cytochrome *c* reductase with thiosulphate. The acceleration effect of cytochrome *c*-555 on the thiosulphate-cytochrome *c* reduction increases with the amount of the cytochrome added until the molar ratio of the cytochrome to the enzyme reaches unity. These facts seem to mean that the enzyme and cytochrome *c*-555 may make a functional complex. As cytochrome *c*-555 is not reduced by the enzyme, it seems unlikely that the cytochrome mediates the electrons between cytochrome *c*-551 and the enzyme by the valency change of its haem iron. The cytochrome may act as an effector for the enzyme, although there may be the possibility that the cytochrome is required for elimination of the reaction product.

Cytochrome *c*-555 is not directely reduced by thiosulphate-cytochrome *c* reductase but is reduced rapidly in the presence of a small amount of cytochrome *c*-551, while cytochrome *c*-551 reacts slowly with the enzyme even in the absence of cytochrome *c*-555. Therefore, it seems that electrons from the enzyme are first transferred to cytochrome *c*-551 and then to cytochrome *c*-555, while cytochrome *c*-555 participates in the reduction of cytochrome *c*-551 catalysed by the enzyme in some fashion.

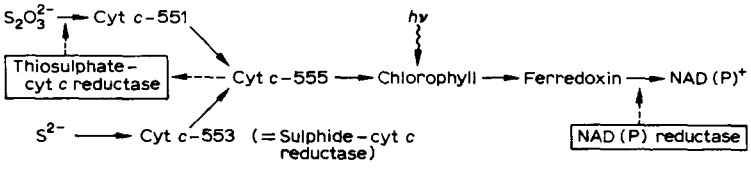
The enzyme does not show a rhodanese activity, and sulphite is not oxidized by the enzyme with cytochromes *c*-551 and *c*-555 as the electron acceptor system but inhibits strongly the reaction catalysed by it. Therefore, it seems unlikely that sulphite occurs as the reaction intermediate. However, so far as tested, polythionates are not detected as the reaction product in the thiosulphate-cytochrome *c* reduction catalysed by the enzyme.

The cytochrome *c*-553 molecule has one molecule of the covalently-bound flavin, probably FMN (ref. 5). The absorption peaks or shoulders at 450 and 480 nm are attributable to the bound flavin. A similar cytochrome with the covalently-bound flavin, cytochrome *c*-552 is obtained from *Chromatium* D (ref. 5). Their function was not elucidated.

In the present investigation, cytochrome *c*-553 is found to catalyse the re-

duction with sulphide of cytochrome *c*-555 and yeast cytochrome *c*. The catalytic activity is lost by heating the cytochrome and is inhibited by cyanide and atebrin. Therefore, cytochrome *c*-553 is an enzyme, sulphide-cytochrome *c* reductase. Cytochrome *c*-552 of *Chromatium* will probably show the similar activity, because its molecular features resemble those of cytochrome *c*-553. In our preliminary study, *C. thiosulphatophilum* cells cultivated in the medium where all the thiosulphate was replaced by sulphide contained more cytochrome *c*-553 than those cultivated in the medium where thiosulphate was the major electron donor to the organism.

Cytochrome *c*-555 is very similar to the *f*-type cytochrome of algae in various respects[3]. Therefore, it seems to act just as the *f*-type cytochrome does in algae although its midpoint redox potential is fairly low as compared with that of the latter type of cytochrome[2,15]. Thus, cytochrome *c* (555, *Chloropseudomonas ethylica*) has been found to be immediately oxidized *in vivo* by illumination of the cells[16]. In our previous study, we have elucidated the reduction mechanisms of pyridine nucleotide in *C. thiosulphatophilum* with a purified preparation of an NAD(P) reductase[17]; $NAD(P)^+$ is reduced by the enzyme in a quite similar way as the reduction of the pyridine nucleotide in higher plants and algae. On the basis of the facts described above, the enzymatic mechanisms of the photosynthetic pyridine nucleotide reduction in *C. thiosulphatophilum* with thiosulphate and sulphide as the electron donor will be presented as follows:



where chlorophyll means *Chlorobium* chlorophyll and/or bacteriochlorophyll.

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