Characterization of Cytochromes in Lithotrophically and Organotrophically Grown Cells of *Thiobacillus* A2

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The dominant cytochrome in thiosulphate-grown *Thiobacillus* A2 was found to be of the *c*-type with a reduced α -band at 548 nm (c_{548}). This c_{548} component did not constitute an integral part of the membrane carrier system. It did, however, appear to be part of a large complex not tightly bound to membranes. Reconstitution experiments showed that cytochromes of the membrane 'abc' system could be reduced by the c_{548} component and *vice versa*. The reduction of membrane cytochromes of either lithotrophic or organotrophic origin by thiosulphate electrons was achieved, but it required the presence of a soluble fraction containing cytochrome c_{548} . Evidence tending to rule out a reductive cleavage as the first step of the thiosulphate oxidation pathway in *Thiobacillus* A2 was obtained by following the reduction of partially purified c_{548} particles under various conditions.

INTRODUCTION

A seemingly general characteristic of lithotrophic micro-organisms, including the thiobacilli, is their strikingly high cytochrome content (Smith & Hoare, 1977; Tonge *et al.*, 1974; Charles, 1971; Tikhonova *et al.*, 1967; Aleem, 1965; Trudinger, 1961). A case in point is *Thiobacillus* A2. Growth of this facultatively chemolithotrophic bacterium on thiosulphate induces much higher levels of cytochrome *c* than does corresponding culture on an organic substrate (Taylor & Hoare, 1969; Kula *et al.*, 1975; Loya *et al.*, 1982). In contrast to other cellular cytochromes, synthesis of the cytochrome c_{548} component specifically associated with lithotrophic cells does not resume after adaptation of such cells to organotrophic growth on succinate (Loya *et al.*, 1982). Direct evidence for participation of cytochrome c_{548} in the lithotrophic energy metabolism of *Thiobacillus* A2 is presented below.

METHODS

Growth of bacteria and preparation of extracts. Growth of aerobic cultures of *Thiobacillus* A2N on sodium thiosulphate (20 mM) or sodium succinate (20 mM) has been described elsewhere (Loya *et al.*, 1982). Cells harvested from thiosulphate cultures (at densities of about 5×10^7 ml⁻¹) are referred to as 'lithotrophic cells', while those recovered from succinate cultures (at densities of about 5×10^8 ml⁻¹) are designated 'organotrophic cells'. Harvested cells, washed twice with potassium phosphate buffer (100 mM, pH 7.0) containing 1 mM-MgCl₂ and suspended in the same buffer at densities up to 500-fold greater than in the original culture, were disrupted by sonication (Loya *et al.*, 1982). After centrifugation of sonicates at 20000 *g*, 4 °C for 30 min, the resulting 20000 *g* supernatant, designated the 'crude extract', was either stored frozen at -40 °C or further fractionated by centrifugation at 144000 *g*, 4 °C for 2 h (Loya *et al.*, 1982). The resultant 144000 *g* supernatant and pellet (the latter resuspended in 1 vol. phosphate/magnesium buffer), referred to as the 'soluble' and 'membrane' fraction respectively, were also stored frozen at -40 °C (Loya *et al.*, 1982).

Gel chromatography. Wet beads of Sepharose 6B (Pharmacia), suspended in phosphate buffer (100 mM, pH 7.0) were packed in a glass column (1.8 cm diam.) to a height of 95 cm. The void volume of the column, determined with Blue Dextran 2000 (Pharmacia) was 77 ml. Apparent particle weights of components eluted from the column were estimated by reference to the elution of the markers bovine serum albumin (Sigma, mol. wt 63000) and horse heart cytochrome *c* (Sigma, mol. wt 13000). A sample of 25.9 mg soluble protein in 2.0 ml phosphate/magnesium

buffer was loaded and eluted (with phosphate buffer) at a flow rate of 15 ml h⁻¹. All operations were performed at 4 °C and fractions of 3.2 ml collected. The relative protein and cytochrome content of each fraction was estimated by measuring the absorbance at 280 and 410 nm, respectively.

Spectrometry. Absolute absorption spectra of samples containing 2–12 mg extract protein in 0.5–2.0 ml phosphate/magnesium or phosphate buffer (see above) were recorded from 650 to 500 nm in a single-beam spectrophotometer linked to a minicomputer (Loya *et al.*, 1982). Reduced minus oxidized difference spectra were computer-generated from the absolute spectra of the reduced and oxidized samples. Reduction was achieved with dithionite, succinate or thiosulphate. In the last case, thiosulphate oxidizing activity was measured by following the reduction kinetics of cytochrome *c* endogenous to the sample tested. The reaction was started by addition of reductant and scans were repeated at timed intervals until further rises in α -band peak heights were no longer observed. The time required for maximal cytochrome reduction by thiosulphate, a function of sample protein content, was routinely 30–45 min.

RESULTS

Chemical reduction of cytochromes in cell-free extracts

The dithionite-reduced minus oxidized difference spectrum of a crude extract from lithotrophic cells (Fig. 1, trace A) was similar to that previously obtained with intact cells (Loya *et al.*, 1982). Based on peak location, the presence of a *c*-type cytochrome with an α -band at 548 nm (cytochrome *c*₅₄₈), and of a cytochrome oxidase of the *a*-type with an α -band at 603 nm (cytochrome *a*₆₀₃) was indicated. Following high-speed centrifugation (144 000 *g*, 2 h) cytochrome *c*₅₄₈ was found almost exclusively in the high-speed supernatant (Fig. 1, trace B). In contrast, cytochrome oxidase was confined to the pellet (Fig. 1, trace C). In the absence of the masking effect of cytochrome *c*₅₄₈, two additional cytochromes were resolved in the pellet fraction on the basis of their α -bands: an apparent *c*-type cytochrome with an α -band at 550 nm (cytochrome *c*₅₅₀), and an apparent *b*-type cytochrome with an α -band (a shoulder in the spectrum) around 560 nm (cytochrome *b*₅₆₀; Fig. 1, trace C). The membrane-associated cytochrome *c*₅₅₀ represented about 16% of total cytochrome *c* content in lithotrophic cells (Table 1).

The dithionite-reduced minus oxidized difference spectrum of a crude extract from organotrophic cells was markedly different from that of a corresponding preparation from lithotrophic cells. It did, however, resemble the spectrum of the lithotrophic membrane fraction. Based on specific absorbance at peak wavelength, the concentration of *c*-type cytochromes was 10-fold lower in succinate-grown cells than in thiosulphate-grown cells (Table 1). Furthermore, the absorption maximum of the major *c*-type cytochrome in succinate-grown cells was 550 nm rather than 548 nm. Indeed, the major *c*-type cytochrome in succinate-grown cells was spectrally similar to the *c*-type cytochrome found in the pellet fraction of thiosulphate-grown cells. In addition to the absorption band at 550 nm observed in the reduced minus oxidized difference spectrum of crude extract from organotrophic cells, there were at least three additional reduced α -bands; at about 555 nm, 560 nm and 603 nm. The 560 nm band probably represents a *b*-type cytochrome, while the 603 nm band is the α -band of cytochrome oxidase. The 555 nm band could either be that of a long-wavelength *c*-type or a short-wavelength *b*-type cytochrome.

Of the total *c*-type cytochrome present in a crude extract from organotrophic cells (Fig. 2, trace A) 90% appeared in the membrane fraction following high speed centrifugation (Fig. 2, trace B; Table 1). Qualitatively, the membrane fractions from lithotrophic (Fig. 1, trace C) and organotrophic cells (Fig. 2, trace B) resemble one another. Similarly, the soluble fraction from succinate-grown cells contained a component with spectral properties not unlike those of the lithotrophic *c*₅₄₈ component (Fig. 2, trace C). Nevertheless, in contrast to the lithotrophically grown bacteria, the amount of *c*₅₄₈-like cytochrome in the organotrophic soluble fraction was small.

Substrate reduction of cytochromes in cell extracts

Sonication of lithotrophic cells resulted in drastic loss of their capacity to oxidize thiosulphate. The rate of thiosulphate-promoted oxygen consumption per mg cell protein was characteristically 600 nmol min⁻¹ prior to cell rupture, and only 1–3 nmol min⁻¹ after sonication. Nonetheless, it remained possible to follow thiosulphate-dependent reduction of cytochromes in cell-free extracts.

Fig. 1

Fig. 2

Fig. 1. Dithionite-reduced minus oxidized difference spectra of extracts from lithotrophic cells. Washed cells from 6 l exponential culture in 5 ml phosphate buffer (100 mM, pH 7.0) were disrupted by sonication and fractionated as described in Methods. The amounts of protein used were 5.6 mg, 4.2 mg and 1.3 mg, respectively, from crude (A), soluble (B) and membrane (C) extracts.

Fig. 2. Dithionite-reduced minus oxidized difference spectra of extracts from organotrophic cells. Washed cells from 1 l exponential culture in 4.5 ml phosphate buffer (100 mM, pH 7.0) were processed as described in Methods. The amounts of protein used were 3.3 mg, 1.6 mg and 1.7 mg, respectively, from crude (A), membrane (B) and soluble (C) extracts.

Table 1. *Distribution of cytochrome c in extracts prepared from lithotrophic and organotrophic cells*

The specific absorbance values tested refer to absorbance divided by the appropriate quantities of crude-extract protein in each case. Absorbance values were obtained from the dithionite-reduced minus oxidized difference spectra in Figs 1 and 2. Crude lithotrophic and organotrophic protein values also derive from Figs 1 and 2 respectively.

Cell state	Fraction	Absorbance of cytochrome <i>c</i>	
		$10^3 \times \text{Specific absorbance}$ (<i>A</i> units mg^{-1})	Relative absorbance
Lithotrophic	Crude extract	17.3	1.00
	Membrane	2.8	0.16
	Soluble	14.4	0.83
Organotrophic	Crude extract	1.4	1.00
	Membrane	1.3	0.93
	Soluble	0.2	0.14

The addition of thiosulphate to a crude lithotrophic extract resulted in reduction of both cytochrome c_{548} and cytochrome a_{603} (Fig. 3a). Observed maximal reduction levels in each case (after 30–40 min) were at least 60% of corresponding dithionite-reducible levels. Thiosulphate did not serve as a reductant of cytochromes associated with lithotrophic membranes in the absence of cytochrome c_{548} and/or other soluble components of thiosulphate-grown cells (Fig. 3b). Extensive reduction of cytochrome c_{548} by thiosulphate readily occurred in a preparation freed of membranes by high-speed centrifugation (Fig. 3c). Furthermore, electron flow from thiosulphate to cytochrome a_{603} was observed after reconstitution of the soluble and membrane

Fig. 3. Thiolsulphate-reduced minus oxidized difference spectra of cytochromes in crude (a), membrane (b), soluble (c) and reconstituted membrane plus soluble (d) fractions from lithotrophic cells. The total protein concentrations were 11.2 mg, 2.6 mg, 8.4 mg and 2.6 plus 8.4 mg, respectively. Note the differences in computer attenuation of the data presented. Reduced spectra were recorded after establishment of anaerobiosis with reductant at 30 min. Additional experimental details are given in Methods.

Table 2. Extent of cytochrome reduction by 20 mM-succinate in cell-free lithotrophic and organotrophic preparations

Data were derived from reduced minus oxidized spectra with dithionite or succinate as reductant. In the latter case, scans were repeated until peak absorbancies remained stable.

Fraction tested	Succinate:dithionite reduction ratio			
	Cyt a_{603}	Cyt b_{560}	Cyt c_{550}	Cyt c_{548}
Organotrophic membranes	0.92	0.44	0.73	—
Lithotrophic membranes	0.88	0.38	0.76	—
Lithotrophic soluble	—	—	—	0
Lithotrophic soluble + organotrophic membranes	0.94	—	—	0.91
Lithotrophic soluble + lithotrophic membranes	0.93	—	—	0.86

fraction (Fig. 3d). Likewise, cytochrome a_{603} associated with organotrophic membranes was reduced by thiolsulphate in the presence of cytochrome c_{548} and other soluble components of lithotrophic origin.

Succinate proved to be almost as efficient a reductant of cytochromes as dithionite in both lithotrophic and organotrophic membranes (Table 2). Extensive reduction of soluble cytochrome c_{548} by succinate was also obtained, but only in the presence of membrane-associated intermediates (Table 2). The latter results confirm and extend earlier studies on succinate oxidation in cell-free extracts (Loya *et al.*, 1982).

Partial characterization of thiolsulphate: cytochrome c oxidoreductase activity in membrane-depleted preparations from lithotrophic cells

The reduction of soluble cytochrome c_{548} by thiolsulphate in a membrane-depleted preparation lacking detectable amounts of other cytochromes obeyed Michaelis-Menten kinetics, and

Fig. 4. Sepharose 6B gel chromatography of soluble components from lithotrophic cells. A 2 ml sample of soluble fraction containing 25.9 mg protein was loaded on a 1.8×95 cm column of Sepharose 6B equilibrated at 4°C with potassium phosphate buffer (100 mM, pH 7.0) and eluted with the same buffer. Fractions (3.2 ml) were collected at a flow rate of 15 ml h^{-1} and then analysed for protein (A_{280} , ---) and cytochrome (A_{410} , —). The column was calibrated with Blue Dextran and standard proteins as described in Methods: VV, void volume.

was subject to inhibition by even modest concentrations of atabrine ($3 \times 10^{-4}\text{ M}$). Apparent K_m and $V_{\max}$ values calculated from initial (5 min) rates of reduction in the presence of various thiosulphate concentrations in oxygenated medium were essentially the same [about 20 mM and $1\text{--}3\text{ nmol min}^{-1}(\text{mg protein})^{-1}$ respectively] as values derived under strictly anaerobic conditions (i.e. air evacuated and replaced by pure argon). Dialysis of the soluble fraction against 100 mM-phosphate buffer at pH 7.0, 4°C , for 6 h neither enhanced nor inhibited the rate of c_{548} reduction. Addition of various electron carriers, including NAD and crystalline cytochrome *c*, or electron donors such as reduced glutathione and reduced pyridine nucleotides did not affect reaction rates under either aerobic or anaerobic conditions.

Size resolution of soluble cytochromes by gel filtration

Passage of the soluble fraction from lithotrophic cells through a column of Sepharose 6B yielded four distinct peaks of material with absorbance at 280 nm (Fig. 4, A_{280}). In and just behind the void volume was material exceeding 1000 kDal in size (peak A). A second group of particles (peak B) eluted in the region where ribulose-1,5-biphosphate carboxylase and other large protein molecules were expected to emerge (Charles & White, 1976). A third A_{280} band was detected in the 15–80 kDal region (peak C), whereas the fourth (and last) band to emerge (peak D) consisted of molecules of less than 15 kDal. Based on the A_{410} , cytochromes were associated with all but the smallest particles (i.e. peak D) recovered from the column (Fig. 4). The dithionite-reduced minus oxidized difference spectrum of pooled fractions from size region A (pool I) showed α -absorption maxima at 603 nm, 560 nm, and 550 nm; these fractions failed to accept thiosulphate electrons. These latter cytochromes are of little interest since they appear to be intimately associated with fragments of cell membranes which were not removed during preliminary high-speed centrifugation of the large-scale preparation. By contrast, pools of fraction containing A_{410} material from size regions B (pool II) and C (pools III, IV, V) were uniquely of the *c*-type of cytochrome and capable of undergoing reduction by thiosulphate to varying extents (Table 3). The percentage of thiosulphate-reducible soluble cytochromes appeared to increase with decreasing particle size and only particles less than about 50 kDal exhibited α -maxima at 548 nm (Fig. 4, Table 3). It should be stressed that none of the soluble *c*-type cytochromes in lithotrophic extracts of *Thiobacillus A2N* eluted with or behind soluble cytochrome *c* of mammalian origin. Moreover, purified complexes containing *c*-type carriers exhibited the same thiosulphate:cytochrome *c* oxidoreductase activity under anaerobic and

Table 3. *Characterization of soluble c-type cytochrome after size resolution of lithotrophic soluble fraction on Sepharose 6B*

Fraction pool tested*	Size region*	α -Absorption maximum (nm)	Extent of reduction by thiosulphate (%)†
II	B	549	8
III	C	550	47
IV	C	548	67
V	C	548	77

* See Fig. 4.

† Percentage of dithionite-reducible cytochrome reduced after prolonged exposure to 20 mM-thiosulphate.

aerobic conditions in the total absence of additional reductants. Considerable efforts were made to dissociate soluble lithotrophic complexes containing *c*-type cytochromes into subunits of smaller size by treatment with various detergents. None of these efforts were successful. For example, chromatography of lithotrophic soluble material on Sepharose 6B in the presence of the non-ionic detergent Brij 30 (0.1% v/v in 0.1M-KCl) had no significant effect on the elution profile of cytochromes in the B and C size regions (see Fig. 4), but did effect drastic changes in the elution of bulk A_{280} material. Similar results were obtained with anionic, cationic and other non-ionic detergents.

Due to the relatively low concentrations of *c*-type cytochromes in the soluble fraction of organotrophically cultured cells, it was necessary to work with much higher inputs (196 mg extract protein) and a larger (3.5 × 95 cm) column in order to obtain a comparable size-resolution profile. As before (Fig. 4), cytochrome-containing membrane fragments eluted with and immediately behind the void volume. However, in contrast to the result obtained with the high-speed soluble fraction from lithotrophically grown cells, region B proteins did not constitute a distinct peak. Nor, based on the A_{410} , were cytochrome-containing particles detectable in this region of the chromatogram. In fact, all of the soluble cytochromes present in high-speed supernatants of organotrophic origin emerged in the C region (i.e. 15–80 kDal). Moreover, the α -absorption maximum of the proximal (i.e. largest) cytochrome-containing particles to elute in region C was at 556 nm, suggesting the possible presence of a soluble *b*-type cytochrome (Poole & Haddock, 1975). Nevertheless, here too, the smallest cytochromes detected in the 40–15 kDal sub-region absorbed maximally at 548 nm and underwent extensive reduction by thiosulphate.

DISCUSSION

The present studies confirm previous conclusions reached on the basis of studies with intact cells (Loya *et al.*, 1982), namely that cytochromes of lithotrophically and organotrophically cultured *Thiobacillus* A2 differ mainly in that the former contain much larger amounts of soluble cytochrome c_{548} . Furthermore, our data suggest that the cytochrome c_{548} carrier is not an integral protein of the membrane '*abc*' system, but is part of either an independent complex (or complexes) not bound to membranes, or a peripheral complex weakly associated with the membrane system. In any case, reconstitution experiments indicated that membrane-bound cytochromes could be reduced by the c_{548} component and *vice versa*. Accordingly, electrons derived from thiosulphate must flow through cytochrome c_{548} in order to reduce the membrane '*abc*' system. However, the growth history of the cells from which the membrane fraction was derived did not matter. Similarly, succinate-derived electrons were able to reduce c_{548} only in the presence of the membrane fraction. The implication for similar *in vivo* processes is obvious. Given that the redox potentials of the pertinent haem carriers have not yet been measured, it is difficult to sequence them. Nevertheless, it seems reasonable to conclude that cytochrome c_{548} mediates the electron flow from thiosulphate to oxygen for the purpose of energy generation, and is also a constituent of the system involved in reverse electron flow to NAD. Schemes basically similar to the one cited above have already been proposed in *Thiobacillus neapolitanus* (Saxena & Aleem, 1972) and *T. novellus* (Aleem, 1966).

It was shown previously that lithotrophic cells of *Thiobacillus* A2N in the process of adapta-

tion to growth on succinate fail to synthesize cytochrome c_{548} at a detectable level. However, in contrast to other constituents of the thiosulphate oxidation system, programmed destruction of cytochrome c_{548} does not occur during this process (Loya *et al.*, 1982). Taken together with our present demonstration of significant (albeit low) amounts of cytochrome c_{548} in cells cultured on succinate for many generations, the above findings suggest a possible role for this particular component in organotrophic metabolism in *Thiobacillus A2*. In this regard it may be significant to note that Silver & Kelly (1976) have reported the presence of sulphite:cytochrome *c* oxidoreductase activity in extracts of the same bacterium after pre-growth on an organic substrate (i.e. formate).

The c_{548} cytochrome is apparently part of a macromolecular complex containing thiosulphate-oxidizing activity. Similar findings were reported in *T. novellus* where the purified thiosulphate-oxidizing system was found to be associated with membrane vesicles (Aleem, 1965; Oh & Suzuki, 1977*a, b*). In *Thiobacillus A2*, on the other hand, the particles containing *c*-type cytochrome appeared to be devoid of major lipid content since detergents normally expected to release proteins from lipoprotein complexes (Helenius & Simons, 1975) and to disrupt protein-protein interactions, failed to dissociate them into smaller subunits.

The partial inhibitory effect of atabrine on the thiosulphate oxidation process, together with apparent saturation kinetics of thiosulphate:cytochrome c_{548} oxidoreductase activity, suggest that non-haem carriers are located in the thiosulphate respiratory chain prior to cytochrome c_{548} and are part of the complex. Also of interest was the fact that purified particles were capable of lithotrophic oxidation under anaerobic as well as aerobic conditions in the absence of additional electron sources. Assuming that the *in vitro* particles studied here are valid models for the situation *in vivo*, these latter observations tend to rule out reductive cleavage (Peck, 1960; Peck & Fisher, 1962) as the first step of thiosulphate dissimilation in *Thiobacillus A2*. Conclusive elucidation of the sulphur oxidation pathway in *Thiobacillus A2* will have to await development of a cell-free system capable of a high rate of terminal oxidation of thiosulphate.

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