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OXIDATION-REDUCTION POTENTIALS AND SPECTRAL PROPERTIES OF SOME CYTOCHROMES FROM *THIOBACILLUS VERSUTUS* (A2)

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Cytochromes *c*-550 (acidic), *c*-550 (basic), *c*-551 and *c*-552.5 from *Thiobacillus versutus* have been highly purified and characterized. Their spectral properties at 77 K are described. Oxidation-reduction titrations of cytochromes *c*-550 (acidic) and *c*-550 (basic) showed them to exhibit Nernst values of $n = 1$, with single redox centres in the cytochromes, and to have midpoint redox potentials at pH 7.0 ($E_{m,7}$) of 290 and 260 mV, respectively. Cytochrome *c*-551 contained two separately titratable redox components, each giving $n = 1$. The low potential centre (55% of titratable cytochrome) and the high potential centre (45%) had $E_{m,7}$ values of -115 and $+240$ mV, respectively. Cytochrome *c*-552.5 also contained at least two redox centres. One (65% of titratable cytochrome) had $n = 1$ and $E_{m,7} = 220$ mV. The remaining 35% appeared to be a low potential component with an $E_{m,7}$ possibly as low as -215 mV. the roles of these cytochromes in respiratory thiosulphate oxidation are discussed.

Introduction

We recently described a thiosulphate-oxidizing multienzyme system from *Thiobacillus versutus*, consisting of four major components ('enzyme A', 'enzyme B' and cytochrome *c*-551 and *c*-552.5) which can effect the complete oxidation of thiosulphate to sulphate in the presence of mammalian cytochrome *c* and a terminal oxidase system such as that present in the membrane fraction of *T. versutus* [1]. The role of the two cytochromes in vivo was postulated to be the coupling of electron flow from oxidations catalysed by enzymes A and B to the membrane-bound cytochrome *c*-552 [1]. Two further soluble *c*-type cytochromes (*c*-550 (basic) and *c*-550 (acidic)) were also partially purified from *T. versutus* [2], but could not be assigned specific functions in thiosulphate metabolism. The cytochromes *c*-551 and *c*-552.5 were unusual as each appeared to have two redox centres, one being reduced by ascorbate, the other by dithionite [2]. Both were rather large molecules and contained two (in *c*-552.5) or 4–5 (in *c*-551) haem groups [1,2].

We now present definitive evidence for the purity of the cytochrome preparations based on their spectra at liquid nitrogen temperatures and for the midpoint redox potentials of all four cytochromes. These were determined for cytochromes *c*-551 and *c*-552.5 with a view to establishing the possible sequence of these two carriers in thiosulphate-dependent electron transport and the possible role of the dual redox centres in each cytochrome. The difference spectra at liquid nitrogen temperatures

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of the membrane fraction of *T. versutus* were also examined to gain further information about the membrane-associated cytochrome systems.

Materials and Methods

Growth of the organism and preparation of membrane and soluble cell-free fractions. These were as described previously [1–4].

Purification of cytochrome c-551, cytochrome c-552.5, cytochrome c-550 (basic) and cytochrome c-550 (acidic). These were purified as described previously [1,2]. For nomenclatural simplicity, all the cytochromes are referred to using the numbers given here, which represent the maxima of their α-peaks in room temperature difference spectra.

Spectral analysis at 77 K. Difference spectra at liquid nitrogen temperatures were obtained as described by Salmon and Poole [5] using a Pye Unicam SP1700 spectrophotometer, fitted with an accessory constructed in the Department of Microbiology, Queen Elizabeth College. This consisted of a Dewar flask, of which the lower 3 cm was silvered, and which was positioned close to the photomultiplier. A brass cuvette holder with two perspex cuvettes (0.5 ml capacity, 2 mm pathlength), was held about 5 cm from the photomultiplier with its lower end immersed in liquid nitrogen in the Dewar flask. Solutions of cytochromes, either reduced with dithionite or oxidised with ammonium persulphate, were pipetted into the cuvettes and then immediately frozen by immersion of the holder with the cuvettes in liquid nitrogen. Spectra were obtained by scanning at 1 $nm \cdot s^{-1}$ with a spectral bandwidth of 1.2 nm.

Oxidation-reduction titration of cytochromes at 298 K (25°C). This method was based on that of Dutton [6]. Absorption spectra were recorded with a Johnson Research Foundation dual-wavelength scanning spectrophotometer (SDB-3) having two monochromators: one was set at a fixed reference wavelength of 540 nm, and the other was used to scan the sample. Spectra of samples at different potentials could be automatically subtracted from each other by means of a digital memory computer which stored any selected spectrum and subtracted it from the subsequent spectra. An anaerobic glass cuvette of approx. 1 cm pathlength and 9 ml working volume was constructed as described by Dutton [6]. Oxidation-reduction potentials were measured with a Beckman model 4500 pH/mV meter using combination platinum and calomel electrodes. The meter was calibrated by immersing the electrode at 25°C in a mixture of $K_4Fe(CN)_6$ (10 mM)/$K_3Fe(CN)_6$ (9 mM) of $E_h = 429$ mV. This gave a meter reading of 184 mV and all subsequent potential values read from the meter were corrected by adding −245 mV to obtain the actual E_h values, relative to the standard hydrogen electrode reference. Titrations were carried out at 25°C, constant temperature being maintained by water circulation to the cuvette base. To achieve mediation between the platinum electrode and the cytochrome under test, the following dyes (E_m 7.0 in mV and concentration in μM) were used: ferricyanide (+430, 20); quinhydrone (+280, 20); *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (+260, 40); 1,2-naphthoquinone-4-sulphonate (+215, 40); 1,2-naphthoquinone (+143, 20); trimethylhydroquinone (+115, 200); *N*-methylphenazonium methosulphate (PMS) (+80, 200); 2-methyl-1,4-naphthoquinone (+10, 400); tetramethyl-*p*-benzoquinone (+ 5 mV, 2400); 2-hydroxy-1,4-naphthoquinone (−145, 25); riboflavin phosphate (−219, 25); anthraquinone-2-sulphonate (−225, 25); benzyl viologen (−311, 2); methyl viologen (−430, 1). Tetramethyl-*p*-benzoquinone and 2-hydroxy-1,4-naphthoquinone were dissolved in ethanol, and the others in distilled water. After mixing the cytochrome sample and mediators in 0.1 M phosphate buffer, (pH 7.0) in the cuvette, the solution was sparged with moistened argon for 15 min before commencing the titration. Gassing was continued during titrations to maintain a slight overpressure in the system. Sodium dithionite or potassium ferricyanide, in 0.1 M phosphate (pH 7.0), were used as reductant or oxidant, respectively. Additions of these were made with a 25 μl Hamilton microsyringe and observation of each titration step was terminated when the absorbance had become constant following a 20–40 mV change in oxidation-reduction potential.

Interpretation of oxidation-reduction titration data. Titration data enabled calculation of percentage reduction of a cytochrome at a given E_h value by reference to the maximum absorbance difference, seen using the specified wavelength pair

between the fully oxidized and fully reduced forms. Two plots were used [6] to examine the data: (a) the ratio of oxidized to the reduced form was plotted on a logarithmic scale against oxidation-reduction potential for each value tested; (b) percentage reduction was plotted against E_h. For simple systems, such as cytochromes with single midpoint potentials, a simple straight line or smooth curve relationship, respectively, was given by these procedures (e.g., Fig. 4). If, however, a cytochrome has two midpoint potentials, a sigmoidal curve results from procedure (b) and a sigmoid plot with two disconnected slopes is obtained by procedure (a) (e.g., Fig. 6). From the sigmoidal curves of reduction (%) against E_h, estimates of the proportions of the two potentiometrically distinct species were made and their midpoint potentials deduced. The Nernst curves for one- and two-electron ($n = 1$ or 2) redox couples were calculated using a simple programme for a Hewlett-Packard HP-33E calculator. These were superimposed on the plotted data to estimate best fit, and E_m values and proportions of the two redox species were optimised by eye on a trial and error basis. These values were similarly estimated from the log plots of oxidized/reduced ratios against E_h.

Effect of carbon monoxide on the difference spectra of the c-type cytochrome. The sample solutions were sparged with CO at room temperature for 10 min before scanning with the oxidized sample as the reference spectrum using the dual wavelength spectrophotometer.

Reagents. All chemicals were of the highest grades commercially available.

Results

Difference spectra at 77 K of the purified cytochromes and membrane fraction of T. versutus

Reduced minus oxidized difference spectra of cytochromes *c*-551 and *c*-552.5 each exhibited single sharp α-bands (Fig.1), confirming the high degree of purification previously claimed [2]. The α-maxima were at 548.5 and 550.5 nm, respectively, indicating a shift of about 2 nm towards the blue region of the spectrum, as described on numerous occasions for 77 K spectra of cytochromes [5]. Several peaks were seen in the complex β-region spectra for both cytochromes (Table I) as has been found for purified cytochrome *c* from other sources [5,7]. Since the bands in the Soret

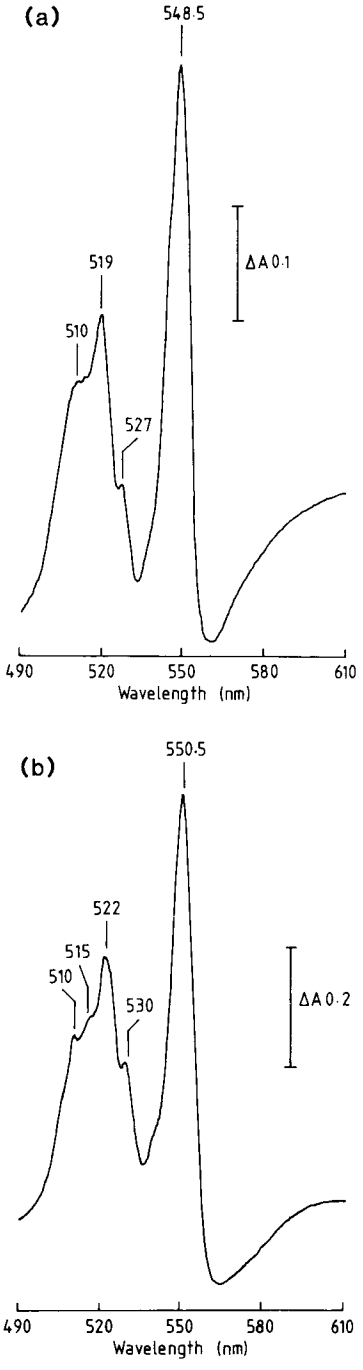


Fig. 1. Difference spectra at 77 K of (a) cytochrome *c*-551 (6 mg protein·ml^{-1}) and (b) cytochrome *c*-552.5 (4 mg protein·ml^{-1}).

TABLE I

PROPERTIES OF THE SOLUBLE *c*-TYPE CYTOCHROMES OF *THIOBACILLUS VERSUTUS*

Measurements of data not newly reported in the text are given in Ref. 2.

Cytochrome	Absorption maxima (nm) of reduced-oxidized difference spectra					Molecular weight		Isoelectric point (p*I*)	Midpoint potentials ($E_{m,7}$) (mV)	Haem content (mol/mol of native form)	Absorbance ratio $\frac{A_\alpha\,(red)}{A_{280}\,(ox)}$	Proportion of crude extract [a] protein (%, w/w)
	at 298 K			At 77 K		native	subunit					
	α	β	γ	α	β							
c-552.5	552.5	523	418	550.5	510 515 522 530	56000	29000	4.8	+220 −215 [b]	2	0.75	1.5
c-551	551	522	418	548.5	510 519 527	260000	43000	5.2	+240 −115	4–5	0.27	1.0
c-550 (acidic)	550	522	415	546 548	508 513 520 527	25000	14000	5.0	+290	1	0.83	0.3
c-550 (basic)	550	521	415	545 547	507 510 518 524	15000	–	8.0	+260	1	0.75	0.4

[a] Prepared as described previously [4].
[b] See text for derivation of this value.

region do not sharpen significantly at 77 K and given no new information, they are not shown.

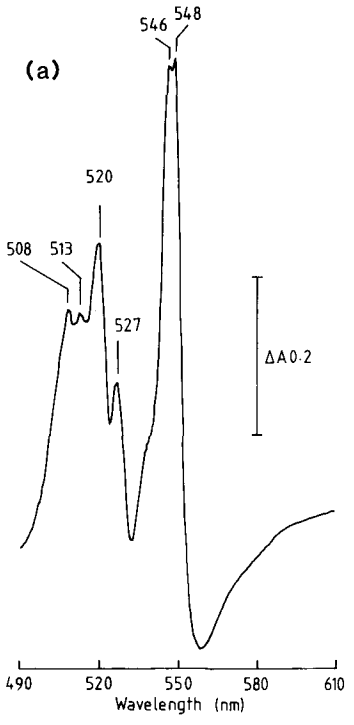
The 77 K difference spectra of cytochromes *c*-550 (acidic) and *c*-550 (basic) were found to give α-bands that were split into two peaks at 548 and 546 nm and at 547 and 545 nm, respectively (Fig. 2). These double peaks are thought to be characteristic of the individual cytochromes as only one *c*-type cytochrome was believed to be presence in either preparation although small amounts of contaminating proteins were present.

A difference spectrum was obtained at 77 K for the A65% fraction [1,2,4], which served as the source for the purification of all four of the cytochromes [2]. Only two peaks were resolved in the α-region (548 nm with a 'shoulder' at 545 nm) and β-region peaks at 510, 519 and 524 nm. This indicates that the 77 K spectral technique does not enable the unequivocal identification of mixtures of spectrally similar cytochromes in as crude a preparation as the 44–65% saturated ammonium sulphate fraction [4].

The membrane fraction [3] from *T. versutus* exhibited a complex difference spectrum at 77 K (Fig. 3). Peaks at 603 and 590 nm were probably due to cytochrome aa_3 and a_1, respectively. The peak at 556 nm with a shoulder at 560 nm corresponded to the α-bands of *b*-type cytochrome(s). in contrast, the 298 K spectra in the 590–608 nm range showed single α-band peaks of 607 and 560 nm for *a* and *b*-type cytochromes [3,8]. A single α-band peak at 549 was seen for the membrane-bound cytochrome *c*, possibly confirming the earlier oxidation-reduction titration indications [8] that only one *c*-type cytochrome was present in the membrane. The multiple peaks in the β-region are contributed to by all the *b* and *c* components and cannot be usefully interpreted. The cause of the peak at 536 nm is unknown.

Effect of carbon monoxide on the cytochromes

The 298 K difference spectra of all four purified cytochromes were identical with and without CO, indicating that none contained any binding sites for CO.



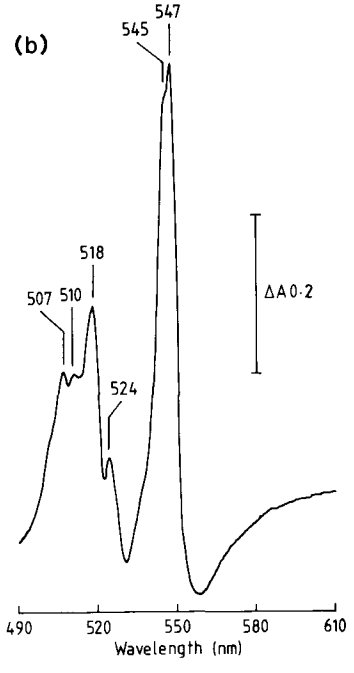


Fig. 2. Difference spectra at 77 K of (a) cytochrome *c*-550 (acidic) (1.6 mg protein · ml^{-1}) and (b) cytochrome *c*-550 (basic) (2.9 mg protein · ml^{-1}).

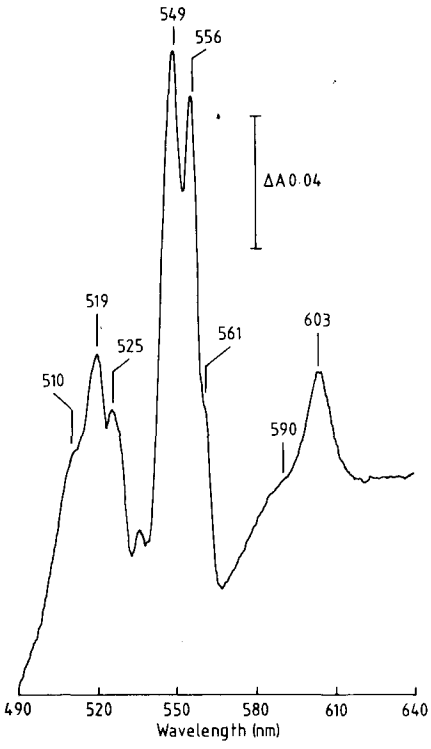


Fig. 3. Difference spectrum of the membrane fraction of *T. versutus* at 77 K. Membrane fraction (5.5 mg · ml^{-1}) was prepared as described in Ref. 3.

Oxidation-reduction titration of cytochromes c-550 (acidic) and c-550 (basic)

'Classic' titration curves were obtained for both cytochromes (Fig. 4), consistent with Nernst curves for one-electron redox couples and single redox centres in the cytochromes. Standard midpoint redox potentials at pH 7.0 ($E_{m,7}$) for the cytochromes were +290 mV for cytochrome *c*-550 (acidic) and +260 mV for cytochrome *c*-550 (basic). As a check on the experimental procedures employed, horse heart cytochrome *c* (Sigma, Type III) was titrated under identical conditions to give an $E_{m,7}$ of 280 mV and a Nernst curve equivalent to $n = 1$. The procedures were thus considered reliable.

Oxidation-reduction titration of cytochromes c-551 and c-552.5

Simultaneous measurements of potential and absorbance changes were made for cytochrome *c*-551 over a potential range of −400 to +340 mV

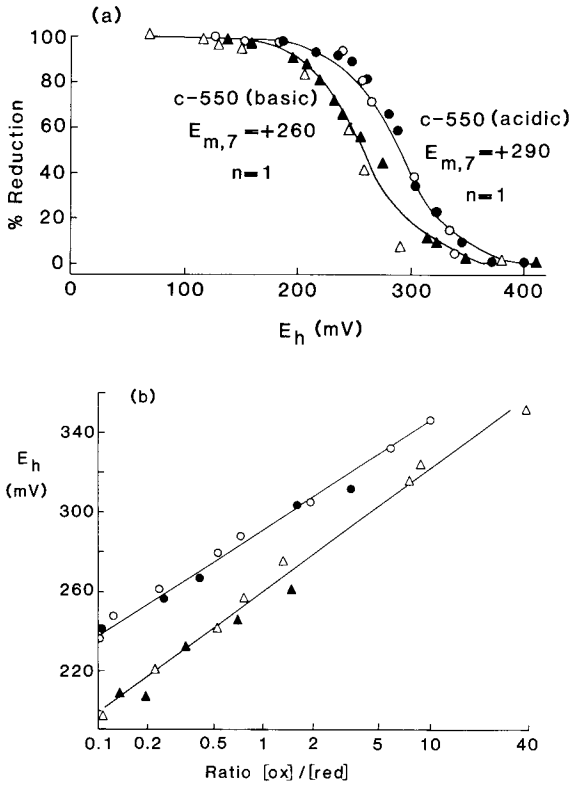


Fig. 4. Dependence of the state of reduction of cytochrome *c*-550 (acidic) (○ and ●) and cytochrome *c*-550 (basic) △ and ▲) on the oxidation-reduction potential (E_h). (a) Reduction is expressed as percentage of the fully reduced form for oxidative redox titrations with ferricyanide (●,▲) and reductive redox titrations with dithionite (○, △), using 10 mg *c*-550 (acidic) or 4.5 mg *c*-550 (basic) in a final 8.2 ml 0.1 M phosphate buffer (pH 7.0). Curves fitted to the data were computed for values of $n = 1$ and the midpoint potentials shown, as described in Materials and Methods. (b) Ratio of the concentrations of the oxidized to reduced forms of the cytochromes at different E_h values. The slopes of both lines are 60 mV (for a 10-fold change in ratio), indicating $n = 1$, and give midpoint potentials at pH 7.0 of 260 and 290 mV as shown in Fig. 4a.

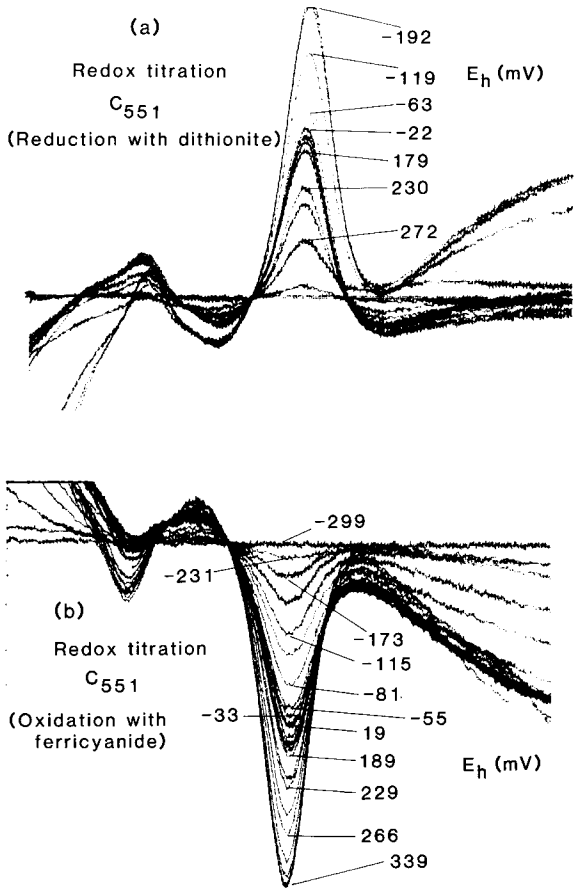


Fig. 5. Absorbance changes accompanying the redox titration of cytochrome *c*-551. (a) Reductive titration: the cytochrome solution (6 mg protein in 7.8 ml) was adjusted to 340 mV with ferricyanide and the spectrum recorded as a baseline. The potential was then lowered stepwise with dithionite to a final value of −400 mV; the spectrum at each step was recorded against the baseline spectrum at +340 mV. (b) Oxidative titration: the baseline spectrum was established at −400 mV after reduction with dithionite and the potential progressively increased by stepwise addition of ferricyanide. The final pH of each titration system was 7.0.

(Figs. 5 and 6). Both reductive and oxidative titration showed two phases, with ready oxidation or reduction up to E_h values between about −60 to +180 mV; relatively small change in absorbance for large changes of E_h occurred within this range. This indicated that two redox centres of different E_m might be present. When the ratio of oxidized/reduced cytochrome was plotted logarithmically against E_h, a sigmoidal curve was obtained (Fig. 6a), indicating the presence of two midpoint potentials in the cytochrome. The plot also shows large Nernst slopes of about 120 mV, which would normally be interpreted to indicate a value of $n = 0.5$. This is an anomaly due to the presence of two midpoint redox centres, as was also found for a cytochrome *b* [9]. The inflexion point on Fig. 6a indicates the separation of the high and low redox forms, and this and the plot of Fig. 6b is consistent with the estimation of their relative contri-

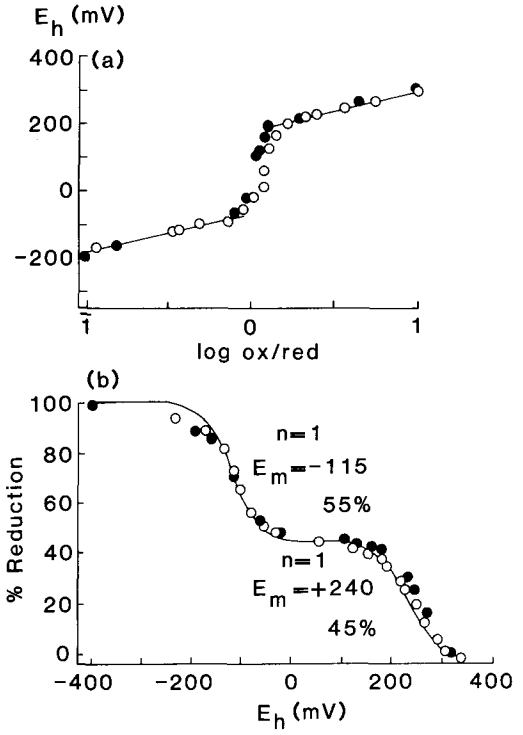


Fig. 6. Analysis of redox titrations of cytochrome *c*-551: oxidative (●) and reductive (○) titration data from Fig. 5. (a) Relationship of E_h and log of ratio of oxidized to reduced form of cytochrome; (b) fitting of the experimental data for percentage of reduction at each E_h value to a calculated curve based on there being two redox components, both having $n = 1$ and having midpoint potentials of -115 mV (55% of total titratable cytochrome) and $+240$ mV (45% of total).

butions to the overall absorbance changes as 45% (high E_m) and 55% (low E_m, respectively. From Fig. 6b, the best estimates of the midpoint potentials were taken to be $+240$ and -115 mV. Using these values, with the components being present at 45 and 55%, respectively, and $n = 1$, the curve drawn through the data (Fig. 6b) was computed as described in Materials and Methods. As can be seen, the curve is an excellent fit, indicating the probable accuracy of the $E_{m,7}$ values. Poor or nonsense fits were obtained when other values of n and E_m were adopted, or when attempts were made to fit the data to the assumption of one or three, rather than two redox forms. The same results were obtained on repeating the titrations with higher concentrations of the redox mediators.

Similar anaerobic potentiometric titration spectra were obtained for cytochrome *c*-552.5. The log plot of oxidized/reduced components (at 552.5 nm relative to 540 nm as reference wavelength) against measured E_m also exhibited a sigmoid curve from the high potential region (Fig. 7a). From this, an apparent overall $E_{m,7}$ of $+195$ mV and a Nernst slope of 85 mV could be deduced. The titration curves obtained for the potential range from -100 to -400 mV were, however, poor (Fig. 7a). The percentage reduction of cytochrome *c*-552.5 relative to redox potential is given in Fig. 7b and shows that the high potential component contributed about 65% of the overall absorbance change and had an $E_{m,7}$ of $+220$ mV. Using these data and taking a value of $n = 1$, the curve fitting the data (Fig. 7b) was computed as for the other cytochromes.

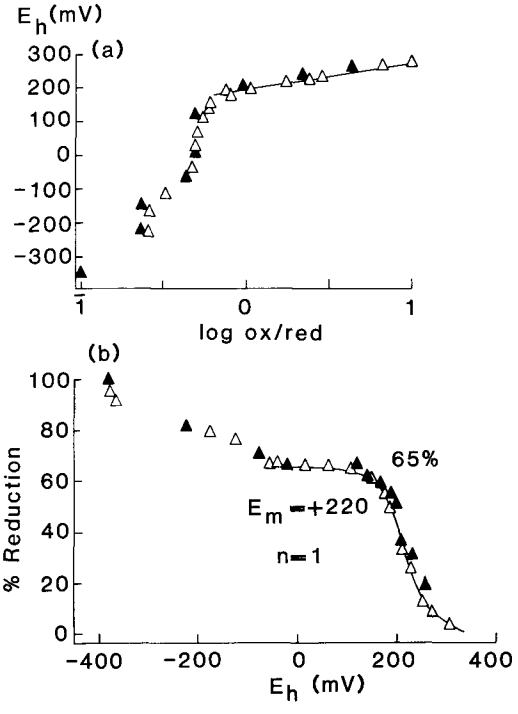


Fig. 7. Oxidative (▲) and reductive (△) titrations of cytochrome *c*-552.5 (2.1 mg protein) conducted as described in Fig. 5 and Materials and Methods. (a) Relationship of E_h and absorbance change of cytochrome *c*-552.5 at 552.5 minus 540 nm, expressed as log of oxidized/reduced ratio. (b) Plot of percentage reduction of total cytochrome against E_h for the whole potential range employed. The curve drawn is a theoretical one based on one redox component comprising 65% of the total and having values of $n = 1$ and midpoint potential = 220 mV.

A number of attempts were made to obtain better values in the low potential range, using different concentrations of some mediators, but improved data were not obtained. The titrations were technically difficult between -200 and −350 mV, in which range there was a large gap in E_m values between anthraquinone-2-sulphonate (−225 mV) and benzyl viologen (−350 mV), and small additions of dithionite or ferricyanide caused large changes in potential and in absorbance. Assuming cytochrome *c*-552.5 to contain only two redox centres, one of which was a high potential component comprising 65% of the total, and the other a low potential component making up the remaining 35%, the data of Fig. 7 were recalculated to express percentage reduction of each redox component relative to redox potential [8,9]. Percentage reduction of each component was calculated by assuming absorbance change over the range of E_h values from +308 to +19 and from −20 to −380 mV to be due to the high potential and low potential components, respectively. Plotting these data confirmed the E_m for the high potential component to be +220 mV (Fig. 8). the slope of the

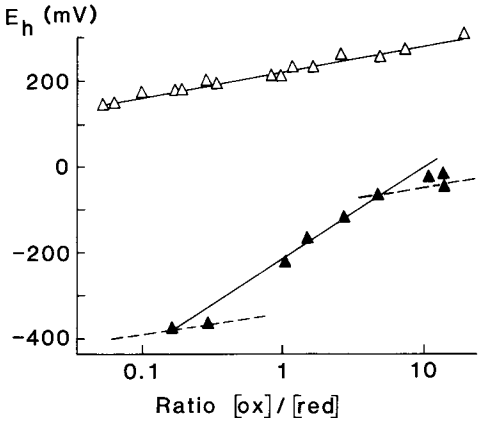


Fig. 8. Recalculation of redox titration data for cytochrome *c*-552.5 from Fig. 7 assuming there are two components: a high potential fraction (△, 65% of total) and a low potential fraction (▲, 35%). Percentage reduction of each fraction was calculated and the ratio of concentration of the oxidized and reduced forms plotted against E_h. The high potential fraction gives a slope of 60 mV ($n = 1$) and $E_m = 220$ mV, as in Fig. 7(b). The slope of the low potential data gives a mid-range slope of about 215 mV. The broken lines indicate slopes of 60 mV ($n = 1$). The E_m is indicated to be about −215 mV by this plot.

[ox]/[red] versus E_h plot (Fig. 8) was 60 mV (for a 10-fold change in [ox]/[red]), consistent with a value of $n = 1$ [6]. The low potential data give results by these plots (Figs. 7 and 8) that are so far from standard one-electron titrations as to make their interpretation conjectural; for example, the slope of the low potential plot (Fig. 8) is over 200 mV. Plotting percent reduction of the low potential component against E_h indicated an apparent $E_{m,7}$ of about −215 mV, consistent with Fig. 8. Our data do not at this stage allow us to speculate further on this fraction.

Discussion

The widely varied properties of the four *c*-type cytochromes we have purified from *T. versutus* are summarised in Table I. The two *c*-550 cytochromes have as yet no know function in inorganic sulphur oxidation and cannot currently be discussed further. It is, however, notable that the *c*-550 (basic) cytochrome is similar in M_r, midpoint potential and p*I* to the cytochrome *c*-550 described earlier from *Thiobacillus novellus* [10]. This cytochrome was later implicated in sulphite oxidation [11], so a similar role may eventually be found for the *T. versutus* *c*-550 cytochromes.

This study has also confirmed that the cytochromes *c*-551 and *c*-552.5 from *T. versutus* each contain two redox forms of widely different midpoint potentials. These two dual-redox centre cytochromes are essential components of the thiosulphate-oxidizing system demonstrated in *T. versutus* [1–4]. the high midpoint potentials of *c*-551 and *c*-552.5 (+240 and +220 mV) are similar to that of the membrane-bound cytochrome *c*-552 (+205 mV; [8]), suggesting that electron transfer from thiosulphate to the membrane electron-transport system may be mediated by these three cytochromes [1,2]. The differences in E_m between them cannot be taken as significant, as electron flow is of course dependent on the absolute concentrations of the components and on the ratio of their oxidized to reduced forms, especially when their E_m values differ by less than 60 mV. The E_m data support the view that electron transfer from the thiosulphate-oxidizing system to oxygen involves cytochrome *c*-551 and *c*-552.5 functioning in parallel rather than in sequence,

each one transferring electrons from different component reactions of the overall thiosulphate-oxidation process. This is consistent with their cooperativity in the reconstituted thiosulphate-oxidizing system [1,2].

While a role for the high E_m centres in cytochromes *c*-551 and *c*-552.5 can thus be inferred, the function of their extremely electronegative components is less evident. The difference between the midpoint potentials in each cytochrome is very large: 335 mV in *c*-551 and possibly as much as 435 mV in *c*-552.5. Such multicentre cytochromes are not unique; the dihaem cytochrome *c*-552.5 from *Pseudomonas perfectomarinus* contained two midpoint potentials at −180 and +175 mV [12]. The multihaem cytochrome c_3 from *Desulfovibrio* contains several different redox centres of differing E_m, ranging from −284 to −319 mV [13–15], but the role of this multiplicity is not known.

In general, the E_0' values for oxidation reactions involving thiosulphate are not believed to be sufficiently electronegative to couple to cytochromes with E_m values as low as −220 mV [16], the E_0' for the thiosulphate/sulphite couple possibly being as high as −20 or +15 mV (Ref. 16, and unpublished observations). Published figures for the E_0' of the sulphite/sulphate couple have, however, been calculated to be as low as −480 to −516 mV [17]. Consequently, electrons from sulphite oxidation could be expected to couple directly to the low potential centre in *c*-551 ($E_{m,7} = -115$ mV). Partial reduction of this component has been observed using *T. versutus* sulphite dehydrogenase (Lu, W.-P. and Kelly, D.P., unpublished data). Since the component reactions in the oxidation of the sulphane group of thiosulphate to sulphite are not known, but must involve the transfer of three electron-pairs, one or more of these reactions could also be sufficiently negative to couple to the low potential centres in these cytochromes. It is therefore conceivable that electrons received by the low potential centres could be transferred to the high potential centres and thence to the membrane-bound cytochrome system. The other electron transport process that the thiobacilli must effect is the energy-dependent flow of electrons from cytochrome *c* to NAD [16]. A possible role for the dual redox-centre *c*-type cytochromes of *T. versutus* is for the high midpoint potential centres to be involved in energy-conserving electron transport to oxygen while the low potential centres supply electrons for cytochrome *b* and subsequently NAD reduction.

Finally our observations confirm the report of Kula et al. [8] that the membrane-bound cytochrome *b* of *T. versutus* exhibits two α-maxima at 77 K, which our rather more sharply defined spectra indicate to be at 556 and 560 nm. We have not resolved whether a single cytochrome *b* with two midpoint potentials is present in *T. versutus* or whether multiple forms of *b*-type cytochromes are present, as has been found in mitochondria [8].

Acknowledgements

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