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OXIDATION-REDUCTION POTENTIALS OF RESPIRATORY CHAIN COMPONENTS IN *THIOBACILLUS* A_2

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(1) Cells of *Thiobacillus* A_2 grown chemoautotrophically on thiosulfate or heterotrophically on succinate with oxygen contained *b*-, *c*-, *o*-, *a*- and a_3-type cytochromes. The amount of cytochrome per mg of cell protein was much greater in thiosulfate-grown cells and differences in the relative concentrations of cytochromes were observed for the different growth conditions. (2) The half-reduction potentials at pH 7.0 ($E_{m,7.0}$) and spectral maxima of *c*-, *b*-, *a*- and a_3-type cytochromes were similar in cells grown aerobically with thiosulfate or with succinate as the growth substrate. (3) The half-reduction potential of the 'invisible', or high-potential copper, as determined from the potentiometric behavior of the carbon monoxide-reduced cytochrome a_3 complex at pH 8.0, was 365 mV. (4) Reducing equivalents from thiosulfate appear to enter the respiratory chain at the cytochrome *c* level; however, studies in cell-free extracts were limited due to a loss in respiratory activity with thiosulfate as a substrate upon cell disruption.

Introduction

Thiobacillus A_2, isolated and described by Taylor and Hoare [1], is a facultative chemolithotroph. It grows autotrophically with thiosulfate as an oxidizable substrate and the sole energy source and with CO_2 as the sole source of cell carbon. Under autotrophic growth conditions the organism is an obligate aerobe and is thus unable to use alternate electron acceptors such as nitrate. The latter is utilized as the respiratory final electron acceptor when this bacterium is grown heterotrophically on a variety of organic substrates (Aleem, unpublished data). Little information is available on the respiratory components that mediate aerobic and anaerobic respiration in *Thiobacillus* A_2. Cytochromes participate in the dissimilatory oxidation of inorganic sulfur compounds by organisms in the *Thiobacillus* genus [2,3] and in the dissimilatory reduction of inorganic oxides of nitrogen which serve as final electron acceptors in many microorganisms [4,5]. A knowledge of the oxidation-reduction potentials of respiratory chain components is essential to the understanding of oxidative phosphorylation in *Thiobacillus* A_2. This report deals with the potentiometric and spectral characterization of cytochromes in *Thiobacillus* A_2 grown autotrophically on thiosulfate and heterotrophically on succinate.

Materials and Methods

Preparation of cell-free extracts. Cultures grown on thiosulfate and succinate were harvested in the

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Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

logarithmic phase of growth as described previously [1,2,11]. Cells were starved in growth medium lacking substrate for 4 h, washed twice in 0.05 M phosphate buffer, pH 7.0, and stored at −20°C. Frozen cells were suspended in a small volume of the same phosphate buffer and disrupted by three passages through a french pressure cell operated at 15000 lb/inch^2. The disrupted cells were centrifuged at 20000 × *g* for 29 min. The 20000 × *g* supernatant (20S) was centrifuged at 100000 × *g* for 3 h. The 100000 × *g* pellet ($100P_1$) was resuspended in buffer and centrifuged at 100000 × *g* for 3 h. The pellet ($100P_2$) was resuspended in buffer and centrifugation was repeated. The resulting pellet was designated the $100P_3$ fraction.

Quantitation of cytochromes b and c in intact cells. Since the high concentration of cytochrome *c* masked the cytochrome *b* absorption maximum in autotrophically grown cells, the acid/acetone extraction procedure of Falk [6] was used to separate protoheme from heme *c*.

Absorption spectra. Absorption spectra of samples (reduced minus oxidized) at room temperature were recorded with an Aminco DW-2 spectrophotometer or Cary 17 spectrophotometer with 1 cm path-length cuvettes. Difference spectra of samples at near liquid nitrogen temperatures were recorded with an Aminco DW-2 spectrophotometer with 0.2 cm path-length cuvettes using samples containing 50% glycerol (v/v). CO was added by sparging or by adding aliquots of buffer saturated with CO (1.2 mM).

Potentiometric titrations. Absorption spectra of samples at defined oxidation-reduction potentials were recorded with a Johnson Research Foundation dual-wavelength spectrophotometer fitted with a scanning attachment. Spectra of samples at different potentials could be automatically subtracted from each other by means of a digital memory unit which stored any selected spectrum and subtracted it from the following spectra [7]. Potentiometric titrations were conducted at room temperature. The oxidation-reduction potentials were measured with a Corning pH/mV meter using Radiometer platinum and calomel electrodes. The oxidation-reduction mediators used and their half-reduction potentials at pH 7.0 ($E_{m,7.0}$) were potassium ferricyanide, 420 mV; phenazine methosulfate, 88 mV; phenazine ethosulfate, 55 mV; diaminodurene, 240 mV, duroquinone, 30mV; pyocyanine perchlorate, −30 mV; and 2-hydroxy-1,4-naphthoquinone, −140 mV. Duroquinone and 2-hydroxy-1,4-naphthoquinone were dissolved in ethanol while the others were dissolved in distilled water [8]. The sample was suspended in buffer and maintained at −400 mV for 10–15 min prior to titration. A dilute solution of sodium dithionite served as the reductant while potassium ferricyanide was used to supply oxidizing equivalents. Titrations were terminated when the absorbance remained constant over a 20–30 mV oxidation-reduction potential change. The graphic representation of log oxidized/reduced as a function of the oxidation-reduction potential measured at 30°C for a single redox component yielded a straight line with a slope of 60 for $n = 1$, as described by the Nernst equation [9]. When the two components with similar spectral properties but different half-reduction potentials (greater than 70 mV) were present, a sigmoid curve resulted. The sigmoid curve was resolved arithmetically into two components [8].

Reagents. All reagents were of the highest grade commercially available.

Electron spin resonance. ESR spectra were recorded for samples at defined oxidation-reduction potentials cooled to near liquid helium temperature with a Varian E-4 spectrometer [10].

Results

The cytochrome system of Thiobacillus A_2 as revealed by use of respiratory inhibitors

Extraction of intact cells with acidic acetone separated the heme of *b*-type cytochromes from heme *c* [6]. Thiosulfate-grown cells contained 3-times as much as cytochrome *b* (0.076 nmol/mg dry wt. vs. 0.025 nmol/mg dry wt.) and 16-times as much cytochrome *c* (1.2 nmol/mg dry wt. vs. 0.075 nmol/mg dry wt.) when compared to cells grown aerobically with succinate as the substrate. The respiratory rate with thiosulfate as the substrate was 0.8–1.5 mol O_2/min per mg dry wt. with thiosulfate-grown cells. 2 mol O_2 were consumed per mol thiosulfate presented, i.e., 0.05 mol $S_2O_3^{2-}$/0.103 mol O_2 corrected for endogenous respiration.

The inhibitors of cytochrome $a + a_3$, cyanide and azide, inhibited the oxidation of thiosulfate in

thiosulfate-grown cells and the oxidation of succinate and malate in cells grown aerobically with succinate as the growth substrate. In contrast, amytal, rotenone and theonyltrifluoroacetone were more effective inhibitors of succinate and malate oxidation than thiosulfate oxidation. Similarly, when compared to thiosulfate oxidation, the oxidation of succinate and malate was more sensitive to inhibition by antimycin A and 2-*N*-heptyl-4-hydroxyquinoline *N*-oxide, inhibitors which block electron transfer between the ubiquinone-cytochrome *b* and -cytochrome *c* segments of the respiratory chain. Although atabrine (0.94 mM) inhibited succinate oxidation by 70%, endogenous respiration was stimulated 2–3-fold, attaining 40% of the respiratory activity with succinate in the absence of atabrine. Atabrine, a flavoprotein inhibitor, at 0.94 mM inhibited the oxidation of thiosulfate by 90%. Similar patterns were observed for the inhibition of succinate and thiosulfate oxidation when thiosulfate-grown cells were induced to oxidize succinate [11]. Addition of thiosulfate to suspensions of starved thiosulfate-grown cells caused reduction of cytochrome *c* (552 and 420 nm) and cytochromes *a* (606 and 445 nm). No reduction of cytochrome *b* was observed but the high concentration of cytochrome *c* could have masked the cytochrome *b* maximum.

In cell-free extracts of thiosulfate-grown cells (20S), less than 1% remained of the respiration rate stimulated by thiosulfate in intact cells. Respiration was not restored by horse heart cytochrome *c*, ADP, ATP, AMP, cysteine, glutathione, Mg^{2+}, F^-, cell-free extracts of succinate-grown cells or by varying the ionic strength, pH or buffers (Tris or phosphate). When thiosulfate-grown cells were freeze-thawed four times then treated with deoxyribonuclease to decrease the viscosity of the cell suspension, less than 1% remained of the respiratory activity with thiosulfate as the substrate. Repeated washing of the freeze-thawed cells which were treated with deoxyribonuclease did not restore the respiratory activity.

Thiosulfate completely reduced cytochrome *c* in the $100S_1$ fraction of thiosulfate-grown cells. Absorption maxima attributed to cytochromes *b* and $a + a_3$ were not detected in this fraction. In the $100P_1$ fraction, 15–20 min were required for partial reduction (less than 10%) of cytochromes *c* (550 nm), *b* (558 nm) and $a + a_3$ (605nm) by thiosulfate. A similar spectrum was obtained in the presence of antimycin A.

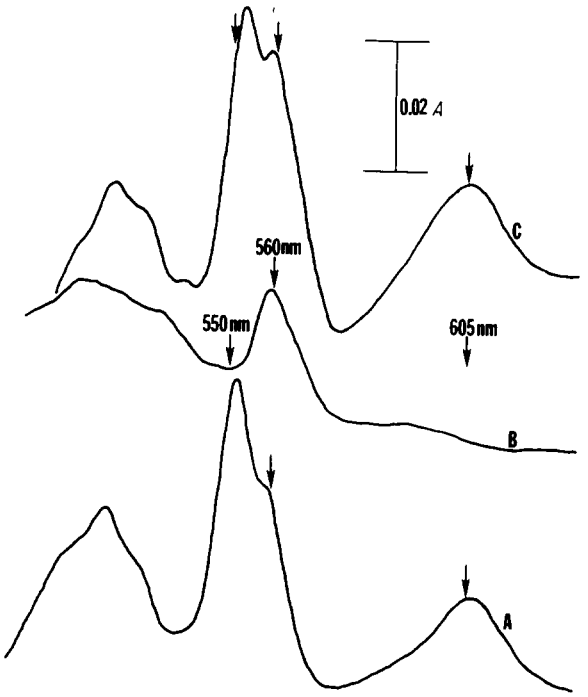


Fig. 1. Absorption spectra of the cytochrome of the 20S fraction of succinate-grown *Thiobacillus* A_2. The 20S fraction was suspended at 20 mg protein/ml in 50 mM phosphate buffer, pH 8.0. Untreated samples (aerobic and substrate deficient) were placed in the reference cuvette and the presented spectra represent the difference between that of the untreated sample and that of a sample treated as follows. (A) Succinate (10 mM) and the sample allowed to become anaerobic. (B) Succinate (10 mM) and antimycin A (30 μg/ml). (C) Dithionite.

In cell-free extracts ($100P_1$) of thiosulfate-grown cells, the presence and participation of *b*-type cytochrome(s) in succinate oxidation was indicated by the appearance of an absorbance band at 560 nm at room temperature. Cytochromes $a + a_3$ (reduced maxima, 607 and 445 nm) and cytochrome *c* (reduced maxima, 552 and 420 nm were reduced completely and thus presumably participate in the transfer of reducing equivalents from succinate to oxygen. In the presence of antimycin A, the maximum at 560 nm at room temperature was split, with maxima at 557 and 563 nm at liquid nitrogen temperature suggesting the presence of multiple *b*-type cytochromes.

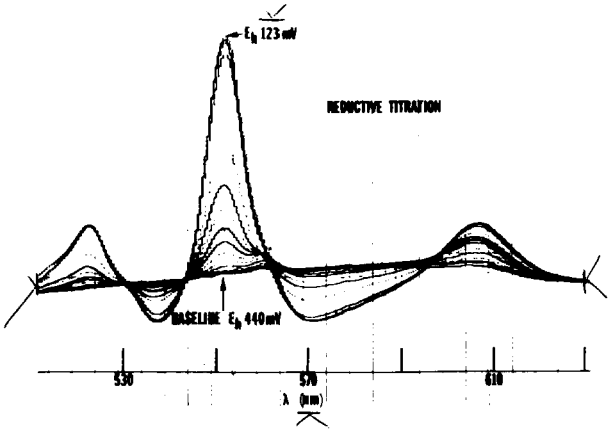
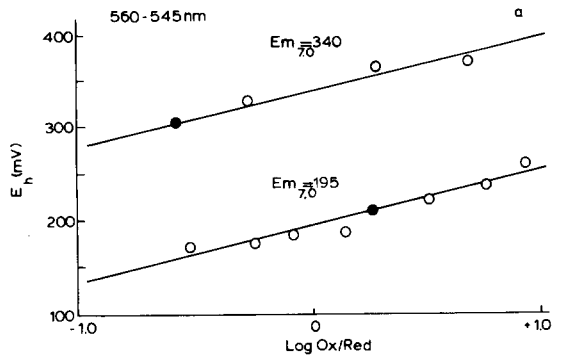


Fig. 2. The absorbance changes which accompany reduction of the cytochromes in the $100P_3$ fraction of thiosulfate-grown *Thiobacillus* A_2 cells. The suspension contained 45 mg protein/ml in 0.1 M phosphate buffer, pH 7.0. The oxidation-reduction mediators used were potassium ferricyanide (40 μM), phenazine methosulfate (40 μM), phenazine ethosulfate (40 μM), diaminodurene (40 μM), duroquinone (40 μM), pyocyanine perchlorate (5 μM) and 2-hydroxy-1,4-naphthoquinone (20 μM). The aerobic suspension was adjusted to 440 mV and the spectrum recorded. The potential was then lowered stepwise to 123 mV and the presented spectra represent the absorption of the sample minus that of the sample at 440 mV.



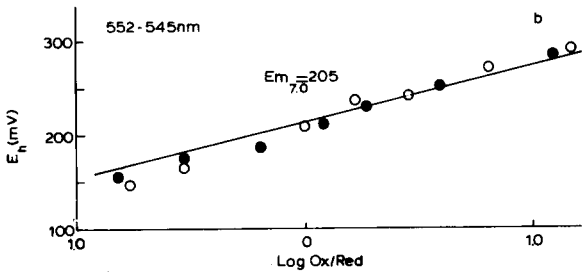


Fig. 3. The oxidation-reduction potential dependence of reduction of the *b*- and *c*-type cytochromes. The potentiometric titrations were carried out as described in the legend to Fig. 2 except that the cytochrome oxidation and reduction were measured using a dual-wavelength spectrophotometer set for the indicated wavelength pair. The protein concentration was approx. 3 mg/ml and the pH 7.0. All titrations were carried out in both the oxidative and reductive directions.

Antimycin A inhibited reduction of *c*- and *a*-type cytochromes by succinate or NADH in cell-free extracts (20S) of cells grown aerobically on succinate (Fig. 1b). Dithionite (Fig. 1c) reduced the *b*-type cytochromes to a greater extent than did succinate or NADH (Fig. 1a). The cytochrome(s) with absorption maxima at 557 and 563 nm were not readily reduced by ascorbate + TMPD (data not shown).

Dependence of the cytochrome b and c reduction in anaerobic samples on oxidation-reduction potential

Simultaneous measurements of absorbance changes and oxidation-reduction potentials were carried out for anaerobic samples over the potential range from −290 mV to +123mV. The two more negative *b*-type cytochromes were fully oxidized at +123 mV. Cytochromes with absorption maxima at 607, 560 and 552 nm undergo reduction over the range of E_h values from 440 to 123 mV (Fig. 2). At fixed dual wavelengths (560 nm minus 540 nm), the titration curve was sigmoid, indicating a mixture of components with different half-reduction potentials. The sigmoid curve was arithmetically resolved [8] into two components, each with an *n* value of unity (Fig. 3a). The half-reduction potential of the high-potential component was 340 mV while that of the low-potential component was 195 mV. Analysis of absorbance changes as a function of E_h over the oxidation-reduction potential range +400 to +275 mV indicated that the component absorbing at 560 nm is the high-potential component (Fig. 3a). The component with spectral properties of cytochrome *c* (absorption maximum 552 nm) titrated as a single component with a half-reduction potential of 205 ± 20 mV (Fig. 3b).

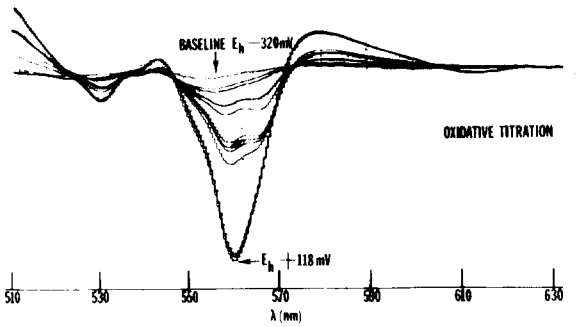


Fig. 4. The spectral properties of the *b*-type cytochromes with $E_{m,7.0}$ values of −90 and 30 mV. The $100P_3$ fraction of thiosulfate-grown *Thiobacillus* A_2 was suspended and titrated as described in the legend to Fig. 2.

With a reference spectrum obtained at an E_h value of 120 mV stored in the digital memory unit, a component absorbing at 562 nm in the reduced form undergoes reduction with a slight shift in absorption maximum to shorter wavelengths at more negative oxidation-reduction potentials suggesting the presence of more than one component. When the reference spectrum stored in the digital memory was obtained for a sample at an E_h value of −320 mV, a component with absorption maxima at 558 and 566 nm was observed to become oxidized as E_h was changed from −50 to −40 mV (Fig. 4). The sum of the spectra of the component with a maximum at 562 nm and the component with maxima at 558 and 566 nm yielded the reduced minus oxidized spectrum of these *b*-type cytochromes.

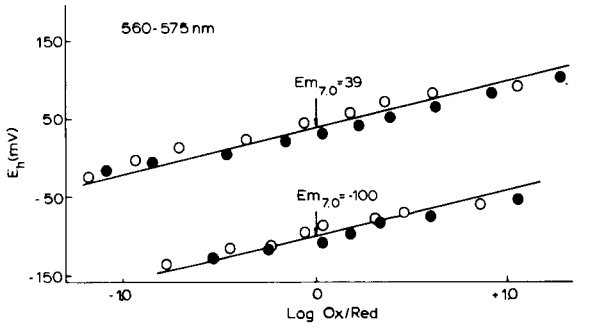


Fig. 5. The oxidation-reduction potential dependence of the reduction of the *b*-type cytochromesin *Thiobacillus* A_2. Potentiometric titrations were as described in the legend to Fig. 2. The absorbance change at the indicated fixed dual wavelengths over the oxidation-reduction potential range −150 to +150 mV was monitored. The titration curve as obtained is sigmoid and this figure gives the data after resolution into its two components.

Titrations over the E_h range −150 to 125 mV with the wavelength pair 560 nm minus 575 nm was sigmoid. The sigmoid curve resolved into two components with half-reduction potentials of 39 and −100 mV (Fig. 5). In multiple titrations, the half-reduction potential of the high-potential component (absorption maximum 562 nm) was 30 ± 15 mV while that for the low-potential component (absorption maxima at 558 and 566 nm) was −90 ± 15 mV. The low-potential and high-potential components contributed 25 and 75%, respectively, to the total absorbance change at 560 nm minus 575 nm.

Dependence of reduction of the a-type cytochromes on oxidation-reduction potential

The sigmoid curve for the oxidation-reduction potential dependence of the 606 nm minus 620 nm absorbance change (Fig. 6a) was resolved into components with half-reduction potentials ($E_{m,7.0}$) of 230 and 380 mV (Fig. 6b). In multiple titrations the half-reduction of the low-potential component was 210 ± 20 mV while that for the high-potential component was 390 ± 15 mV. Each component contributed equally to the total absorbance change.

ESR absorption was measured in samples frozen at defined oxidation-reduction potentials and the sample temperature was lowered to near that of liquid helium. The signal in the *g* 6 region was low in aerobic samples and increased as E_h was lowered, attaining a maximum value at 325 mV, and then decreased to a low value at an E_h value of 100 mV (Fig. 7a). The approximate half-formation potential ($E_{m,7.0}$) for appearance of the *g* 6 signal was 390 mV while that for the disappearance of the signal was 200 mV (Fig. 7b).

The difference in absorption between samples treated with cyanide + dithionite and that of samples treated with dithionite showed absorption maxima at 593 and 453 nm indicating the presence of a reduced cytochrome a_3-cyanide complex [12,13].

The differences in absorption between the reduced and oxidized forms of the high- and low-potential *a*-type cytochromes were measured and found to be similar to those of their mammalian counterparts. The high-potential component (295

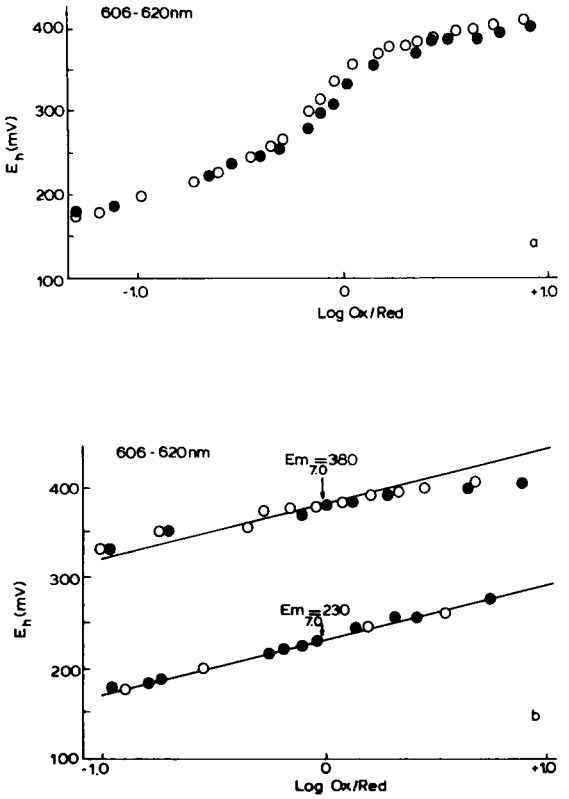


Fig. 6. The oxidation-reduction potential dependence of the reduction of the *a*-type cytochromes of *Thiobacillus* A_2. The $100P_3$ fraction of thiosulfate-grown *Thiobacillus* A_2 was suspended at 2.7 mg protein/ml, pH 7.0, and titrated as described in the legend to Fig. 2 except that cytochrome reduction and oxidation were measured using a dual-wavelength spectrophotometer set for the indicated wavelength pair.

mV minus 440 mV) had an asymmetric absorption band with a maximum at approx. 608 nm. Each contributed approx. one-half of the total reduced minus oxidized absorbance change.

The oxidation-reduction potential dependence of the absorbance change was alsomeasured for samples at pH 8.0. At this pH the half-reduction potentials of the high- and low-potential components were 345 and 190 mV, respectively. The 45 mV shift in half-reduction potential of cytochrome a_3 suggested that this component, like its counterpart in mammalian mitochondria, accepts a proton upon reduction when the pH is more alkaline than 7.0 [14].

At -20 mV, an E_h value at which both cytochrome *a* and a_3 were reduced, addition of 100 μM CO decreased the absorbance at 606 nm by approx. 12% and a shoulder appeared at shorter wavelengths. Difference spectra indicate that the CO compound has an absorption maximum at 595 nm (Fig. 8). Approx. 8% of the decrease in absorbance could be attributed to dilution resulting from the addition of buffer saturated with CO.

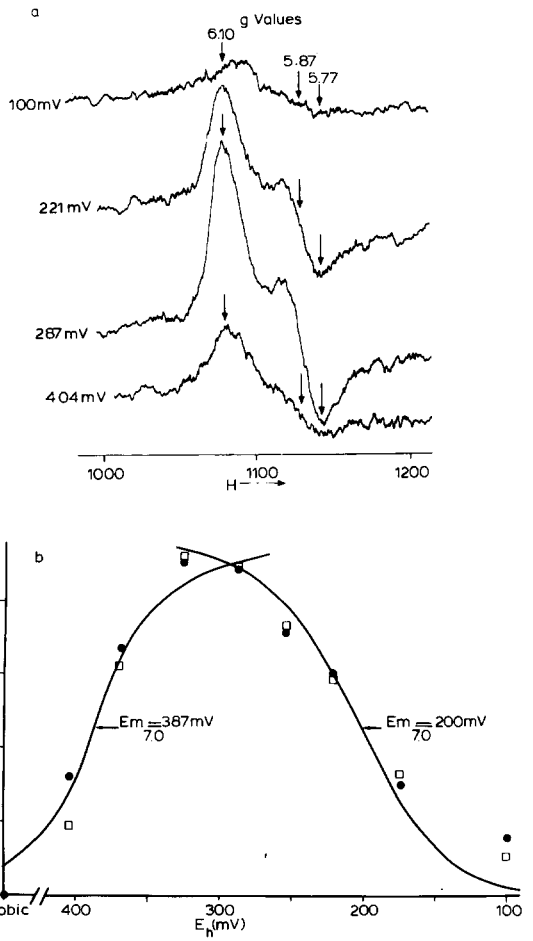


Fig. 7. (a) The ESR spectra of the high-spin hemes of the $100P_3$ fraction of succinate-grown *Thiobacillus* A_2. The fraction was suspended as described in the legend to Fig. 2 and aliquots at the indicated oxidation-reduction potential were transferred anaerobically to ESR sample tubes and frozen as described in Ref. 11. The ESR spectra were measured with the sample at 8 K and an instrument power setting of 40 mV (Varian E4 spectrometer). (b) Dependence of the measured signal height at *g* 6.1 on the oxidation-reduction potential of the sample prior to freezing. The measured height of the *g* 6.1 signal is plotted against the sample E_h value for duplicate experiments (● and □). The solid lines are $n = 1.0$ curves approximately fitted to the appearance and disappearance of the signal.

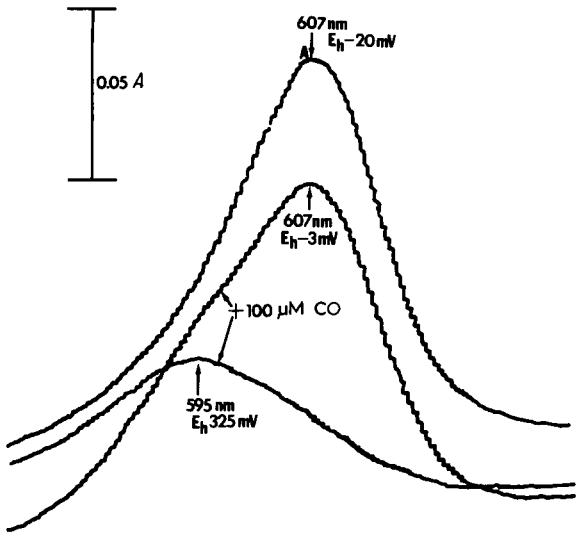


Fig. 8. The spectral properties of the CO complex of cytochrome a_3. The $100P_3$ fraction of thiosulfate-grown cells was suspended at 55 mg protein/ml in 0.1 M phosphate buffer, pH 8.0. Ferricyanide (100 μM), phenazine methosulfate (40 μM) and diaminodurene (40 μM) were added and the oxidation-reduction potential adjusted first to 440 mV and then to the indicated values. The presented spectra are for the absorption of the sample at the indicated E_h value minus the absorption of the sample at 440 mV. Trace A is for the sample in the absence of CO while the other traces are for the sample in the presence of 100 μM CO. Trace A is offset by a constant absorbance value throughout the spectral region in order to prevent confusion due to intersection of the traces.

Titration to an E_h values of 325 mV demonstrated an absorption maximum at 595 nm, that of the CO complex (Fig. 8). In the presence of 100 μM CO, 75% of the total absorbance at 607 nm was due to the component oxidized at an E_h value of 325 mV and 25% was due to the CO complex. Therefore, the addition of CO resulted in a 50% increase in the extinction coefficient of cytochrome *a*.

The dissociation constant (K_d) of CO from the CO complex was determined by titration of the absorbance change at 445 nm minus 460 nm with CO while the E_h value was approx. 0 mV. The data are plotted in Fig. 9. The slope of the line was −1.0, indicating that the binding of CO with cytochrome a_3 has a 1:1 stoichiometry and a K_d of 1.2 μM. Similar results were obtained using the wavelength pair 595 minus 620 nm.

Potentiometric titration of the formation of the reduced cytochrome a_3-CO complex

Since the half-reduction potential of the CO complex at pH 7.0 would be too positive for effective mediation by the ferricyanide couple ($E_{m,7.0}$ = 430 mV, pH independent), titrations were performed at pH 8.0 which lowered the half-reduction potential of the CO complex sufficiently for its oxidation by ferricyanide [15]. The titration curve with 3 μM CO was nonlinear with a midpoint potential of 335 mV (Fig. 10). In the presence of 30 μM CO, the titration curve approached linearity with an *n* value of 1.8 and a half-reduction potential of 393 mV. Theoretical curves were drawn through the experimental points according to the equation of Lindsay et al. [7, 16] using an E_m value for cytochrome a_3 of 350 mV and a K_d value of 1.2 μM. The calculated half-reduction potential of the high-potential copper in the presence of 30 μM CO as obtained by curve fitting was 365 mV.

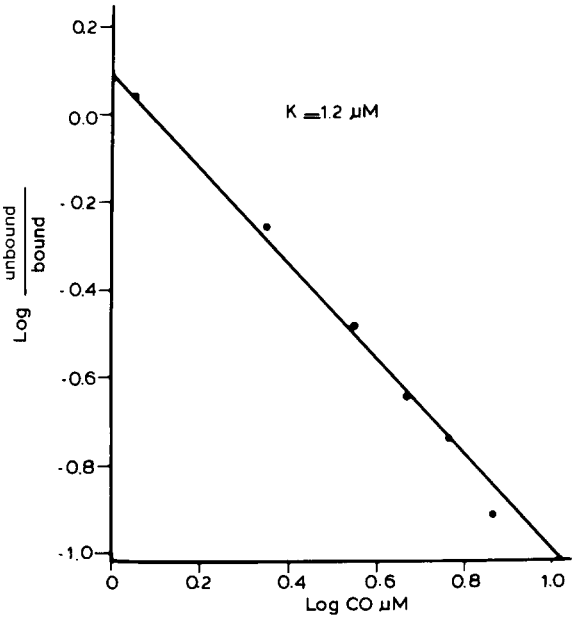


Fig. 9. The CO concentration dependence of the formation of the reduced cytochrome a_3-CO complex. The $100P_3$ fraction of succinate-grown cells was suspended at 2.75 mg protein/ml in 0.1 M phosphate buffer, pH 8.0. The cytochromes were reduced using a minimal amount of dithionite and CO was added as aliquots of buffer saturated with CO at atmospheric pressure. The formation of the CO complex was measured by the absorbance change at 445 nm minus 460 nm.

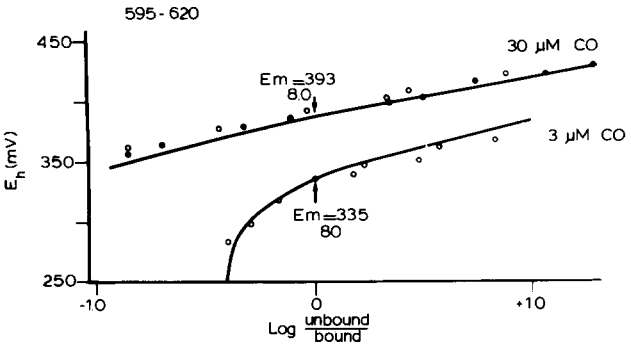


Fig. 10. Dependence of the formation of the reduced cytochrome a_3-CO complex on oxidation-reduction potential. The $100P_3$ fraction of succinate-grown cells was suspended at 2.8 mg protein/ml in 0.1 M phosphate buffer, pH 8.0. The titrations were carried out as previously described [7] in the presence of 3 and 30 μM CO. The formation of the CO complex was followed by the absorbance change at 595 minus 620 nm. The solid lines are theoretical curves fitted to the data as described by Lindsay et al. [7]. This fit gives an $E_{m,8.0}$ value of 365 mV for the invisible copper.

General observations

Addition of CO to the 20S fraction of either thiosulfate- or succinate-grown cells resulted in the appearance of an absorption maximum at 435 nm and a minimum at 447 nm indicative of the presence of cytochrome a_3 [18]. Prolonged sparging with CO (20 min) resulted in a spectral change characteristic of *o*-type cytochromes. This did not occur when the $100P_3$ fraction was treated with CO, suggesting that this component was removed during preparation of the membrane fraction. The presence of a soluble *o*-type cytochrome has been reported by Scholes and Smith [17].

It should be emphasized that the cytochromes in extracts of cells grown aerobically on thiosulfate and succinate were found to have the same absorption maxima and half-reduction potentials. The quantities of the cytochromes were the only difference noted. The regulation of cytochrome content in cells grown anaerobically on succinate with nitrate as the terminal electron acceptor was apparent from the observed absence of cytochromes $a + a_3$.

Attempts to detect phosphorylation of ADP during the oxidation of succinate or thiosulfate in 20S cell-free extracts of thiosulfate-grown *Thiobacillus* A_2 using the luciferin-luciferase methods [19] were unsuccessful.

Discussion

A higher content of cytochrome *c* in autotrophically grown when compared to heterotrophically grown *Thiobacillus* A_2 was reported by Taylor and Hoare [1]. A portion of the cytochrome *c* in cell-free extracts was not sedimented by centrifugation at $96000 \times g$ for 2 h. Similar results were reported in *T. novellus* by Charles [20], Cole [19] and Aleem [21]. The content (nmol cytochrome/mg dry wt. cells) of cytochromes *c* and *b* in autotrophically grown *Thiobacillus* A_2 was similar to that reported for *T. novellus*. However, in *T. novellus* grown on succinate the content of these cytochromes was 5-times greater than that found in *Thiobacillus* A_2 [19].

Numerous investigators [19,21,23–25] reported that intact thiobacilli use approx. 2 mol O_2 per mol thiosulfate utilized. The loss in respiration with thiosulfate as the substrate after autotrophically grown cells were disrupted was an enigma. Complete reduction of endogenous cytochrome *c* by thiosulfate in cell-free extracts suggests that the terminal oxidase was affected. However, reduction of endogenous cytochromes *c* and $a + a_3$ as well as respiration was easily demonstrated with succinate. The low recovery of respiration with thiosulfate as the substrate in cell-free extracts of cells grown autotrophically was observed in other thiobacilli [19,22,23]. In addition to the low respiratory rates, the stoichiometry of thiosulfate utilized to oxygen consumed was affected [23]. However, cell-free extracts of *T. novellus* [21] and *T. thiooxidans* [26] oxidized thiosulfate completely to sulfate.

The presence of multiple *b*-type cytochromes in animal mitochondria was established by potentiometric methods [8]. Two *b*-type cytochromes were reported for rat liver and pigeon heart mitochondria with $E_{m,7.2}$ values of +30 and −30 mV [27,28] while three components were detected in beef heart submitochondrial particles [29]. The participation of the additional high-potential cytochrome *b* ($E_{m,7.0} = 120$ mV) of beef heart submitochondrial particles in electron transport has been questioned as it is not present in intact mitochondria or isolated cytochrome b-c_1 complex [28]. Preliminary experiments suggested that it binds CO and may represent a denatured hemopro-

tein [28]. The cytochrome *b*-561 in pigeon heart mitochondria with $E_{m,7.2}$ +30 mV had an absorption maximum at 561 nm while the cytochrome *b*-565 ($E_{m,7.2}$ −30 mV) had absorption maxima at 558 and 565 nm [30]. The high-potential cytochrome with an $E_{m,7.0}$ value of 340 mV and absorption maximum at 560 nm is only tentatively identified as a *b*-type cytochrome.

The determination of the special properties of three *b*-type cytochromes in *Thiobacillus* A_2 as facilitated by using a digital memory unit which stored any selected spectrum at a defined oxidation-reduction potential and automatically subtracted it from later spectra taken at other potentials [7]. The low-potential cytochrome *b* ($E_{m,7.0}$ −90 mV) of *Thiobacillus* A_2 had a higher absorbance at 558 nm when compared to the cytochrome *b*-565 ($E_{m,7.2}$ −30 mV) in pigeon heart mitochondria [27,28]. The half-reduction potential of this cytochrome in mitochondria shifted approx. 240 mV more positive in the presence of ATP suggesting its role as a transducer of electrochemical energy into chemical energy at Site II of the respiratory chain [8,31]. The cytochrome *b* with $E_{m,7.0}$ +30 mV of *Thiobacillus* A_2 and pigeon heart mitochondria had the same absorption maximum, 562 nm.

Both of the *b*-type cytochromes ($E_{m,7.2}$ +30 and −90 mV) in *Thiobacillus* A_2 were reduced by succinate and the observed reduction of cytochrome *b* and oxidation of cytochromes *c*, *a* and a_3 in the presence in the presence of antimycin A suggested an antimycin A inhibitory site very similar to that of mammalian mitochondria [32,33].

Rotenone and amytal inhibit electron transfer between pyridine nucleotides and the cytochromesin mammalian mitochondria. Succinate oxidation was also inhibited in intact cells of *Thiobacillus* A_2, however, suggesting that succinate was metabolized to malate from which the reducing equivalents entered the electron-transport chain at the pyridine nucleotide level. In contrast, thiosulfate oxidation was not affected by rotenone or amytal, indicating that the transfer of reducing equivalents from thiosulfate to the electron-transport chain occurred at a higher oxidation-reduction level than that of pyridine nucleotides. Inhibitor and spectra data indicated the oxidation of thiosulfate was mediated by flavoprotein, cytochromes *c* and $a + a_3$. The high cytochrome *c* concentration in intact cells would have masked the *b*-type cytochromes. The enzymatic reduction of cytochromes of *b*, *c* and *a* types occurred at the expense of electrons transferred from thiosulfate, sulfide and sulfite in *T. denitrificans*. Either oxygen or nitrate (under anaerobic conditions) could serve as oxidant for the cytochrome system [24,34]. This demonstrated the role of O_2 and NO_3^- as terminal electron acceptors for the cytochromes of *c* and types in the mediation of thiosulfate oxidation. In *T. novellus*, the process was insensitive to amytal, atabrine and antimycin A [21]. Purified cytochrome *c* from *T. novellus* has been reported to have a half-reduction potential at pH 7.0 of +276 mV [35], and to act as electron acceptor for the sulfite oxidoreductase [36].

Reduction of endogenous cytochrome *c* by thiosulfate in cell-free extracts of *Thiobacillus* A_2 without a significant respiratory rate suggested that the terminal oxidase of this respiratory chain was inhibited. That the 'thiosulfate oxidase' and the 'succinate oxidase' electron-transport chains might be different structurally and/or functionally could be suggested from the observation that the reduction of the endogenous cytochromes *c*, *b* and *a* and a_3 as well as respiration with succinate was demonstrated in cell-free extracts of thiosulfate-grown cells.

The components of cytochrome oxidase in *Thiobacillus* A_2 were remarkably similar to those of mammalian mitochondria. Two cytochromes, *a* and a_3, were present and were similar to the mammalian enzyme with respect to spectral properties [12–14,37,38] and half-reduction potentials. Moreover, the heme ESR absorptions were comparable with a signal due to a high-spin ferric heme in an asymmetric environment appearing as the high-potential cytochrome was reduced and disappearing as the low-potential cytochrome was reduced [11,39,40]. Mammalian cytochrome oxidase contains two copper atoms (per cytochrome $a + a_3$). In the present study, the 'visible' copper could not be unequivocally demonstrated by ESR measurements due to the presence of interfering signals. The presence of the invisible copper, however, could be readily detected and its oxidation-reduction properties measured by its effect on the formation of the reduced cytochrome a_3-CO complex

[7,16]. As was observed for mammalian cytochrome oxidase, during potentiometric titrations the formation of the reduced cytochrome a_3-CO complex required two reducing equivalents per CO complex formed. From the measured dependence of the half-reduction potential for the formation of the CO complex on CO concentration the half-reduction potential of the invisible copper at pH 8.0 could be calculated to be 365 mV. This copper atom has been considered to be an essential part of the active site for oxygen reduction, participating in the electron reduction of oxygen to form a bridged peroxide intermediate [7,38]. It appears that a similar mechanism is present in *Thiobacillus* A_2.

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