Terminal Electron Transport in *Treponema pallidum*

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Reduced-minus-oxidized difference spectra of sonically treated virulent *Treponema pallidum* disclosed cytochromes of the b and c types as well as large amounts of flavoprotein. Difference spectra of the carbon monoxide-binding pigment identified cytochrome o as the terminal oxidase. Physiological reduction of the cytochromes indicated that the cytochrome system was functional and established the capability of *T. pallidum* for aerobic respiration. The potential significance of these findings is discussed.

Numerous attempts to cultivate *Treponema pallidum* anaerobically in vitro have failed (22, 28). This treponeme has been considered to be an anaerobe (Bergey's Manual, 8th ed.). However, Cox and Barber (5) recently reported O₂ uptake by *T. pallidum*, which occurred at a rate comparable to that of the known aerobic *Leptospira*. Oxygen uptake was observed to be a function of treponeme concentration, and found to be cyanide sensitive, and was shown to be inhibited by azide, chlorpromazine, and amytal. Cyanide, at concentrations higher than inhibitory levels, established transient increases in respiration, suggesting that electron transport was coupled to oxidative phosphorylation. Recently, Schiller and Cox (23) have presented evidence that glucose was degraded via the Embden-Meyerhof-Parnas and hexose monophosphate pathways and were unable to demonstrate a functioning Krebs cycle. The latter would seem to place severe restrictions on the ability of *T. pallidum* to generate adenosine 5'-triphosphate unless aerobic respiration involved oxidative phosphorylation coupled to a terminal electron transport system. This report represents the results of our search for terminal electron transport mechanisms in *T. pallidum*.

**MATERIALS AND METHODS**

Virulent *T. pallidum* was maintained by serial passage in rabbits as previously described (5). Testicles from exsanguinated rabbits were removed aseptically, trimmed, and cut once longitudinally with scissors. The edges of the testicles were snipped several times with scissors to provide exit channels for the treponemes. Testicles were dipped into extraction medium to remove excess testicular debris and then added to extraction flasks containing 10 ml of fresh extraction medium per testicle. The extraction medium consisted of 0.14 M NaCl, 0.01 M Na₂HPO₄, and 0.06% reduced glutathione and was adjusted to pH 7.3 with NaOH. Extractions were carried out aerobically with shaking for 1 h at room temperature followed by 1 h at 4°C. Separation of *T. pallidum* from tissue components was approached as previously described (23; Fig. 1). The use of 0.8-µm Nucleopore filters to separate treponemes from tissue cells has been shown by Schiller and Cox (23) to be highly effective. With this procedure, treponemal preparations were found to contain <200 tissue cells per ml, which was the limit of detection. The centrifugation at 300 × g was performed in a Sorvall RC2-B centrifuge equipped with 50-ml swinging buckets. All other centrifugations were carried out in a Sorvall RC2-B centrifuge using an SS34 rotor. High-speed pellets of filtered and centrifuged treponemes were not dispersed, but were rinsed in 40 ml of extraction medium, pooled, and recentrifuged. This pellet was suspended in 0.02 M phosphate buffer (pH 7.3), and the cells were ruptured by sonic oscillation. The sonic extract was divided into supernatant (SS) and pellet (SP) fractions by centrifugation at 32,000 × g for 20 min. These were the treponemal fractions that were examined for cytochromes.

Four controls were derived using the scheme in Fig. 1. The tissue cell control consisted of low-speed pellets suspended in high-speed supernatant fluid to a concentration of 10⁶ tissue cells per ml, which was at least 500 times the actual concentration of tissue cells, as mentioned previously. The high-speed supernatant fluid (C4) also served as a control for soluble components from tissue cells and/or treponemes. Controls were also derived from filtered treponemal suspensions that had been further filtered to remove the treponemes. The 0.8-µm filtrate (T2) was passed by negative pressure through successively smaller Nucleopore and Millipore filters, with a final filtration through a 0.2-µm Nucleopore membrane to obtain C1. This filtrate was centrifuged to obtain supernatant fluid (C2) and pellet (C3) controls. This procedure effectively removed essentially all of the treponemes. All four controls were sonically extracted and centrifuged at 32,000 × g as described for the treponemal pellet T3. The supernatant fraction of each control was then examined spectrophotometrically for cytochromes.

Cytochrome difference spectra were measured at ambient temperature with a Cary model 14 record-
RESULTS

A typical cytochrome spectrum of the supernatant fraction (SS) from sonically treated *T. pallidum* is presented in Fig. 2. NADH reduced-minus-oxidized spectra yielded \( \beta \)-maxima at 520 nm and \( \gamma \) (Soret)-maxima at 426 nm, indicating a combination of \( b \)- and \( c \)-type cytochromes. In the \( a \)-region there was a clear distinction between a \( c \)-type cytochrome at 553 nm and a \( b \)-type cytochrome at 558 nm. The trough in the 455-nm region indicated that there were large amounts of flavoproteins in *T. pallidum*, as have been observed in the other spirochetes *Spirochaeta aurantia* (3), *Treponema hyodysenteriae* (R. A. Harris, D. L. Harris, and J. M. Kinyon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, D43, p. 58), and *Leptospira* sp. (1). Reduction with dithionite increased both the height of the cytochrome peaks and the depth of the flavoprotein trough. The addition of ferricyanide to the reference cuvette did not increase the peak heights, which showed that the preparation was already fully oxidized.

![Schematic diagram for the selective removal of tissue cells and treponemes from infected testicular extracts in order to obtain tissue-free treponemes (T3) and controls. The treponemal pellet (T3) was sonically treated and centrifuged at 32,000 \( \times g \) to obtain supernatant and pellet fractions for cytochrome scans. Controls included tissue cells from the low-speed pellet (LSP) suspended in high-speed supernatant (HSS), HSS alone (C4), and the supernatant fluid (C2) and pellet (C3) fractions derived from C1. All controls were sonically treated and centrifuged as for the treponemal pellet T3, and the supernatant fraction from each centrifugation was scanned for cytochromes.](http://iai.asm.org/)
An oxidase test was performed according to the method of Kovac as described by Steel (26), using a fresh prepared solution of 1% tetramethyl-p-phenylenediamine dihydrochloride. The treponemal pellet T3 turned purple within 2 s when applied to a filter paper soaked in fresh oxidase reagent, which indicated the presence of a terminal oxidase.

Carbon monoxide difference spectra of SS preparations (Fig. 2) revealed a Soret maximum at 419 nm accompanied by both a prominent trough at 432 nm and maxima at 539 and 572 nm, all of which indicated that cytochrome o, a CO-binding pigment, was the terminal cytochrome oxidase. The peak at 455 nm was due to the slight reoxidation of flavoproteins. There was no evidence for an α-type cytochrome in the CO spectra, yet a small broad peak did exist around the 600-nm region in reduced-minus-oxidized spectra. It is unclear at this time whether or not this may be due to an α-type cytochrome.

Difference spectra of the pelleted fraction (SP) of sonically treated T. pallidum were difficult to obtain due to light scattering. Maxima at 430 and 560 nm plus a shoulder around the 530-nm region indicated the presence of the β-type cytochrome. Flavoproteins were also present, but there was no indication of a cytochrome c. As with the SS fraction, there was a small broad peak around the 600-nm region, which may be due to an α-type cytochrome.

The physiological reduction of cytochromes and flavoproteins by NADH was 80 to 90% of the level obtained with dithionite (Fig. 2) and indicated that T. pallidum had a functioning electron transport system. Cytochromes were also reduced with reduced NADP, glucose-6-phosphate plus NADP, 6-phosphogluconate plus NADP, glyceraldehyde-3-phosphate plus NAD, malate plus NAD, isocitrate plus NADP, and lactate plus NAD (Fig. 3). There was no reduction when using either succinate or pyruvate with or without cofactors or when malate, isocitrate, or lactate was used without pyridine nucleotides. When malate, isocitrate, or lactate plus pyridine nucleotides were used to physiologically reduce the cytochromes, a previously undetected cytochrome c with an α-maximum at 550 nm appeared (Fig. 3). There was no appearance of a trough at 455 nm, which would have indicated flavoprotein reduction. The cytochrome c550 was stable in an 8.5 mM concentration of H2O2 and could be repeatedly reduced after being oxidized by H2O2. Cytochromes c553 and b558 could not be physiologically reduced again after oxidation with H2O2, and, after their inactivation, only cytochrome c550 was reduced with NADH. However, cytochromes c553 and b558 were able to be reduced with dithionite after H2O2 oxidation. Cytochrome c550 did not appear to bind CO and therefore did not seem to function as a terminal oxidase.

The extinction coefficients that were used to determine cytochrome concentrations were arbitrarily selected, since no values exist for T. pallidum cytochromes. However, it was felt that these coefficients would be a close approximation and that the calculations would be of value, perhaps for comparison with future calculations of cytochrome concentrations of treponemes grown in vitro. The cytochrome concentrations are presented in Table 1. All of the cytochromes were detected in the SS fraction, whereas the SP fraction contained only b558.

Washing whole treponemes with high salt (200 mM NaCl) removed T. pallidum cytochromes. A single high-salt wash removed all

![Fig. 3. Difference spectra of supernatant fluids of sonically treated T. pallidum. (A) Glucose-6-phosphate plus NADP reduced-minus-oxidized spectrum; similar curves were obtained with 6-phosphogluconate plus NADP, with glyceraldehyde-3-phosphate plus NAD, and with reduced NADP. (B) Malate plus NAD reduced-minus-oxidized spectrum; similar curves were obtained with isocitrate plus NADP and with lactate plus NAD. (C) Curve B after addition of 8.5 mM H2O2; only cytochrome c550 could be physiologically reduced after this treatment.](http://iai.asm.org/)
detectable c-type cytochromes from the SS fraction. Two high-salt washes accompanied by vigorous agitation on the Vortex mixer effectively removed the b-type cytochrome as well. In both cases, treponemal cells remained intact as observed by dark-field microscopy, and flavoproteins were detected in the usual amounts.

The possibility of tissue contamination of the treponemal preparations was strongly considered and studied. NADH reduced-minus-oxidized spectra of the tissue cell control and controls C2 and C4, adjusted where possible to the protein concentrations of the treponemal preparations, failed to show the presence of any cytochromes. C3 was found to contribute only minutely to the T3 pellet and contained such a small amount of protein that no cytochromes could have been detected had they been present. Extracts of harshly ground infected rabbit testicles, prepared in the same manner as C2, C3, and C4, also failed to show evidence of tissue cytochromes. The presence of rabbit hemoglobin was a major concern, since its CO-bound spectrum was identical to that of cytochrome o, due to the presence of protoporphyrin IX as the prosthetic group for these heme proteins. Controls included a lysate of rabbit erythrocytes and a commercial preparation of crystallized rabbit hemoglobin (Sigma Chemical Co.). Hemoglobin was judged by difference spectroscopy to be present in the C2 and C4 supernatant controls, as well as in the tissue cell control, after comparison with scans of the prepared hemoglobin solutions. However, when either hemoglobin or the control preparations were treated with NADH, they were found to be reduced to only 5% of the level obtained with dithionite. Since the treponemal preparations derived from T3 were reduced 80 to 90% with NADH, we concluded that rabbit hemoglobin, if at all present, could not be a significant source of error.

**DISCUSSION**

The lack of an a- or c-type cytochrome in the CO-bound spectrum suggests that cytochrome o functions as the only cytochrome oxidase in *T. pallidum*. Cytochrome o is a b-type cytochrome (7) and may, in fact, be identical to cytochrome b$_{558}$, as has been proposed for *S. aurantia* (3). However, there is the possibility that another b-type cytochrome is present in addition to cytochrome o. Such a possibility has been discussed for the spirochetes *S. aurantia* (3) and *Leptospira* (1). The presence of c-type cytochromes in *T. pallidum*, along with cytochrome b$_{558}$ places this treponeme in an intermediate position between the "unicytochrome" of *S. aurantia* (3) and the leptospires that have c-, a-, and a-type cytochromes as well (1).

*T. pallidum* c-type cytochromes are able to be readily released from the cells as has been described for *Haemophilus parainfluenzae* cytochrome c$_1$ (25) and various other gram-negative bacterial as well as mitochondrial c cytochromes (10-12). It is unknown at the present time what part cytochrome c$_{550}$ may play in electron transport. NADH has been shown to reduce this cytochrome if the preparation is first treated with H$_2$O$_2$ to prevent the reduction of cytochromes c$_{553}$ and b$_{558}$, but it is unknown whether c$_{550}$ participates in electron transport during normal NADH oxidation, since it could easily be masked by the larger c$_{553}$ peak. The ability of c$_{550}$ to be repeatedly oxidized by H$_2$O$_2$ and then reduced physiologically suggests that it might function with a cytochrome c peroxidase as has been described for *Pseudomonas fluorescens* (17, 18). In the latter system, the reduced cytochrome c donates electrons to H$_2$O$_2$ to yield two H$_2$O. It was suggested by those authors that cytochrome peroxidase functions optimally at low O$_2$ tensions. Whether this system is present in *T. pallidum* must await the cultivation of the treponemes in vitro so that enzyme systems can be isolated.

The fact that malate plus NAD and isocitrate plus NADP were able to physiologically reduce the cytochrome c$_{550}$ argues for the importance of malate and isocitrate dehydrogenases in an otherwise absent tricarboxylic acid cycle (23). The high levels of malate dehydrogenase (EC 1.1.1.37) found by Schiller and Cox (23) could mean that this enzyme is a major source of reducing power.

It is important to note that there is no concomitant flavoprotein reduction when cytochrome c$_{550}$ is reduced by malate, isocitrate, or lactate plus pyridine nucleotides. A lactate dehydrogenase that donates electrons directly to cytochrome c has been noted in acetic acid bacteria (8, 9), which are rich in tightly bound dehydrogenases. A malate dehydrogenase in *Micrococcus lysodeikticus* (13) donates electrons directly to vitamin K$_2$. The malate-vitamin K reductase of *Mycobacterium phlei* (4) donates electrons directly from malate into the respiratory chain and also bypasses the flavoproteins. If the *T. pallidum* cytochrome c$_{550}$ acts as in the above systems, it may be the electron acceptor of a membrane-bound dehydrogenase or quinone. Since cytochrome c$_{550}$ does not appear to be a terminal oxidase, it is difficult to explain where the electrons go from the reduced state. The possibility of interaction with a cytochrome peroxidase has been mentioned. Another possibility is that cytochrome
c₅₅₀ normally interacts with c₂₃₃ and b₂₅₈, but that during sonic oscillation, the dehydrogenases and c₅₅₀ may become separated from the other cytochrome components. It is known that some dehydrogenases form complexes with c-type cytochromes (15, 21) and that a spatial disorientation caused by ultrasound, lysis, or osmotic shock can prevent the oxidation of substrates and subsequent cytochrome reduction (13, 24, 27).

It is interesting to note that both NADH and reduced NADP are able to reduce the cytochromes. The results of the physiological reductions suggest that glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12), phosphogluconate dehydrogenase (EC 1.1.1.43), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), as well as the previously mentioned dehydrogenases, provide the treponemes with a major portion of reducing power. The presence of these enzymes has been determined by Schiller and Cox (23) and was part of the accumulated evidence that indicated that T. pallidum degraded glucose by both the Embden-Meyerhoff-Parnas and hexose monophosphate pathways. T. pallidum would therefore seem to possess both "NADH oxidase" and "NADPH oxidase" activity.

The inability of pyruvate to physiologically reduce cytochromes fits the lack of a tricarboxylic acid cycle (23). The inability of succinate to physiologically reduce cytochromes fits the lack of oxoglutarate dehydrogenase (EC 1.2.4.2) and succinate dehydrogenase (EC 1.3.99.1) (23) but constitutes an enigma, since succinyl-coenzyme A is instrumental in protoporphyrin synthesis (16). If T. pallidum is unable to synthesize heme, tissue porphyrins may serve as the source of ready-made prosthetic groups. Preliminary data indicated that harshly ground testicular preparations possessed hemoglobin along with the treponemes, which is the reason we employed a less disruptive extraction procedure. In addition, the hemoglobin present in these ground extractions disappeared with time, indicating the possibility of a protease. The action of proteolytic enzymes that degrade hemoglobin could cause the removal of the porphyrin prosthetic group for modification and reassembly into new treponemal heme proteins. Heme may well be needed for in vitro growth.

The presence of large amounts of flavoproteins indicates that T. pallidum may be heavily dependent on flavin-linked enzymes. Various flavoproteins can catalyze the reduction of ferric citrate chelates. Baseman et al. (2) observed an inhibition of protein synthesis after the addition of ferric ammonium citrate and suggested the interuption of an energy-generat-

ing electron flow. An artificial electron acceptor acting as a flavoprotein oxidant would certainly divert electrons needed for energy generation and protein synthesis. The stimulation of CO₂ release from pyruvate by ferric ammonium citrate under anaerobic conditions could also be explained by the oxidation of a flavoprotein that would facilitate the reaction by accepting electrons from NADH, which in turn could be generated during the decarboxylation of pyruvate. Flavoproteins could serve as effective electron carriers during these periods of low O₂ tension.

The presence of cytochrome o as a terminal oxidase explains the exquisite sensitivity of T. pallidum to micromolar levels of cyanide, which has been previously reported (5). Baseman et al. (2) demonstrated the sensitivity of protein synthesis to millimolar levels of cyanide but not to micromolar levels. Whereas protein synthesis might not be as sensitive as O₂ uptake to cyanide, the inability of Harris et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, D4, p. 52) to demonstrate sensitivity of O₂ uptake to micromolar levels of cyanide is perplexing. In this investigation we have demonstrated that c-type cytochromes are eluted by 200 mM NaCl, which is not uncommon for a bacterial system. It is possible that the use of hypertonic extraction media could inadvertently remove cytochrome c, effectively perturbing electron transport and cyanide sensitivity.

The results of this investigation leave no doubts in our minds as to the capabilities of virulent T. pallidum for aerobic respiration. The evidence supports the concept of a functioning flavoprotein-cytochrome electron transport system driven by the oxidation of NADH and reduced NADP. Previous reports of O₂ toxicity may be explained on the basis of substrate or cofactor depletion or by the oxidative formation of toxic intermediates. How well T. pallidum can circumvent these problems may directly relate to in vitro survival and growth.

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LITERATURE CITED


