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# THE RESPIRATORY CHAIN OF *PARAMECIUM TETRAURELIA* IN WILD TYPE AND THE MUTANT $Cl_1$

## I. SPECTRAL PROPERTIES AND REDOX POTENTIALS

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## Summary

1. Purified mitochondria have been prepared from wild type *Paramecium tetraurelia* and from the mutant $Cl_1$ which lacks cytochrome $aa_3$. Both mitochondrial preparations are characterized by cyanide insensitivity. Their spectral properties and their redox potentials have been studied.

2. Diffference spectra (dithionite reduced minus oxidized) of mitochondria from wild type *P. tetraurelia* at 77 K revealed the $\alpha$ peaks of *b*-type cytochrome(s) at 553 and 557 nm, of *c*-type cytochrome at 549 nm and *a*-type cytochrome at 608 nm. Two $\alpha$ peaks at 549 and 545 nm could be distinguished in the isolated cytochrome *c* at 77 K. After cytochrome *c* extraction from wild type mitochondria, a new peak at 551 nm was unmasked, probably belonging to cytochrome $c_1$. The *a*-type cytochrome was characterized by a split Soret band with maxima at 441 and 450 nm. The mitochondria of the mutant $Cl_1$ in exponential phase of growth differed from the wild type mitochondria in that cytochrome $aa_3$ was absent while twice the quantity of cytochrome *b* was present. In stationary phase, mitochondria of the mutant were characterized by a new absorption peak at 590 nm.

3. Cytochrome $aa_3$ was present at a concentration of 0.3 nmol/mg protein in wild type mitochondria and ubiquinone at a concentration of 8 nmol/mg protein both in mitochondria of the wild type and the mutant $Cl_1$. Cytochrome $aa_3$ was more susceptible to heat than cytochromes *b* and $c,c_1$.

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

4. CO difference spectra at 77 K revealed two different CO-cytochrome complexes. The first, found only in wild type mitochondria, was a typical CO-cytochrome $a_3$ complex characterized by peaks at 596 and 435 nm and troughs at 613 and 450 nm. The second, found both in mitochondria of the wild type and the mutant, was a CO-cytochrome *b* complex with peaks at 567, 539 and 420 nm and a trough at 558—549 nm. Both complexes are photo-dissociable.

5. Spectral evidence was obtained for interaction of cyanide with the *a*-type cytochrome (shift of the $\alpha$ peak at 77 K from 608 to 605 nm), but not with the *b*-type cytochrome.

6. The mid-point potentials of the different cytochromes at neutral pH are as follows: cytochrome $aa_3$ 235 and 395 mV, cytochrome $c,c_1$ 233 mV, cytochromes *b* 120 mV.

## Introduction

Although the mitochondrial respiratory chain of protozoa carries out oxidative phosphorylation with the same efficiency as the respiratory chain of mammalian mitochondria does, there appear to exist marked differences between them in the spectral and redox properties of the mitochondrial components [1—5]. The protozoa which have been extensively studied in this respect include *Tetrahymena pyriformis* [1,2], a number of trypanosomatids (cf. Ref. 3) and *Acanthamoeba castellanii* [4]. *Paramecium tetraurelia* [5] is another example of protozoa with some unusual properties of the components involved in mitochondrial respiration. In the present report, we describe the spectra and redox properties of mitochondria isolated from the wild type *P. tetraurelia* and also from the mutant strain $Cl_1$ [6,7]. This mutant is of great interest for study on the respiratory chain of *Paramecium* since it is deficient in cytochrome $aa_3$ and resistant to cyanide.

Beside giving a detailed analysis of the respiratory components of the wild type and the mutant, the following experiments reveal a CO-binding pigment, characterized as a *b*-type cytochrome, and present both in mitochondria of wild type and the mutant.

## Materials and Methods

### *Strains*

The wild type strain used originated from stock d4-2 of *Paramecium tetraurelia* according to the new nomenclature of Sonneborn [8]. The mutant $Cl_1$ was obtained after ultraviolet light mutagenesis from the wild type strain [6]. It is a double mutant carrying a nuclear mutation $Cl_1$ and a mitochondrial mutation $M^{c1}$ [7]. The $Cl_1$ mutation severely disturbs the structure and function of the wild type mitochondria. The $M^{c1}$ mutation partially suppresses this effect.

Maintenance of the strains and preparation of media were described by Sonneborn [9]. Cells were grown at 28°C in a 'Scotch' grass infusion supplemented with $\beta$-sitosterol (3 mg/l) and inoculated the day before use with *Klebsiella pneumoniae*. In these conditions, the generation time of the wild

type was 6 h, that of the mutant $Cl_1$ 8—9 h. Cells were harvested by centrifugation at 20°C for 3 min at 100 × *g* in the 4 times 1000 ml rotor of an International centrifuge in pear-shaped oil-testing centrifuge bottles and washed once with 0.5 M mannitol, 5 mM morpholinopropanesulfonate. Unless otherwise mentioned the wild type cells were obtained from cultures in the stationary phase (about $4 \cdot 10^3$ cells/ml) and mutant cells from cultures in the mid exponential phase (about $1.5 \cdot 10^3$ cells/ml).

*Isolation of mitochondria*

Rupture of cells and isolation of mitochondria were carried out at 4°C as follows. The packed cells were suspended in 30 ml of a medium consisting of 0.5 M mannitol, 0.5% bovine serum albumin, 5 mM morpholinopropanesulfonate, 1 mM EDTA, final pH 7.3 (homogenization medium). The suspended cells were disrupted in a hand homogenizer (Thomas emulsion homogenizer). The homogenate was centrifuged at 600 × *g* in a Sorvall SS1 for 5 min. The pellet was discarded and the supernatant fluid centrifuged at 600 × *g* for 10 min. The mitochondrial pellet was resuspended in the same medium and the suspension centrifuged at 600 × *g* for 4 min to eliminate trichocysts. The supernatant was recentrifuged at 5000 × *g* for 10 min to sediment the mitochondrial particles (crude mitochondrial fraction). The crude mitochondrial fraction was purified by centrifugation through a gradient of sorbitol (40—60%, w/w) containing 5 mM morpholinopropanesulfonate buffer and 1 mM EDTA, pH 7.3, at 150 000 rev./min for 1 h in a SW 25-2 Spinco rotor. After centrifugation the layer corresponding to the mitochondrial particles was carefully removed and diluted 10-times with the homogenization medium. For further spectroscopic and potentiometric studies, it was essential that the mitochondrial preparations should not be contaminated by bacteria initially present in the growth medium. Significant contamination of *Paramecium* mitochondria by *Kl. pneumoniae* can be ruled out on the basis of the following observations. First, the reduced spectrum of *Kl. pneumoniae* markedly differs from that of *Paramecium* mitochondria, especially by a well-characterized peak at 635 nm; this peak is absent in preparations of *Paramecium* mitochondria. Second, in the stationary phase of growth, most of the bacteria in the growth medium have been ingested and digested by *Paramecium* cells which then become starved; this is not the case of *Paramecium* cells in the exponential phase of growth, which are still ingesting actively and digesting bacteria; yet in both cases the spectra of mitochondria were identical. Third, electron microscopy of pellets of mitochondrial preparations showed small contamination (10—20%) by trichocystes and peroxysomes arising from the *Paramecium* cells, but failed to detect contamination by bacteria higher than 2%.

Submitochondrial particles were obtained by sonication of purified mitochondria in a Branson sonifier at maximal output for 2 min at 2°C. Unbroken mitochondria were removed by centrifugation at 10 000 × *g* for 15 min. The submitochondrial particles present in the supernatant were sedimented by centrifugation at 100 000 × *g* for 1 h and the pellet was resuspended in the homogenization medium. For spectrophotometric and potentiometric assays, the suspensions of mitochondria or sonicated particles were diluted in 0.5 M mannitol, 5 mM $MgCl_2$ and 10 mM phosphate buffer, pH 7.2.

*Spectrophotometric measurements*

Difference spectra of mitochondria or mitochondrial fragments were recorded with a Cary model 15 Spectrophotometer either at 77 K or at room temperature [10]. The optical paths of the cuvettes were 3 mm for spectral analysis at 77 K and 1 cm at room temperature. Reduction of the sample cuvette was obtained by addition of a few crystals of dithionite or by respiratory substrates. Oxidation of the reference cuvette was obtained by 1 mM $H_2O_2$ (catalase present in the mitochondrial preparations generates $O_2$ from $H_2O_2$). For CO difference spectra, the mitochondria were reduced with dithionite in the sample and the reference cuvettes, the sample cuvette being flushed with CO for 5 min; then the two cuvettes were frozen at 77 K. In photodissociation assays, light from a 375 W projector lamp equipped with an appropriate filter was focused on the cuvette containing the mitochondrial suspension at 77 K.

*Potentiometric titrations*

Potentiometric titrations were carried out at 20°C in an anaerobic chamber of 6 ml volume and an optical path of 1 cm, under a constant flow of purified argon (oxygen content <1 ppm), as described by Dutton [11]. Potentials were measured with a platinum electrode against a calomel standard. Simultaneous measurements of oxidation-reduction potentials and of absorbance with a dual wavelength spectrophotometer were carried out, using potassium ferricyanide as the oxidant and freshly prepared dithionite as the reductant. The medium consisted of 50 mM Tris-HCl, 10 mM phosphate buffer, and 2 $\mu$M FCCP, final pH 7.2. Purified mitochondria were employed at a concentration of 2—3 mg/ml. The following concentrations of redox mediators were used: *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD) 5 $\mu$M, diaminodurene 20 $\mu$M, phenazine methosulfate 30 $\mu$M, phenazine ethosulfate 30 $\mu$M, duroquinone 10 $\mu$M, pyocyanine 5 $\mu$M, 2 hydroxy 1,4-naphtoquinone 5 $\mu$M. Separation of composite curves into individual components was performed as described by Wilson and Dutton [12].

*Extraction of cytochrome c*

After centrifugation, the mitochondria were resuspended in cold distilled water to a final concentration of 2 mg/ml and left to stand for 5 min at 2°C. Then KCl was added to a final concentration of 0.25 M. After 15 min at 2°C, the mitochondria were sedimented by centrifugation. A second washing by 0.25 M KCl resulted in a complete extraction of cytochrome *c*. The supernatant fluids were recovered and concentrated for spectral analysis of the extracted cytochrome *c*. Oxidation of reduced cytochrome *c*, extracted from *Paramecium* mitochondria, by cytochrome *c*-depleted *Paramecium* mitochondria was carried out, following the same method as that described by Appelmans et al. [13] for mammalian mitochondria.

*Other determinations*

The amount of ubiquinone present in mitochondria and its percentage of reduction in different metabolic states were determined essentially by the method of Redfearn [14]. The molecular extinction coefficient for the differ-

ence in absorption of the oxidized and reduced forms of ubiquinone at 275 nm was assumed to be 12.5 $mM^{-1} \cdot cm^{-1}$. For further characterization, ubiquinone was chromatographed on silica plates impregnated with paraffine oil, using acetone/water (95 : 5) as solvent [15]. The haem *a* content was measured from the absorption spectrum at room temperature as described by Yonetani [16], assuming the same $\Delta\epsilon$ mM 608—630 nm = 16.5 as for mammalian cytochrome *a*.

Thermodenaturation of cytochrome $aa_3$ was carried by heating at 55°C for 4 min wild type mitochondria suspended in 0.5 M mannitol, 5 mM $MgCl_2$ and 10 mM phosphate buffer (pH 7.2) at a concentration of about 10 mg protein/ml.

Protein content was estimated by the biuret method [17].

## Results

*Components of the respiratory chain in mitochondria of wild type and the mutant $Cl_1$*

The spectra shown in Fig. 1 were taken at 77 K. The difference spectrum (dithionite reduced minus oxidized) of wild type mitochondria reveals peaks at 608, 450 and 441 nm which are attributable to cytochromes $aa_3$, by analogy with mammalian cytochrome $aa_3$. Maxima at 557, 553, 526 and 426 nm can be tentatively assigned to *b*-type cytochrome(s) and those at 549, 518 and 419 nm to *c*-type cytochromes (Fig. 1A). At room temperature the $\alpha$ bands of cytochrome *c* and *b* were shifted by 2—3 nm to higher wavelengths. Reduction in anaerobiosis by succinate resulted in a spectrum pattern similar to that obtained with dithionite; the extent of reduction of cytochrome(s) *b* was 70—80% of that obtained by dithionite; it amounted to 90—95% for the *c* and *a*-type cytochromes. The same difference spectrum (dithionite-reduced minus oxidized) was observed for whole mitochondria and submitochondrial particles obtained by sonication. To ascertain the respective assignment of the peaks in the 550—560 nm region to cytochromes *b* and *c*, cytochrome *c* was extracted from mitochondria (see Methods). The spectrum of wild type mitochondria depleted of cytochrome *c* (Fig. 1 trace B) reveals the typical maxima of cytochrome(s) *b* at 553 and 557 nm and a new peak at 551 nm probably belonging to cytochrome $c_1$. The main peaks of the extracted cytochrome *c* after reduction by dithionite are at 549, 545, 518 and 419 nm (Fig. 1 trace E); it may be noted that the $\alpha$ peaks at 549 and 545 nm are clearly resolved at 77 K. The spectrum of mitochondria reduced by ascorbate (Fig. 1 trace C) shows peaks of both cytochrome *c* and cytochrome $aa_3$. The difference spectrum, dithionite minus ascorbate (Fig. 1 trace D), permits a clear identification of peaks of cytochrome(s) *b* and 553 and 557 nm.

Specific interaction of cytochrome *c* and cytochrome $aa_3$ in wild type mitochondria is indicated by the following observations. Reduced cytochrome *c*, isolated from wild type *Paramecium* was easily oxidized by cytochrome *c*-depleted wild type mitochondria. The same preparation of mitochondria was unable to oxidize reduced cytochrome *c* isolated from beef heart or *Saccharomyces cerevisiae* mitochondria.

The difference spectrum of mitochondria of the mutant harvested during the

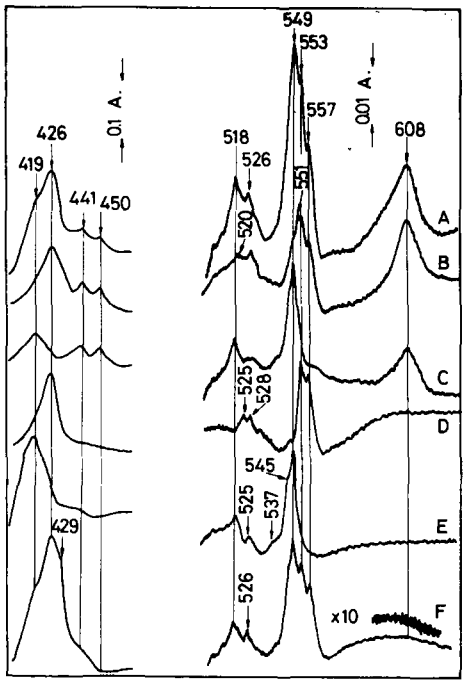


Fig. 1. Difference spectra obtained at 77 K. (A) wild type mitochondria (2.7 mg protein/ml) reduced with dithionite minus oxygenated reference. (B) As (A), but with cytochrome *c* depleted mitochondria (2.5 mg protein/ml). (C) wild type mitochondria (2.2 mg protein/ml) reduced with 3 mM ascorbate plus 50 $\mu$M TMPD minus oxygenated reference. (D) wild type mitochondria (2.2 mg protein/ml) reduced with dithionite minus mitochondria reduced with ascorbate plus TMPD. (E) cytochrome *c* extracted from wild type mitochondria and reduced with dithionite. (F) mitochondria of the mutant $Cl_1$ (1.2 mg protein/ml) reduced with dithionite minus oxygenated reference.

exponential phase of growth differed from that of wild type mitochondria in that cytochrome $aa_3$ was absent (loss of peaks at 608 and 450 nm) and a relative increase in the concentration of *b*-type cytochrome(s) (nearly 2-fold) (Fig. 1 trace F). Furthermore the Soret peak of *b*-type cytochrome(s) was slightly modified. In addition to the 426 nm peak, a small shoulder at 429 nm was observed. A spectrum virtually identical to that of mitochondria of the mutant was obtained with mitochondria of wild type cells grown in the presence of 0.2 mM cyanide.

The ubiquinone content was the same in mitochondria of wild type and the mutant (about 8 nmol/mg protein). In both cases, ubiquinone behaved on silica plates like $Q_7$. The haem *a* content was equal to 0.30 nmol/mg protein in wild type mitochondria. No attempt was made to calculate the contents in cytochromes *b*, *c* and $c_1$ from room temperature spectra because of the overlapping of the absorption peaks.

*Effect of inhibitors*

A special feature of the respiratory chain in *Paramecium* mitochondria is the absence of spectroscopic changes upon addition of antimycin at concentrations as high as 1 $\mu$g/mg protein to either wild type or mitochondria of the mutant

incubated with succinate or pyruvate. Addition of antimycin at concentrations higher than 5 $\mu$g/mg protein resulted in a decreased reduction of all cytochromes. This apparent uncoupling effect has been described for mammalian mitochondria [18]. HQNO at a concentration of 20 $\mu$M could reduce the *b* cytochromes of wild type mitochondria incubated with succinate whereas cytochromes $c,c_1$ and $aa_3$ became more oxidized.

*Identification of CO-binding cytochromes*

CO-difference spectra (dithionite plus CO, minus dithionite) at 77 K of mitochondria of the wild type and the mutant are presented in Fig. 2. The spectrum in trace A (wild type) shows peaks at 596, 567, 539, 435, 420 and 416 nm and troughs at 613, 558—549 and 450 nm, suggesting a cytochrome $a_3$-CO complex and possibly a cytochrome *b*-CO complex. The peaks and troughs are due to both a shift and a decrease in intensity of the peaks of the CO-reactive cytochromes after addition of CO to the reduced mitochondria. When succinate was used as reductant instead of dithionite, a similar pattern of CO-difference spectrum was obtained, although the extent of spectral changes was less, especially for the cytochrome *b*-CO complex. As succinate dehydrogenase is specifically located in mitochondria, this result indicates that the CO-difference spectrum is attributable essentially to mitochondria and not to microsomal contaminants. As compared to spectrum A, the CO-difference spectrum B (mutant $Cl_1$) is characterized by a restricted number of peaks at 571, 539, 420 and 416 nm which are typical of a cytochrome *b*-CO complex.

The cytochrome-CO complexes in wild type mitochondria were further characterized by photodissociation (trace C and D). The difference spectrum

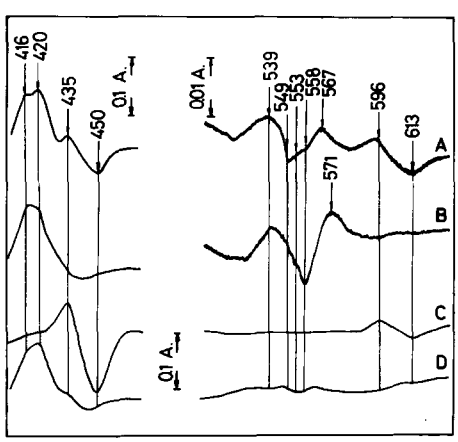


Fig. 2. CO difference spectra at 77 K. (A) wild type mitochondria (5 mg protein/ml); dithionite reduced plus CO, minus dithionite reduced. (B) Same as (A), but with mitochondria of $Cl_1$ (3 mg protein/ml). (C) wild type mitochondria (15 mg protein/ml); dithionite reduced plus CO, minus dithionite reduced plus CO photoirradiated for 30 s by a 375 W lamp through an Eppendorf filter selecting light above 600 nm (photoirradiation above 600 nm results in photodissociation of the CO-cytochrome $aa_3$ complex). (D) Wild type mitochondria (15 mg protein/ml); dithionite reduced plus CO, minus dithionite reduced plus CO photoirradiated for 5 min by a 375 W lamp using two filters to select light between 530 and 560 nm (photoirradiation under these conditions results in photodissociation of the CO-cytochrome *b* complex). Note that the same absorbancy scale is used in (C) and (D) for the whole spectrum.

after photoirradiation at wavelengths higher than 600 nm (reduced CO, minus reduced CO photoirradiated) exhibited peaks at 596 and 435 nm and troughs at 613 and 450 nm typical of cytochrome $a_3$, (trace C). The difference spectrum after photoirradiation between 530 and 560 showed a trough in the 549—558 nm region, and a significant peak centered at 416—420 nm, which probably belongs to a CO-cytochrome *b* complex (trace D). This spectrum and particularly the peak at 416—420 nm resembled the CO difference spectrum of mitochondria of the mutant $Cl_1$ (trace B). As the mutant contains the *b* type cytochrome(s), but is devoid of cytochrome $aa_3$, its CO difference spectrum provides additional evidence in favour of a cytochrome *b*-CO complex.

Comparatively to CO, the effect of cyanide on the dithionite reduced spectrum of wild type mitochondria was minor and restricted to a shift of the band of cytochrome $aa_3$ from 608 to 605 nm at 77 K.

*Heat lability of type a cytochrome*

Cytochrome $aa_3$ in wild type mitochondria differed from the *b* and *c* cytochromes by a high susceptibility to heat. Heating wild type mitochondria for 4 min at 55°C resulted in specific modification of the $\alpha$ and $\gamma$ peaks of cytochrome $aa_3$. The $\alpha$ band was shifted from 608 to 590 nm and the split $\gamma$ band (441 and 450 nm) was converted to a broad shoulder centered at 440 nm (Fig. 3B). Furthermore heating at 55°C selectively prevented the formation of the typical cytochrome $a_3$-CO-complex. On the other hand, reactivity of the *b* type cytochrome(s) to CO was apparently not modified by heating.

As shown above, mitochondria of the mutant have no detectable traces of cytochrome $aa_3$ when they are prepared from cells harvested during the exponential phase of growth. In contrast, when prepared from mutant cells in

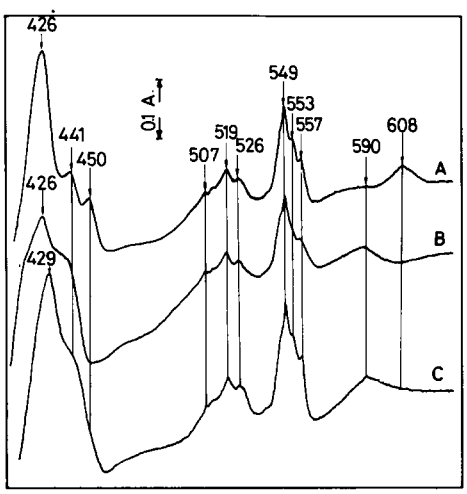


Fig. 3. Heat modification of cytochrome $aa_3$. (A) Difference spectrum at 77 K (dithionite reduced minus oxygenated sample) of wild type mitochondria (11 mg protein/ml). (B) Same as (A) with wild type mitochondria heated for 4 min at 55°C. (C) Difference spectrum at 77 K (dithionite reduced minus oxygenated sample) of mitochondria of the mutant $Cl_1$ in stationary phase of growth (8.2 mg protein/ml).

stationary phase of growth, their difference spectrum reveals peaks at 590 nm and 441 nm (Fig. 3C) similar to those found in wild type mitochondria after thermal denaturation (Fig. 3B).

*Potentiometric titrations*

Redox titrations were carried out with wild type mitochondria at pH 7.2 and at 22—25°C. The redox titration of the *a*-type cytochrome, employing wavelengths at 590 and 608 nm, gave a sigmoidal curve (Fig. 4A) which could be decomposed by the method of Wilson and Dutton [12] into two single curves with a slope of 1, Fig. 4B indicating two components with midpoint potentials of 395 and 235 mV, respectively. The high potential component ($E'_0$ = 395 mV) accounted for about 80% of the absorbance changes and the low potential component ($E'_0$ = 235 mV) for about 20%. These percentages are different from those found for mammalian mitochondria, for example 60 and 40% in rat liver mitochondria [19]. For this difference in *Paramecium* and mammalian cytochrome $aa_3$, we can offer no explanation.

As *Paramecium* mitochondria appear to possess at least two type *b* cytochromes with peaks close to those of cytochrome *c* and $c_1$, a series of redox titrations was performed to more accurately determine the half reduction potential of each cytochrome, using 537 nm as the reference wavelength and varying the other wavelength between 550 and 558 nm. When the second wavelength was between 552 and 558 nm, the titration curves were sigmoidal; each of them could be decomposed into two single curves with a slope of 1. The mid-point potentials of the two components were 220 and 120 mV respectively (Fig. 5). The respective contributions of the high and low potential components to the sigmoidal titration curve depended on the wavelength pair utilized. At 537—552 nm they were about 50%; at 537—558 nm, they were 15 and 85% respectively. Using the wavelength couple 537—550 nm, a single-slope curve

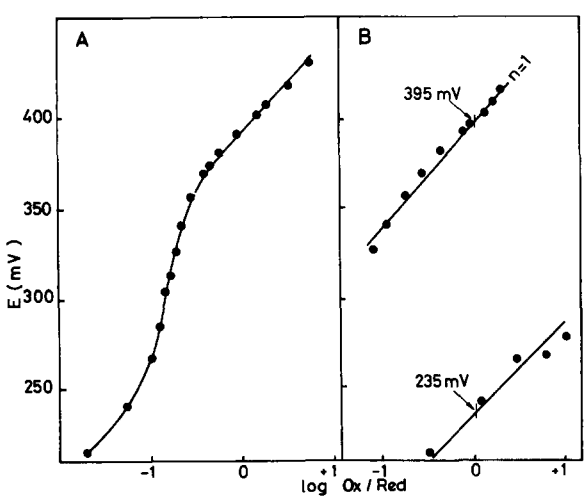


Fig. 4. Potentiometric titration of cytochrome $aa_3$ in wild type mitochondria. (A) The measurements were performed at 20°C and pH 7.2 under argon, as described in Materials and Methods. The wavelengths used were 590 and 608 nm. The protein concentration was 3 mg/ml. (B) Decomposition of the sigmoidal titration curve obtained in (A) (see Materials and Methods).

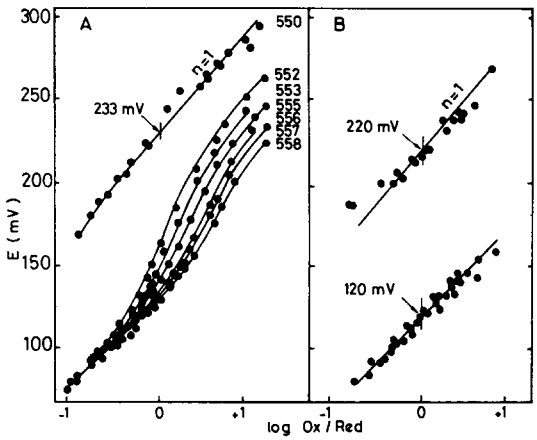


Fig. 5. Potentiometric titration of cytochromes *b* and *c* in wild type mitochondria. (A) Conditions as described in Materials and Methods and in Results. The protein concentration was 2.5 mg/ml. The reference wavelength was 537 nm and the scanning wavelength was varied between 558 and 550 nm. (B) Decomposition of the sigmoidal titration curves obtained in (A) between 537 and 552—558 nm (see Materials and Methods).

was obtained, which corresponded to a midpoint potential of 233 mV, belonging to cytochromes $c,c_1$. Within the accuracy of the titration method, the two values 220 and 233 mV are too close to be ascribed to two different components; in fact they could correspond to the same components, namely the cytochromes $c,c_1$. In that case the low potential component ($E_0$ = 120 mV) would be a *b*-type cytochrome. The lack of evidence, under our test conditions, of cytochrome *b* with a mid point-potential lower than 120 mV does not rule out the possibility that such a cytochrome is present and has escaped detection, because it is present at a too low concentration, or that its mid-point potential is too close to 120 mV.

## Discussion

Three types of cytochromes (*a*, *b* and *c*) are present in mitochondria of *P. tetraurelia*. The possible contribution of microsomal membranes to the *b* region of the 'mitochondrial' spectrum is probably not significant. In fact, all dithionite-reducible cytochromes *b* are 70—80% reduced by typical mitochondrial substrates like succinate. In common with animal and plant mitochondria, *Paramecium* cytochrome *c* could be selectively extracted by saline solutions, indicating that it is loosely bound to the mitochondrial membrane.

Although *Paramecium* cytochrome $aa_3$ consists of components characterized by mid-point potentials of 235 and 395 mV, which closely resemble those found for mammalian mitochondria (220 and 380 mV [20,21]), it differs from cytochrome $aa_3$ from higher organisms by some aspects. It reacts specifically with its own cytochrome *c* but not with cytochrome *c* extracted from beef heart or *S. cerevisiae*. A similar species specificity has been observed for *T. pyriformis* [21,22]. Another characteristic of cytochrome $aa_3$ in wild type *P. tetraurelia* is the presence of two discrete maxima at 441 and 450 nm. This unusual feature of two maxima in the Soret band for cytochrome $aa_3$ has

already been reported for *Astasia longa* [24], *Euglena gracilis* [25], *Neurospora crassa* [26], *Crithidia fasciculata* [27,28] and *Acanthamoeba castellanii* [4].

Mitochondria of the *Paramecium* mutant $Cl_1$ are completely devoid of the *a* type cytochrome during te exponential phase of growth as shown by examination of the reduced spectrum and the CO-difference spectrum at 77 K. When mitochondria of the mutant $Cl_1$ are prepared from cells harvested during the stationary phase of growth, small non-identified, peaks at 590 and 440 nm become detectable. Abnormal peaks have also been observed at 595 nm in the nuclear Cni-1 mutant [29] and at 591 nm in the extranuclear poky mutant [26] of *N. crassa.* These peaks have been attributed to an abnormal integration of the components of cytochrome $aa_3$ into the mitochondrial inner membrane. In the same way, the peaks at 590 and 440 nm in the *Paramecium* mutant could represent a modified, non functional form of cytochrome $aa_3$. In this respect, it is noteworthy that heating wild type *Paramecium* mitochondria for 4 min at 55°C modifies the $\alpha$ and $\gamma$ peaks of cytochrome $aa_3$ to give a spectrum with peaks at 590 and 440 nm, similar to that of the mutant.

Two cytochromes *b* were identified in their reduced form by $\alpha$ peaks at 553 and 557 nm at 77 K. One of them in the reduced form is able to combine with CO and the resulting CO-cytochrome *b* complex is photodissociable. The apparent lack of cytochrome *b* absorbing at higher wavelengths is difficult to reconcile with the classical scheme of electron transfer at site II of the respiratory chain in mammalian mitochondria and with the observation that the P/O ratio for NAD-linked substrates is close to 3 in mitochondria of wild type *Paramecium*, like in mammalian mitochondria. We cannot, of course, exclude the possibility that *Paramecium* mitochondria possess another *b* type cytochrome as a minor component, which has escaped detection by lack of resolution.

There is a number of bacteria in which a cytochrome *b* oxidase, refered as cytochrome *o*, is able to combine with CO and cyanide and to react with $O_2$ (Ref. 31, and for review Ref. 32). The CO difference spectrum of mitochondria of wild type *Paramecium* and of the mutant $Cl_1$ reveals peaks at 567, 530 and 420 nm and a trought at 558—549 nm which resembles those of the bacterial CO-cytochrome *o* complex [31,32]. However, evidence for a role of the CO-reactive cytochrome *b* as a functional terminal oxidase in *Paramecium* mitochondria requires the photochemical action spectrum for the relief of the CO-inhibited respiration (see accompanying paper). In that context it is worth mentioning that Lloyd and Edwards [32] have critically reviewed a number of eukaryotic systems for which it was claimed that cytochrome *o* acts as an alternative oxidase. Actually, photochemical action spectrum has provided evidence for cytochrome *o* essentially in *Trypanosoma mega*, *Blastocrithidia culicis*, and *Leishmania tarentolae* [33], but not in *T. pyriformis* [1,2] and *A. castellanii* [4].

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