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MITOCHONDRIAL RESPIRATORY CHAIN OF *TETRAHYMENA PYRIFORMIS*

THE PROPERTIES OF SUBMITOCHONDRIAL PARTICLES AND THE SOLUBLE *b* AND *c* TYPE PIGMENTS

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SUMMARY

Submitochondrial particles isolated from *Tetrahymena pyriformis* contain essentially the same redox carriers as those present in parental mitochondria: at pH 7.2 and 22 °C there are two *b*-type pigments with half-reduction potentials of −0.04 and −0.17 V, a *c*-type cytochrome with a half reduction potential of 0.215 V, and a two-component cytochrome a_2 with $E_{m7.2}$ of 0.245 and 0.345 V.

EPR spectra of the aerobic submitochondrial particles in the absence of substrate show the presence of low spin ferric hemes with g values at 3.4 and 3.0, a high spin ferric heme with $g = 6$, and a $g = 2.0$ signal characteristic of oxidized copper. In the reduced submitochondrial particles signals of various iron-sulfur centers are observed.

Cytochrome c_{553} is lost from mitochondria during preparation of the submitochondrial particles. The partially purified cytochrome c_{553} is a negatively charged protein at neutral pH with an $E_{m7.2}$ of 0.25 V which binds to the cytochrome *c*-depleted *Tetrahymena* mitochondria in the amount of 0.5 nmol/mg protein with a K_D of $0.8 \cdot 10^{-6}$ M. Reduced cytochrome c_{553} serves as an efficient substrate in the reaction with its own oxidase. The EPR spectrum of the partially purified cytochrome c_{553} shows the presence of a low spin ferric heme with the dominant resonance signal at $g = 3.28$.

A pigment with an α absorption maximum at 560 nm can be solubilized from the *Tetrahymena* cells with butanol. This pigments has a molecular weight of approx. 18 000, and $E_{m7.2}$ of −0.17 V and exhibits a high spin ferric heme signal at $g = 6$.

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INTRODUCTION

The mitochondrial respiratory chain from the ciliate protozoon *Tetrahymena pyriformis* carries out oxidative phosphorylation with the same efficiency as does the respiratory chain of higher animals. The P/O ratio for the oxidation of NAD-linked substrates is 3 and that for succinate oxidation is 2 [1, 2]. The respiratory chain of this organism contains ubiquinone, at least two *b*-type cytochromes ($E_{m7.2} = -0.065$ and -0.15 V), cytochrome *c* ($E_{m7.2} = 0.225$ V) and two *a* type cytochromes: cytochrome a_1 ($E_{m7.2} = -0.085$ V) and a_2 ($E_{m7.2} = 0.245$ V and 0.345 V). The thermodynamic measurements and the photochemical action spectrum demonstrated that the terminal oxidase is cytochrome a_2, a two-heme component enzyme which in this aspect resembles cytochrome aa_3.

Cytochrome *c* of *Tetrahymena pyriformis*, in contrast to a great variety of *c* cytochromes isolated from other organisms, is an acidic protein [3, 4] similar in its structure to cytochrome c_2 of *Rhodospirillum rubrum* [5]. Of the two *b* type pigments, one is easily solubilized from the mitochondra [3] without the aid of any detergent or addition of proteolytic enzymes while the other is tightly linked to the mitochondrial membrane.

It has been reported that the respiration of cytochrome *c*-depleted *Tetrahymena* mitochondria could not be stimulated by the addition of horse heart cytochrome *c* and that the reduced mammalian cytochrome *c* was not oxidized by mitochondria of this protozoon [6–9].

In this paper we describe the preparation of submitochondrial particles isolated from *Tetrahymena pyriformis* mitochondria and characterize the thermodynamic parameters of the redox-components of this preparation. Moreover, we present information concerning the characteristics of the soluble *b* and *c*-type cytochromes of this organism.

METHODS AND MATERIALS

Tetrahymena pyriformis, strain ST, (kindly provided by Dr. Y. Suyama, Department of Biology, University of Pennsylvania) were grown and harvested as described previously [2].

Submitochondrial particles

Mitochondria from *Tetrahymena pyriformis* were isolated as described elsewhere [2] and stored at -18 °C at a protein concentration of approx. 40 mg/ml. Approx. 10 ml of mitochondria were thawed and diluted to 40 ml with cold distilled water. The mitochondria were disrupted mechanically in a French Press (Aminco) at 3000 p.s.i. The fragments were centrifuged in a Spinco Model L refrigerated ultracentrifuge at 100 000 $\times g$ for 45 min. The supernatant and the small amount of black sediment, which adhered very tightly to the bottom of the tube, were discarded and the pellet was resuspended in 0.25 M sucrose/0.05 M morpholinopropane sulfonate (MOPS) pH 7.2.

Extraction of cytochrome c_{553} and b_{560} pigment

The harvested and washed cells were resuspended in a minimal amount of cold

distilled water and stored frozen at −18 °C. Approx. 50 ml of frozen *Tetrahymena pyriformis* cell suspension were rapidly thawed and centrifuged at 12000×*g* for 15 min. The supernatant, which contained cytochrome c_{553}, was decanted off and stored at −18 °C overnight. The pellet was suspended in 50 ml of cold distilled water and the remaining cytochrome c_{553} and b_{560} pigment extracted with butanol according to the method described by Swank and Burris [10]. One butanol/water treatment was found to be sufficient to extract over 95 % of these proteins. The butanol extract was centrifuged at 12000×*g* for 30 min. Four layers were visible after centrifugation. The top butanol layer was pipetted off while the second lipid layer was left intact as a siphon tube was carefully inserted to collect the third aqueous layer which contained cytochromes c_{553} and b_{560} pigment. (Care was taken during syphoning not to disrupt the pellet at the bottom of the tube (cell debris)). The aqueous extract containing the pigments was stored overnight at −18 °C.

Purification of cytochrome c_{553} and b_{560} pigment

Both extracts were thawed, combined and centrifuged at 12000×*g* for 15 min. Ammonium sulfate fractionation of the supernatant was carried out according to the method of Swank and Burris [10]. The final sediment (50–90 % $(NH_4)_2SO_4$ saturation) was dissolved in a minimal amount of cold, distilled water, dialyzed against 500 ml of water for 1 h, and then against 2 l of 0.01 M Tris · HCl, pH 7.6, overnight. The dialyzed solution was centrifuged at 12000×*g* for 15 min to separate any denatured protein, and the sample was loaded on a DEAE column which had previously been equilibrated with 0.01 M Tris · HCl, pH 7.6. The cytochrome c_{553} was absorbed on the column while the b_{560} pigment passed through the column and was collected and stored. The column was washed with 0.01 M Tris · HCl pH 7.6; the absorbed cytochrome c_{553} was eluted with 0.12 M Tris · HCl pH 7.6, collected, and dialyzed against 2 l of 0.01 M Tris · HCl pH 7.6 overnight. The dialyzed solution was centrifuged at 12000×*g* for 10 min and the supernatant containing cytochrome c_{553} was concentrated on a small DEAE column equilibrated with 0.01 M Tris · HCl pH 7.6.

The b_{560} pigment was further purified and concentrated by repeating the ammonium sulfate treatment and DEAE cellulose column chromatography as stated previously.

Analytical methods

Potentiometric titrations. These were carried out at 22 °C in an anaerobic chamber with an atmosphere of ultrapure argon gas. Simultaneous measurements of absorbance (Johnson Foundation dual wavelength scanning spectrophotometer) and oxidation-reduction potentials were carried out as described by Dutton [11]. Submitochondrial particles or purified enzymes were suspended in 0.2 M sucrose/0.05 M MOPS, pH 7.2. Potassium ferricyanide was used as oxidant and freshly prepared sodium dithionite was used as reductant. The following redox mediators were used: phenazine methosulfate, flavin mononucleotide (Sigma Chemical Co.); phenazine ethosulfate, pyocyanine perchlorate, 2-OH-1, 4-naphthoquinone (K and K laboratories); duroquinone and diaminodurene (Aldrich Chemical Co.).

Spectrophotometric measurements. They were performed using a Johnson Foundation dual wavelength spectrophotometer or a dual wavelength scanning

spectrophotometer [12, 13]. The detailed conditions are given in the figure legends.

Absolute spectra. They were determined with a Shimadzu recording split beam spectrophotometer.

Binding affinity. The binding of horse heart cytochrome *c* to *Tetrahymena* mitochondria and the binding of extracted *Tetrahymena* cytochrome c_{553} to *Tetrahymena* mitochondria were carried out as described previously [14]. Millimolar extinction coefficients used for calculations were 19.7 cm^{-1} for horse heart cytochrome *c* and 19.0 cm^{-1} for *Tetrahymena* cytochrome *c*.

Oxygen uptake. It was measured polarographically using a Clark-type oxygen electrode (Yellow Spring Instrument Co., Cleveland, Ohio) at 22 °C in 0.01 M Tris · HCl pH 7.4, with 0.01 M ascorbate and $2 \cdot 10^{-4}$ M *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD) as the substrate, before and after the addition of cytochrome *c*.

Oxidation of reduced cytochrome c. Cytochrome oxidase activity was determined by measuring the rate of oxidation of externally added reduced cytochrome *c*. Isolated cytochrome *c* was reduced with sodium dithionite and filtered through a Sephadex G-10 column to remove excess dithionite. The rates of oxidation were measured in a dual wavelength spectrophotometer by monitoring the decrease in absorption at 553–540 nm and a sample temperature of 22 °C.

Sephadex gel filtration. The Sephadex gel filtration of b_{560} pigment was carried out in 9 × 750 mm columns of Sephadex G-75 and G-50 preequilibrated with 0.01 M Tris · HCl pH 7.6 using blue dextran, bovine serum albumin, and horse heart cytochrome *c* as standards (Sigma Chemical Co.). The elution was carried out with the same buffer and the elution rate was 0.4 ml/h.

Polyacrylamide gel electrophoresis. It was performed in the presence of sodium dodecyl sulfate at 20 °C in cylindrical gels (10 × 0.6 cm) containing 10 % acrylamide and 0.3 % bisacrylamide and the buffer components described by Laemmli [15]. A 1 cm stacking gel containing 3 % acrylamide and 0.08 % bisacrylamide was routinely used.

Protein determination. Proteins were assayed by the biuret method [16] using crystalline bovine serum albumin (Sigma Chemical Co.) as a standard.

EPR. The EPR spectra were measured with a Varian E-109 spectrometer equipped with an Air Products LTD 3-110 liquid helium cryostat.

RESULTS

Respiratory chain carriers in submitochondrial particles isolated from Tetrahymena pyriformis mitochondria

Submitochondrial particles, which are isolated from the mitochondria of *Tetrahymena pyriformis* by essentially the same method used to prepare submitochondrial fragments of avian or mammalian mitochondria [17], contain the respiratory chain carriers tightly linked to the mitochondrial inner membrane. These carriers are represented in Fig. 1 and 2 by their difference spectra obtained during the anaerobic potentiometric titration of the preparation.

In the potential region between 0.400 and 0.100 V, two cytochromes are present in approximately equal concentrations: cytochrome *c* with an α absorption maximum at 554 nm and cytochrome a_2 with an α absorption peak at 617 nm.

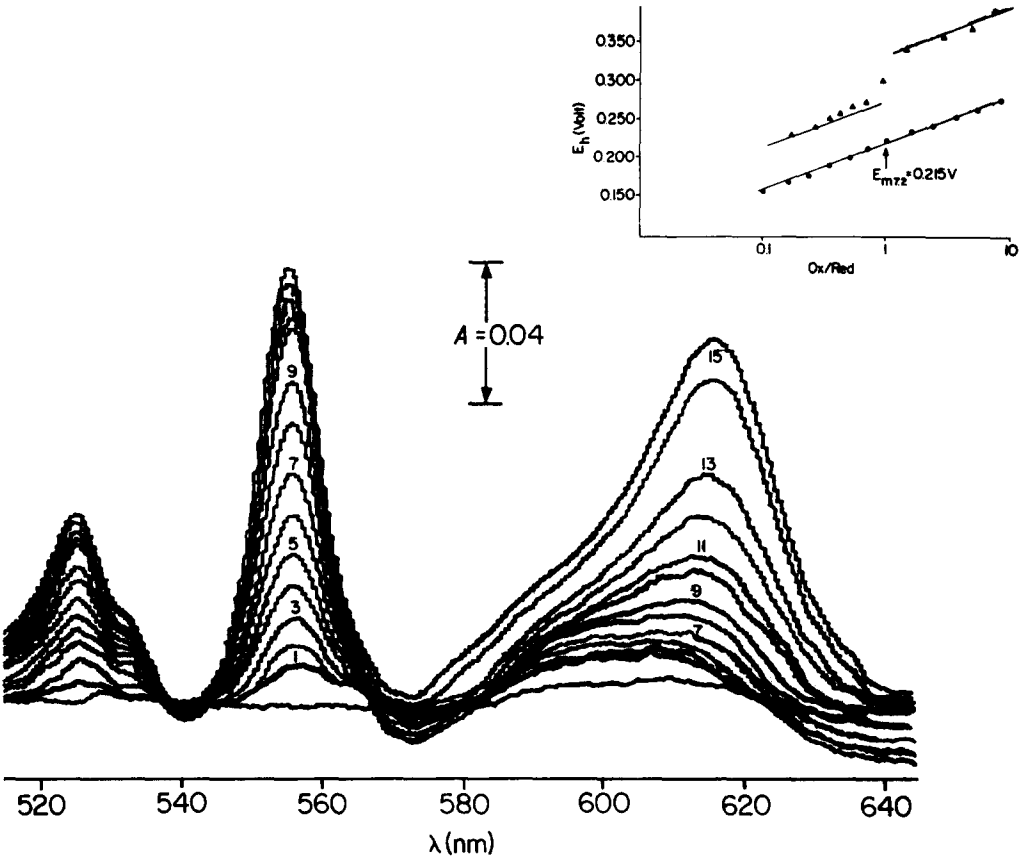

Fig. 1. Difference spectra obtained for cytochrome *c* and cytochrome *c* oxidase of submitochondrial particles isolated from *Tetrahymena*, during anaerobic potentiometric titration. The submitochondrial particles were suspended at approx. 22 mg protein/ml in 0.2 M sucrose/0.050 M MOPS, pH 7.2, buffer. The sample was preequilibrated with ultra-pure argon gas to remove most of the dissolved oxygen. The following redox mediators were used: 20 μM diaminodurol and 40 μM phenazine methosulfate. The sample was made anaerobic and reduced with sodium dithionite to 0.15 V. The spectrum of the reduced anaerobic sample was measured and stored in the memory of a digital computer. The flat baseline is the reduced minus reduced difference spectrum. Spectra 1 through 15 are the difference spectra obtained after stepwise additions of ferricyanide minus the reduced spectrum stored in the computer memory. The reference wavelength was 540 nm. Inset: Potentiometric titration curves of cytochrome *c* and cytochrome a_2 obtained from difference spectra. The curves are plots of the absorbance changes as a function of the oxidation-reduction potential (E_h). ●, Oxidative titration of absorbance changes at 554–540 nm; ▲, oxidative titration of absorbance changes at 617–570 nm. The sigmoid curve shows the presence of two components with half-reduction potentials of 0.245 and 0.345 V. Solid lines represent theoretical $n = 1$.

Cytochrome c_{554} titrates as a single one-electron redox component with a half-reduction potential at pH 7.2 and 22 °C of 0.215±0.010 V. Titration of the 617 nm peak yields a sigmoid curve which can be resolved into two components with half-reduction potentials of 0.245 and 0.345 V at pH 7.2 and 22 °C contributing approx. 35 and 65 %, respectively, to the overall absorbance change at this wavelength (617–575 nm).

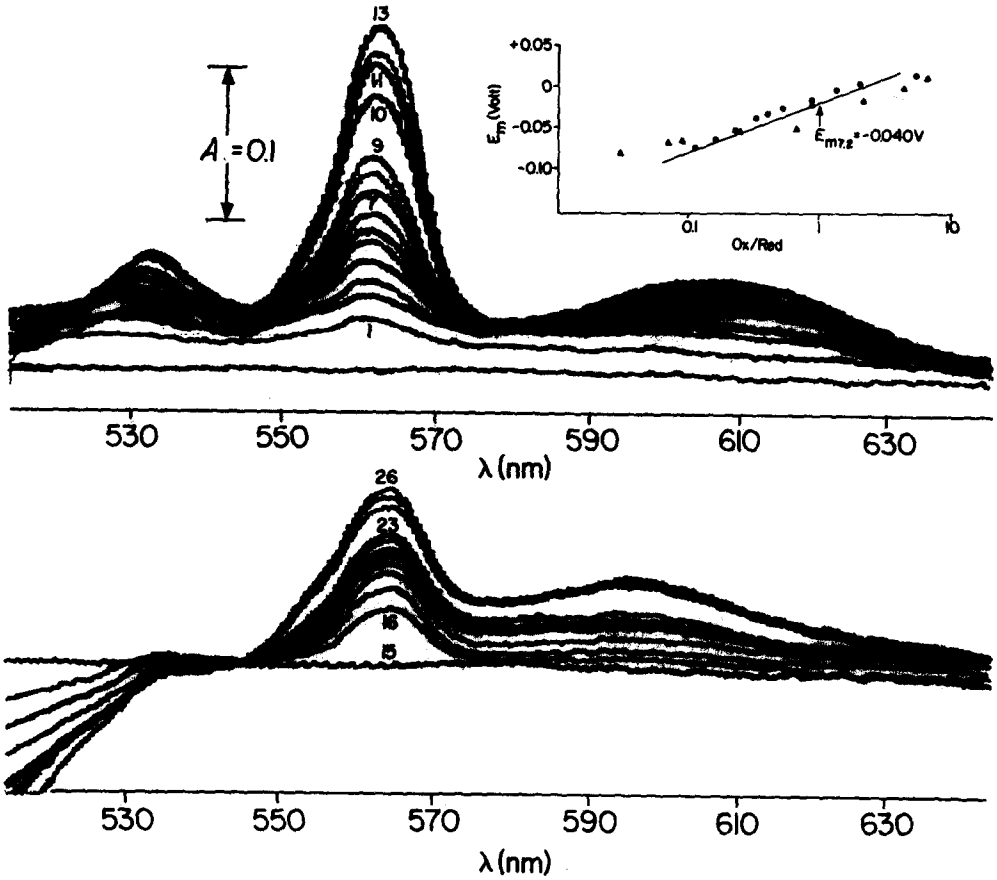

Fig. 2. Difference spectra obtained for the cytochromes *b* of *Tetrahymena* submitochondrial particles during anaerobic potentiometric titration. Conditions are those of Fig. 1. The following redox mediators were used: 40 μM each of phenazine methosulfate, phenazine ethosulfate, duroquinone; 20 μM flavin mononucleotide; 15 μM 2-hydroxy-1,4 naphtoquinone. Upper traces represent the absorbance changes taken in the potential range between 0.100 and −0.120 V and the bottom traces represent absorbance changes between −0.120 and −0.250 V. The reference wavelength was 540 nm. The inset is the potentiometric titration curve of the upper potential cytochrome *b*. The solid line represents theoretical $n = 1$.

In the potential region between 0.100 and −0.25 V (Fig. 2) there are two one-electron redox carriers with α absorption maxima at 560 nm. The half-reduction potential for the upper potential redox component is −0.040 V (inset) and for the lower potential one, below −0.15 V. (The detailed potentiometric titration of the latter pigment is presented below.) There is also a small absorbance change with an α maximum at around 600 nm which titrates with a half-reduction potential of approximately −0.05 V.

Studies on the isolated cytochrome c_{553} and b_{560} pigments

Freezing and thawing of either the cells or mitochondria isolated from *Tetrahymena pyriformis* solubilizes a *c* type cytochrome (cytochrome c_{553}) while treat-

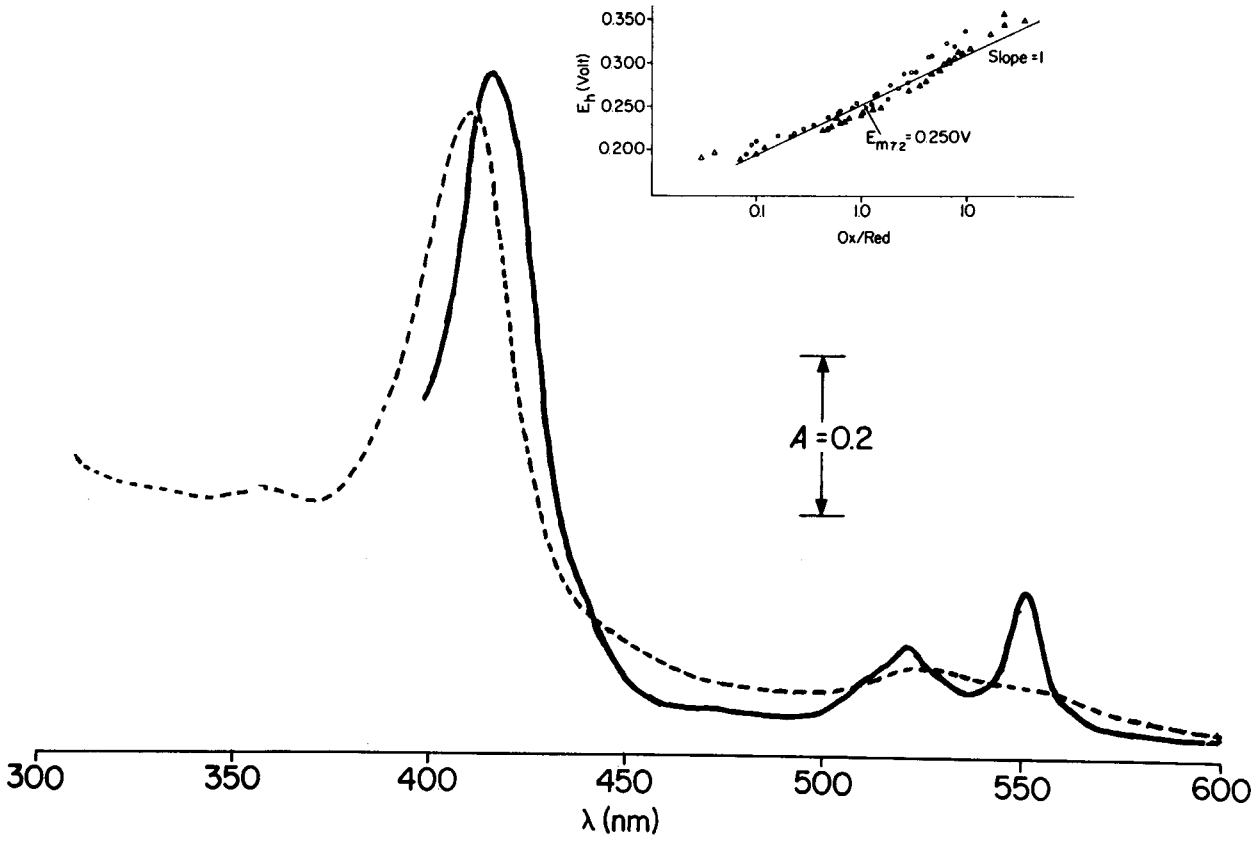

Fig. 3. Absorption spectra and anaerobic potentiometric titration of *Tetrahymena* cytochrome c_{553}. The cytochrome was suspended in 0.01 M Tris · HCl, pH 7.6 (----, oxidized; —, reduced with $Na_2S_2O_4$). For the anaerobic potentiometric titration, cytochrome c_{553} was suspended in 0.20 M sucrose/0.05 M MOPS, pH 7.2, buffer. The following redox mediators were used: 20 μM diaminodurol and 40 μM phenazine methosulfate. The different symbols represent oxidative and reductive titrations carried out on three different preparations.

ment with butanol liberates an additional pigment (b_{560} pigment) with an α-absorption maximum at 560 mn.

Cytochrome c_{553}

Cytochrome c_{553} is a negatively charged molecule with an isoelectric point of 6.2–6.5 (ref. 3 and our unpublished data) composed of 109 amino acids [4]. The absorption spectra of the isolated oxidized and reduced cytochrome c_{553} as well as the anaerobic potentiometric titration of the sample are presented in Fig. 3. The Soret absorption maximum of the oxidized cytochrome is at 410 nm while the reduced form exhibits a Soret peak at 414 nm and visible maxima at 523 nm and 553 nm. Potentiometric titrations reveal the presence of a single one-electron donor/acceptor redox carrier with a half-reduction potential of 0.25 V at pH 7.2 and 22 °C. SDS polyacrylamide gels show the presence of at least 3 additional minor bands in addition to a characteristic brownish-red band of cytochrome *c* which comigrates on the gels with horse heart cytochrome *c*.

The negatively charged *Tetrahymena* cytochrome c_{553}, in contrast to positively charged cytochromes *c* from other organisms, is easily lost from the mitochondria upon freezing and thawing in an isotonic medium. These cytochrome *c*-depleted membranes have an absorption spectrum identical to that of submitochrondrial particles and contain cytochrome c_{554} and cytochrome a_2 in approximately equal concentrations. The depletion procedure as well as other treatments of mitochondria (freezing-thawing, breakage in the French press) lead to over 80 % loss of the capability to oxidize any added substrate including ascorbate (+TMPD). This is most likely due to the lability of cytochrome a_2, the terminal oxidase, noted previously by Turner et al. [1]. Experiments are currently under way to minimize or eleminate this inactivation of cytochrome a_2.

The residual succinate oxidase or ascorbate-TMPD oxidase activity could be, however, stimulated up to two-fold by increasing concentrations of partially purified *Tetrahymena* cytochrome c_{553}. (Activity increased from 9.3 to 16.0 nmol O_2/mg protein per min upon addition of 13 μM *Tetrahymena* cytochrome *c*.) On the other hand, horse heart cytochrome *c* did not stimulate the oxygen uptake of cytochrome *c* depleted *Tetrahymena* mitochondria.

Since binding of cytochrome *c* to its site on the membrane has been implicated as the neceessary prerequisite for its activity, binding of partially purified *Tetrahymena* cytochrome c_{553} to the *c*-depleted *Tetrahymena* mitochondra was investigated and compared with that of horse heart cytochrome *c*. The results presented in the form of Scatchard plots show that while the horse heart cytochrome *c* binds in appreciable quantities to *Tetrahymena* mitochondria, cytochrome c_{553} binds to its "own membrane" in negligible amounts. Three independent experiments (similar to those of Fig. 4) gave almost identical results. There were 0.4–0.5 binding sites for cytochrome c_{553} per mg protein of *Tetrahymena* membranes with a K_D of $\geqslant 0.8 \cdot 10^{-6}$ M. The horse heart cytochrome *c* binding curve was nonlinear which indicates heterogeneity in binding sites. The number of binding sites with higher affinity was approximately 3–4 per mg protein and the binding constant was $1.4 \cdot 10^{-6}$ M. This latter value is 100-fold greater than the K_D value for tight cytochrome binding sites on cytochrome *c*-depleted mammalian or avian membranes [14].

The rates of oxidation of the reduced *Tetrahymena* cytochrome c_{553} and horse

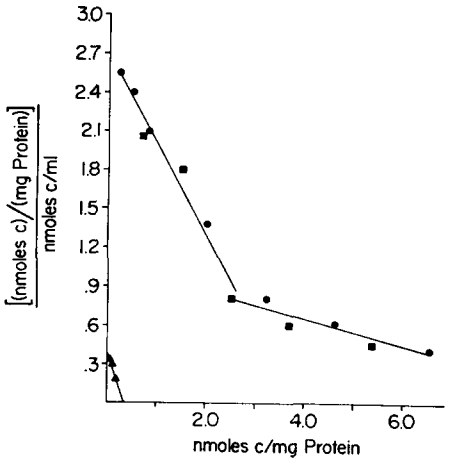

Fig. 4. Binding of extracted *Tetrahymena* cytochrome c_{553} and horse heart cytochrome *c* to cytochrome *c*-depleted *Tetrahymena* mitochondria. Cytochrome *c*-depleted mitochondria (6 mg protein/ml) were incubated in 0.2 M sucrose/0.05 M MOPS pH 7.2 buffer with various concentrations of cytochrome *c* for 5 min at room temperature. The suspensions were centrifuged for 10 min at $8000 \times g$. The pellet was suspended in 0.1 M phosphate buffer, pH 7.2, containing 1 % Triton X-100. The concentrations of cytochrome *c* in the pellet and in the supernatant were determined as described in the methods section. ■, ●, Horse heart cytochrome *c* (two separate experiments); ▲, *Tetrahymena* cytochrome c_{553}.

heart cytochrome *c* by *Tetrahymena* mitochondria and by pigeon heart mitochondria are compared in Table I.

The concentrations of cytochromes *c* were adjusted to approximately the same value in order to obtain comparable results. It can be seen that *Tetrahymena* cytochrome c_{553} is a much better substrate for its own oxidase than it is for the cytochrome aa_3 oxidase of pigeon heart mitochondria. Reduced horse heart cytochrome *c* was consistently found to be oxidized under our experimental conditions by *Tetrahymena* mitochondria, although at a much slower rate than it was oxidized by pigeon heart mitochondria and substantially slower than *Tetrahymena* cytochrome c_{553} was oxidized by *Tetrahymena* mitochondria. The *k* values were found to decrease in each

TABLE I

RATES OF OXIDATION OF THE REDUCED *TETRAHYMENA* CYTOCHROME *c* AND HORSE HEART CYTOCHROME *c*

The first order rate constants for oxidation of reduced *Tetrahymena* cytochrome *c* and horse heart cytochrome *c* by *Tetrahymena* mitochondria; for reduced *Tetrahymena* cytochrome *c* and horse heart cytochrome *c* by pigeon heart mitochondria. The experimental details are as described in the Methods section.

Mitochondria	Concentration of cytochrome *c* (μM)		*k* ($s^{-1} \cdot$ mg protein)	
	Tetrahymena	Horse heart	*Tetrahymena*	Horse heart
Tetrahymena	6.42	7.14	0.450	0.082
Pigeon heart	8.20	7.20	0.104	0.571

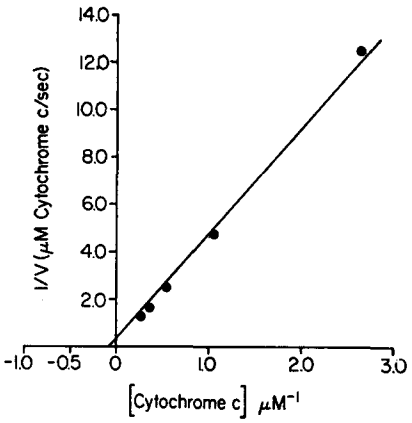

Fig. 5. Activity of reduced *Tetrahymena* cytochrome c_{553} in the cytochrome oxidase reaction. The *Tetrahymena* mitochondria (0.28 mg protein) were suspended in 0.01 M Tris · HCl, pH 7.6, buffer at room temperature. The reaction was started by adding various concentrations of *Tetrahymena* cytochrome c_{553} (between 0.94 μM and 3.75 μM). The rate of oxidation was followed spectrophotometrically at 553–540 nm. The velocity was calculated from the very initial straight line portion of the cytochrome *c* oxidation trace.

case when the concentrations of cytochromes *c* were increased, which is in agreement with the observation of other authors that both the ferri and ferrocytochrome *c* are inhibitory in the cytochrome *c*-cytochrome oxidase reaction [18].

By extrapolation from the initial rates of the oxidation of reduced cytochrome c_{553} by Tetrahymena mitochondria, double reciprocal plots were obtained which allowed us to calculate the K_M for cytochrome c_{553}. The calculated value was 5–10 μM under our experimental conditions (Fig. 5).

Pigment b_{560}

The absorption spectra of the partially purified, oxidized and reduced *b* type pigment (alkaline pyridine hemochromogen peak at 557 nm characteristic of protoporphyrin IX, unpublished data) as well as the potentiomentric titration of the preparation are shown in Fig. 6. The potentiometric titration yields a straight line with an *n* value of 1 and $E_{m7.2}$ of −0.17 V at 22 °C. Attempts were made to determine the molecular weight of the b_{560} pigment. Filtration through Sephadex G-75 and G-50 column showed that this pigment was eluted from the column immediately before the horse heart cytochrome *c*. SDS-polyacrylamide gels revealed the presence of one major band with a molecular weight of approximately 18000 daltons and two minor bands with M_r values of 13000 (slight contamination with cytochrome *c*) and 48000.

EPR spectra of the mitochondrial respiratory chain carriers in Tetrahymena pyriformis

The EPR spectra of partially purified cytochrome c_{553} at two different temperatures, 6.2 K (upper spectrum) and 15 K (lower spectrum), are shown in Fig. 7. The spectrum characteristic of a low spin heme signal exhibits a dominant transition at $g = 3.28$ resonance with a second transition at $g = 3.02$. More of the latter form is seen at higher temperatures, as the size of the $g = 3.02$ peak increases with respect

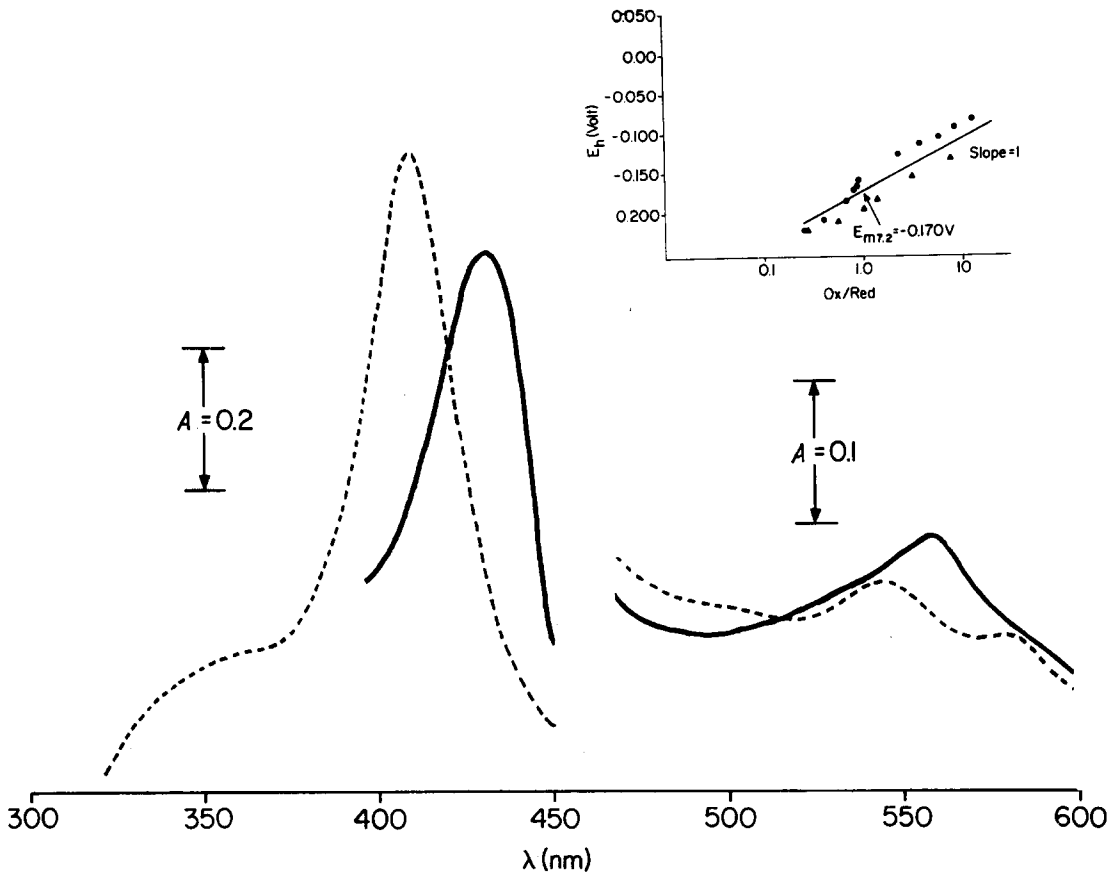

Fig. 6. Absorption spectra and anaerobic potentiometric titration of *Tetrahymena* pigment b_{560}. The b_{560} pigment was suspended in 0.01 M Tris · HCl pH 7.6 (----) oxidized, (—) reduced with $Na_2S_2O_4$. For the anaerobic potentiometric titration, the pigment was suspended in 0.2 M sucrose/0.05 M MOPS, pH 7.2, buffer. The following redox mediators were used: 40 μM each of phenazine methosulfate, phenazine ethosulfate, and duroquinone; 20 μM flavin mononucleotide; 15 μM 2-hydroxy-1, 4-naphtoquinone. ●, Oxidative titration; ▲, reductive titration.

to that of $g = 3.28$. The location of the dominant transition at $g = 3.28$ in *Tetrahymena* cytochrome c_{553} is similar to that of *Rhodospirillum rubrum* c_2 ($g = 3.17$) [19], *Euglena* cytochrome c_{558} ($g = 3.20$) (20), *Paracoccus denitrificans* c_{550} ($g = 3.27$) [20] and differs from the position of the g_1 transition in horse heart [21], yeast and tuna cytochrome *c* [20], all of which exhibit at neutral pH dominant resonance signals at higher magnetic field values ($g = 3.05$–3.06). The $g = 4.3$. signal which is very prominent in the spectrum and a small amount of high spin heme signal arise from contaminating proteins.

In contrast to cytochrome c_{553}, the 560-nm absorbing *b*-type pigment exhibits an EPR signal characteristic of a high spin heme with $g = 6$. There is no low spin heme signal visible in the spectrum and only a small $g = 4.3$ signal (Fig. 8).

The EPR spectrum of oxidized submitochrondrial particles isolated from

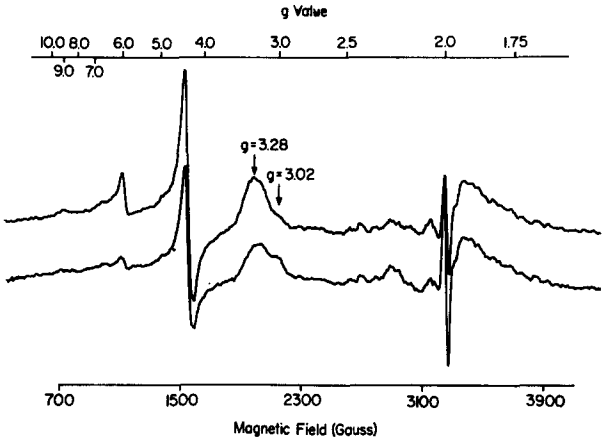

Fig. 7. EPR spectrum of partially purified cytochrome c_{553} at pH 7.4. Cytochrome c_{553} was diluted to approximately 30 μM and frozen in liquid nitrogen. EPR spectrum was obtained with microwave frequency of 9.105 GHz, microwave power of 5 mW at 6.2 K (upper spectrum) and 15 K (lower spectrum). Scanning time was 2 min; time constant, 0.128 s.

Tetrahymena pyriformis mitochondria is shown in Fig. 9. Two low spin heme signals with $g = 3.4$ and $g = 3.0$ are seen as well as a high spin heme signal at $g = 6$. Moreover, the spectrum shows a signal at $g = 4.3$ and a complex signal in the $g = 2$ region. The $g = 3.4$ low spin heme signal can be attributed to the presence of *c*-type cytochromes and mitochrondrial *b*-type cytochromes while the high spin heme signal at $g = 6$ may be predominantly due to the 560 nm absorbing pigment (cf. Fig. 2 and Fig. 6, spectra of submitochondrial particles). The $g = 3.0$ signal is most likely to arise from oxidized heme(s) of cytochrome a_2. (The rationale behind this assignment is given below in the discussion.)

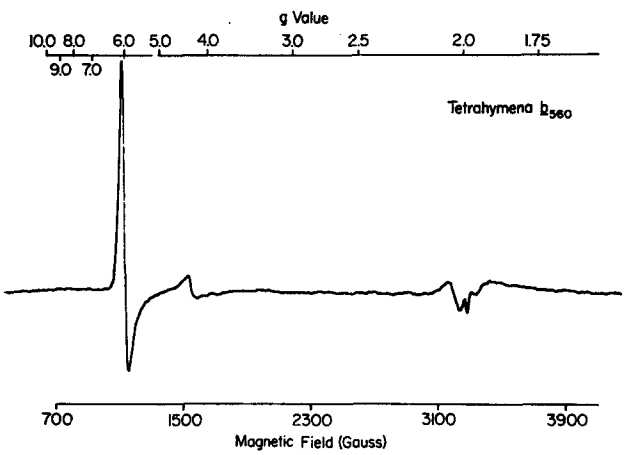

Fig. 8. EPR spectrum of partially purified 560-nm absorbing pigment at pH 7.4. The partially purified 560 nm absorbing pigment was diluted in 0.2 M Tris · HCl (pH 7.4) and frozen in liquid nitrogen. EPR frequency 9.104 GHz, microwave power, 10 mW; temperature, 8.25 K. Scanning time, 2 min; time constant, 0.128 s.

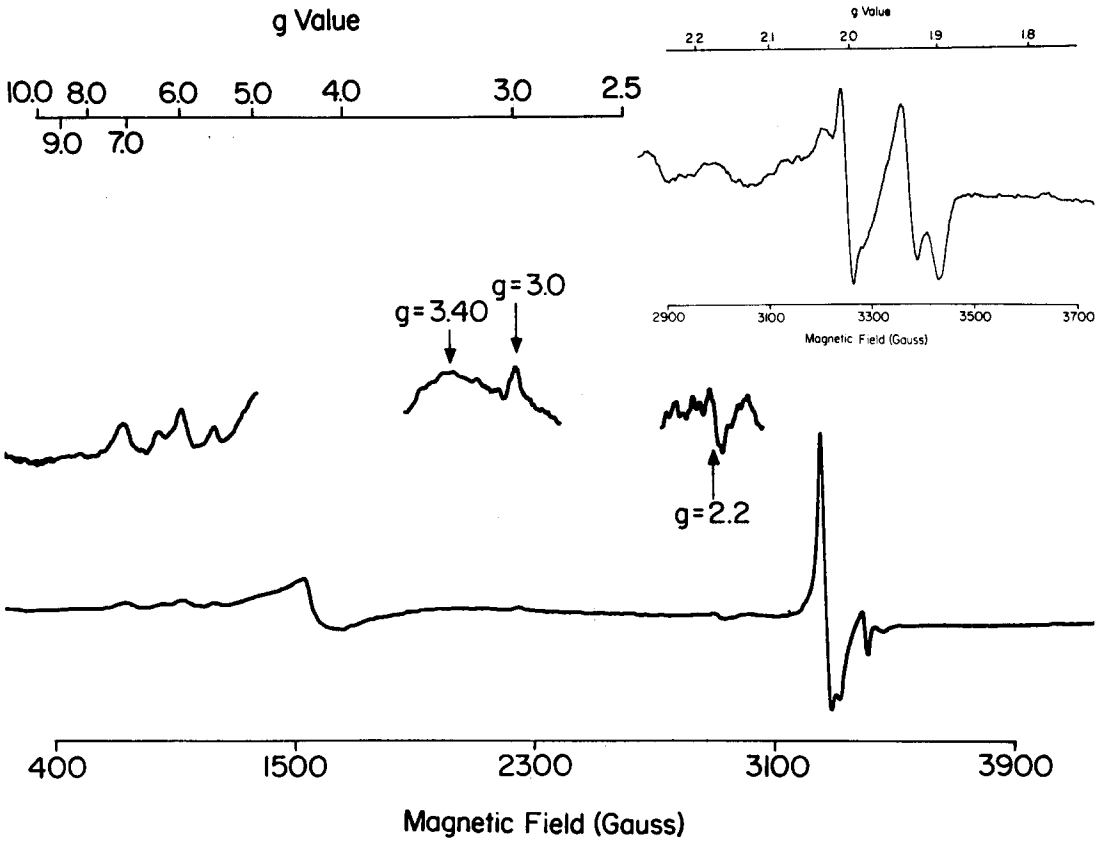

Fig. 9. EPR spectrum of the oxidized submitochondrial particles isolated from *Tetrahymena pyriformis*. Submitochondrial particles were suspended in 0.2 M sucrose/0.05 M MOPS (pH 7.2) buffer in the absence of substrate at a protein concentration of approx. 50 mg/ml. An aliquot was frozen in liquid nitrogen in a 3 mm (internal diameter) quartz EPR tube. EPR frequency of 9.104 GHz, microwave power 5 mW, and temperature 14.5 K. Scanning time 2 min; time constant, 0.128 s. Inset: magnification of the $g = 2$ region.

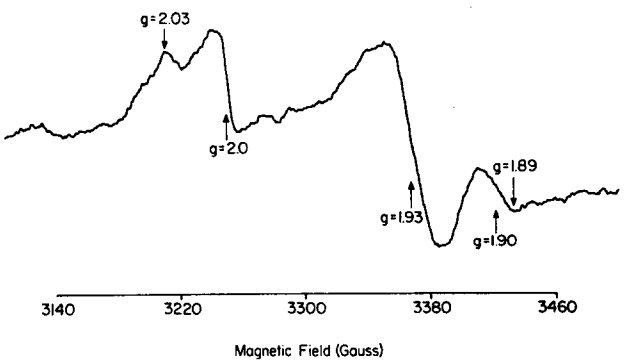

Fig. 10. EPR spectra of iron-sulfur centers in reduced submitochondrial particles isolated from *Tetrahymena pyriformis*. Submitochondrial particles (50 mg protein/ml) were reduced by the addition of dithonite and frozen in liquid nitrogen. EPR frequency, 9.10 GHz; microwave power, 5 mW; temperature, 8.4 K. Scanning time 2 min; time constant, 0.128 s.

In the $g = 2$ region a signal characteristic of oxidized copper can be seen which disappears upon reduction of the sample (inset of Fig. 9). The EPR spectrum of the reduced submitochondrial particles exhibits signals characteristic of iron-sulfur centers. Although more detailed studies are required to identify those signals, the two shown in Fig. 10 with g values at 1.93 and 1.90 indicate that *Tetrahemena pyriformis* iron-sulfur centers are similar to those of mammalian or avian mitochondria [22, 23]. In the latter systems, iron-sulfur centers S-1 (succinic dehydrogenase), N-2 (NADH dehydrogenase), and center 5 (b–c_1 region) exhibit their characteristic EPR signals at $g = 1.94$ while Rieske's iron-sulfur protein is identified by $g = 1.90$ value (24).

DISCUSSION

Submitochondrial particles isolated from *Tetrahymena pyriformis* contain essentially the same redox carriers as those present in the parental mitochondria. At pH 7.2 and 22 °C, there are two b-type pigments with half-reduction potentials of −0.04 and −0.17 V, a two-component cytochrome a_2 with half-reduction potentials of 0.245 and 0.345 V, a cytochrome a_1 ($E_{m7.2} = -0.05$ V), and a c-type cytochrome with an α absorption maximum at 554 nm and a half-reduction potential of 0.215 V. This c-type cytochrome can be considered the analogue of cytochrome c_1 in that its half-reduction potential is more negative than that of the soluble cytochrome c and its α absorption maximum is shifted to a slightly longer wavelength. Cytochrome c with an α absorption maximum at 553 nm and $E_{m7.2}$ of 0.250 V is lost from the particles during their preparation which indicates that it is more loosely bound to the mitochondrial membrane than is cytochrome c_1.

Additional information on the nature of the redox carriers was provided by EPR studies on the particles. The low spin ferric heme signal with $g = 3.0$ was attributed to the oxidized heme(s) of cytochrome a_2. This assignment is based on the fact that a signal with the same g value was found in partially purified cytochrome a_2 isolated from *Tetrahymena* mitochondria (unpublished results). A broad $g = 3.4$ signal arises from tightly bound c and b-type mitochondrial cytochromes the signals of which overlap in this region in mitochondria of higher animals. Therefore, EPR studies combined with anaerobic potentiometric titrations are required to assign unequivocally different peaks to individual cytochromes.

The high spin heme signal at $g = 6$ is mostly due to the 560 nm absorbing pigment. In addition to the heme signals, a $g = 2$ signal characteristic of oxidized copper is observed both in submitochondrial particles and in partially purified cytochrome a_2 which suggests that cytochrome a_2 like cytochrome aa_3 may be a copper-heme enzyme. Studies of the number of oxidizing or reducing equivalents accepted or donated by purified cytochrome a_2 and precise potentiometric titrations of the preparation, which are under way, should clarify the nature of this enzyme. Preliminary results on the iron-sulfur centers show them to be similar to those of mitochondria of higher animals [25–29].

The negatively charged *Tetrahymena* cytochrome c_{553}, in contrast to the whole class of positively charge c type cytochromes, does not bind appreciably to negatively charged lipids or depleted mitochondrial membranes and high concentrations are required to stimulate oxygen uptake of the depleted mitochondria. In calculating the number of cytochrome c_{553} binding sites per cytochrome a_2 it was assumed that

20 cm^{-1} is a reasonable millimolar extinction coefficient for cytochrome a_2 at 615–640 nm. With this assumption and defining cytochrome a_2 as a two component system similar to cytochrome aa_3, cytochrome a_2 is present in a concentration of 0.15–0.2 nmol/mg protein [2]. Therefore the number of cytochrome c_{553} binding sites (0.5 nmol/mg protein) is approx. 2 per cytochrome a_2. This is similar to the number of high affinity binding sites for horse heart cytochrome *c* in cytochrome *c*-depleted mitochondria of higher organisms [14], but the K_D value is almost 100-fold higher. The large K_D value and relative effectiveness of cytochrome c_{553} in restoring the oxygen uptake of the depleted mitochondria may suggest that the specific binding site for cytochrome c_{553} on *Tetrahymena* mitochondria is either modified or partially destroyed during the depletion procedure. In contrast, freshly prepared *Tetrahymena* mitochondria are able to oxidize reduced cytochrome c_{553} at a rate comparable to which pigeon breast cytochrome aa_3 oxidizes reduced horse heart cytochrome *c*. Thus, under the latter conditions reduced exogenous cytochrome c_{553} is able to reach its own oxidase, either directly or indirectly, in such a way that efficient electron transfer can occur.

Experiments done with horse heart cytochrome *c* show that while it does bind to cytochrome *c*-depleted mitochondria isolated from *Tetrahymena*, the tight binding sites with a K_D of 10^{-8}–10^{-7} M normally seen in mammalian or avian mitochondria [14] are absent from *Tetrahymena* mitochondria. The absence of the tight affinity binding sites must be related in some way to the ineffectiveness of horse heart cytochrome *c* in reacting with *Tetrahymena* respiratory chain.

It seems highly unlikely that the b_{560} pigment (a relatively small protein with M_r of about 18 000), which is easily extractable with butanol from *Tetrahymena*, is a respiratory cytochrome of this organism. The b_{560} pigment differs from tightly bound mitochondrial *b*-type cytochromes in that its half-reduction potential, $E_{m7.2} = -0.170$ V, is too negative to make it an effective member of the pool of the redox carriers at site II. Also, EPR results reveal that in the oxidized form it exhibits a high spin ferric heme signal with $g = 6$, while the oxidized forms of all other mitochondrial *b*-type cytochromes have low spin ferric hemes.

In this latter characteristic, it resembles either a hemoglobin (myoglobin) or a peroxidase, both of which exhibit high spin heme signals. Since attempts to demonstrate the formation of a peroxidase ES complex upon addition of ethyl hydrogen peroxide failed (unpublished results), it is unlikely that this *b*-type pigment is a peroxidase.

The presence of a hemoglobin-like pigment in *Tetrahymena* has been reported by Keilin [7] and similar chromophores were recently investigated in the yeast *Candida mycoderma* [30]. It seems likely that the b_{560} pigment found in *Tetrahymena* is an accessory pigment, perhaps of the hemoglobin type which occurs in lower animals, but the function of which is not clear at the present time. The measured stoichiometry and redox properties of the other cytochromes are very similar to those observed for the cytochromes of mammalian mitochondria. This suggests that the observed cytochromes are the analogous components of the mammalian mitochondrial respiratory chain. Cytochromes of advantitious non-mitochondrial membranes must be present at concentrations less than approx. 15 % of that of the mitochondrial cytochromes.

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