A SUCCINATE- AND DPNH-REDUCIBLE o-TYPE CYTOCHROME IN MITOCHONDRIAL PREPARATIONS FROM TETRAHYMENA PYRIFORMIS

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Summary: The presence of an o-type cytochrome--a pigment previously observed in a number of bacteria, several yeasts, and a parasitic protozoon--was detected from reduced plus CO minus reduced difference spectra of mitochondrial preparations of Tetrahymena. The cytochrome was observed when the reducing agent was ascorbate-TMPD, succinate, DPNH or dithionite and showed absorption maxima at 572, 537, and 418 mμ. It is estimated that about 70-85% of the total cellular o-type cytochrome is located in the mitochondrial fraction, and based on dithionite reduction, its concentration ranges from 0.25-0.62 mμmole per mg. protein in the crude mitochondrial fractions of the several Tetrahymena strains studied.

In the protozoon, Tetrahymena pyriformis, an a-type cytochrome was first reported by Baker and Baumberger in 1941 (1). They noted, in cells of the T strain, two main centers of absorption at 587-590 and 616-618 mμ, which they ascribed to cytochromes a_1 and a_2, respectively. Recently, Kobayashi (2) found that mitochondrial preparations from W strain homogenates oxidized ascorbate-TMPD[1], but he observed no a-type cytochrome near 600 mμ by microspectroscopy of dithionite-treated mitochondria. We confirmed and extended (3) Kobayashi's observation with ascorbate-TMPD and also concluded that oxidation of this couple by homogenates and mitochondrial preparations from many Tetrahymena strains proceeds by a cytochrome oxidase-type system, after noting its marked sensitivity to cyanide, azide, and other inhibitors of succinate and DPNH oxidases, insensitivity to such electron transport inhibitors as Amytal, rotenone, HNO, and SN 5949, mitochondrial location, and $QO_2(N)$. Recently, we reported (4) that various substrate- and chemically-reduced minus oxidized difference spectra of T. pyriformis S mitochondria show peaks at 613 and 447 mμ, 561, 554, 523, 429-430, and 421 mμ, corresponding to a and a_3-type cytochromes, and the α-, β-, and Soret bands of

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1 Abbreviations: TMPD, tetramethyl-p-phenylenediamine; HNO, 2-n-heptyl-4-hydroxy quinoline-N-oxide; SN 5949, 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone.

b- and c-type cytochromes*. The spectral changes seen following the addition of various inhibitors and treatment with CO supported these conclusions (4). Similar, though not identical, positions of most of these cytochrome peaks were described for T. pyriformis ST by Turner and Lloyd in an abstract (5) that became available to us after completion of our study (4). The present communication presents evidence for the occurrence in Tetrahymena of a second CO-binding pigment with spectral characteristics similar to the CO-compound studied in detail in several bacterial species and named cytochrome o by Castor and Chance (7,8). The pigment has since been observed in a number of other bacteria (9,10).

Materials and Methods: Reduced minus oxidized difference spectra were obtained with a Cary Model 11 recording spectrophotometer equipped with a 0-1.0 absorbance slidewire. Cuvettes of 1-cm. light path and 3-ml. volume were used. A mitochondria-rich fraction was prepared from cultures (3-5 days old) of T. pyriformis S,WH6, W, GL, HS, and E strains grown in 2% proteose-peptone-0.2% yeast extract medium, supplemented with 1.5 X 10^{-4} M iron (11), in 2-liter Erlenmeyer flasks at 25°. Usually, cells from 6-8 flasks containing 200 ml. medium per flask were rapidly washed three times in glass-distilled water, concentrated in water (5-10 ml. per flask), and disrupted at 10 kc for 15 seconds with a Branson sonifier. Sonicates from S, W, and E cells were centrifuged at 2,264 Xg, others at 20,360 Xg, for 15 minutes. The particulate fraction (P_1), containing most of the mitochondria, was usually reconstituted in water to about 33% of the original sonicate volume, avoiding a blackish material which often sediments at the bottom of the centrifuge tube and adheres firmly to it. In some cases, P_1 was washed several times by resuspension and recentrifugation. Total protein was analyzed by the biuret method (12). The cytochrome content of mitochondrial fractions was usually determined

* In some strains, such as WH6 and GL, the a-peak is located at 611 mμ. These findings will be described in detail elsewhere. It should be emphasized that assignment of the letter "a" to the reduced cytochrome with a peak at 611 or 613 mμ is provisional, although cytochrome a is invariably associated with cytochrome a_3 in animal and plant tissues, and when the two cytochromes occur in bacteria, most of those cases. In 1952, Chin (6) called a pigment with a band at 612 mμ from Acetobacter peroxydans "cytochrome a_4".

in the presence of 50-100 mM phosphate buffer, pH 7.2. Such mitochondrial suspensions were gassed with CO (C.P. grade, Matheson) at room temperature. P_1 preparations exhibit the following enzymatic activities characteristic of the electron-transmitter system: succinate → O_2 (13), DPNH → O_2 (13,14), ascorbate-TMPD → O_2 (3), succinate → phenazine methosulfate (15), DPNH → 2,6-dichlorophenolindophenol (14).

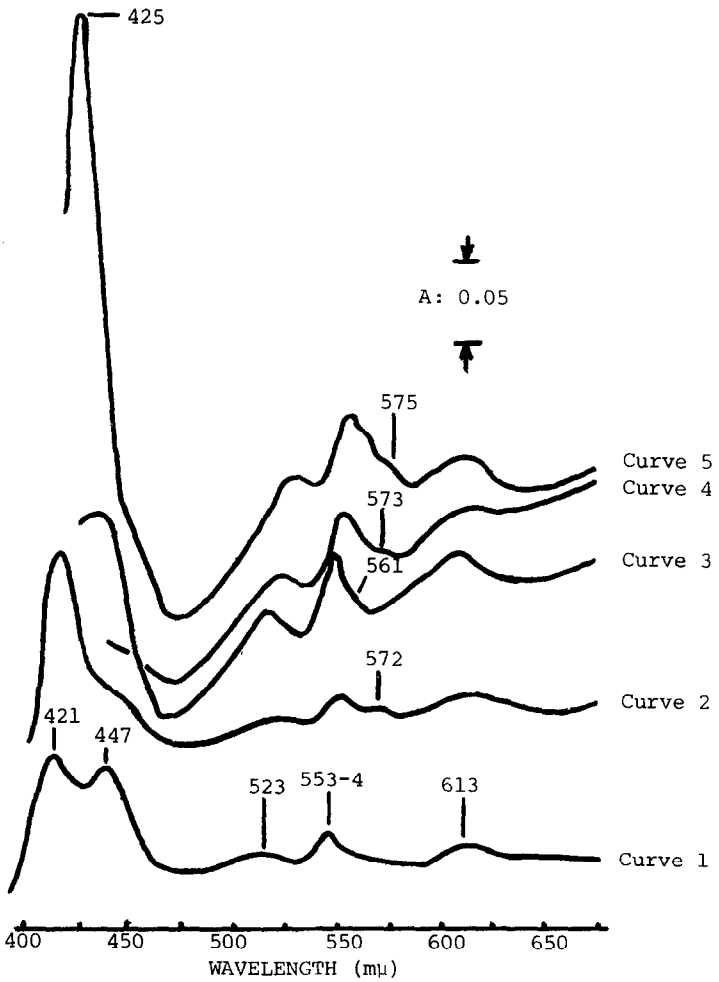

Fig. 1. Reduced plus CO minus oxidized difference spectra of T. pyriformis S cytochromes. Curve 1: Ascorbate-TMPD was added to the sample cuvette containing 6.0 mg. protein per ml. (strain S mitochondrial fraction) in 60 mM K_2HPO_4-KH_2PO_4, pH 7.2. The final concentrations of ascorbate and TMPD were 19 mM and 0.64 mM, respectively. Curve 2: CO was bubbled into the contents of the sample cuvette 4 minutes. Curve 3: Succinate was added to the sample cuvette containing 9.0 mg. protein per ml. The final concentration of succinate was 83 mM. Curve 4: CO into the contents of the sample cuvette 4 minutes. Curve 5: Dithionite was added to the CO-treated contents of the sample cuvette used to obtain Curve 4. In all cases, the contents of the sample and reference cuvettes were identical except that no substrate was added to the reference cuvette.

Results and Discussion: Spectra of ascorbate-TMPD-, succinate-, and dithionite-reduced cytochromes treated with CO for 4 minutes are illustrated in Fig. 1, Curves 2, 4, 5, which represent reduced plus CO minus oxidized tracings. Reduced minus oxidized spectra with ascorbate-TMPD and succinate as substrates are shown in Curves 1, 3, respectively. The γ-band of cytochrome a_3 at 447 mμ largely disappears after gassing with CO (Curve 2), probably forming a CO-compound with a peak near that of cytochrome c, since absorption in the region of c-γ increases considerably. In addition, the spectra of Fig. 1 show the emergence of a new peak at 572-575 mμ (Curves 2,4,5) and a small change at about 535-540 mμ on CO-treatment of the reduced cytochromes. The nature of these changes is clarified in Fig. 2 A-C which represents spectra typical of cytochrome o produced by reduction of both the sample and reference cuvettes with (A) 19 mM ascorbate plus 0.64 mM TMPD, (B) 83 mM succinate, or (C) solid dithionite, and then treating only the sample cuvette with CO for 4 minutes (or 1 minute, not shown). Maxima appear at 572, 537, and 418 mμ (C). The trough at 437 mμ in the dithionite-reduced plus CO difference spectrum probably represents a mixture of the Soret peaks of the reduced cytochromes a_3, o, and possibly b, while the maximum at 418 mμ is probably a mixture of the a_3-CO and o-CO compounds, and possibly the CO-compound of cytochrome b, by analogy with the CO-binding property of cytochrome B-560 which was extracted and purified from acetone-dried Tetrahymena cells by Yamanaka et al. (16). It will be noted that in the ascorbate-TMPD-reduced plus CO minus ascorbate-TMPD-reduced difference spectrum (Fig. 2A), the Soret band is located at 422 rather than 418 mμ. This is probably due to the fact that only partial reduction of the o-type cytochrome is achieved and to the overlapping presence of the a_3-CO compound. Reduction of mitochondria in the sample and reference cuvettes with 0.91 mM DPNH, followed by CO-treatment of the former, indicates that about 50% of dithionite-reducible o-type cytochrome is reduced by DPNH (not shown).

In evaluating the changes in the visible and Soret regions of the spectrum on bubbling with CO, it has been necessary to consider carefully the following

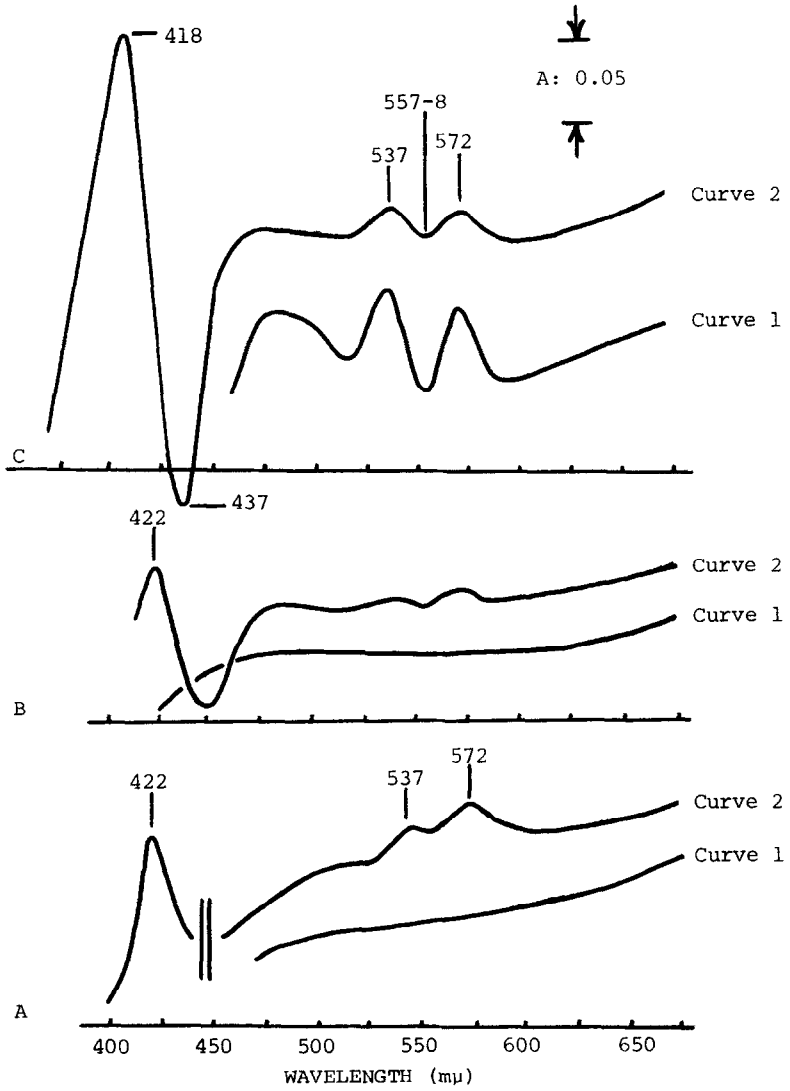

Fig. 2. Reduced plus CO minus reduced difference spectra of T. pyriformis S cytochromes. A. Curve 1: Ascorbate-TMPD was added to both the sample and reference cuvettes containing a mitochondrial fraction from strain S, 18.8 mg. protein per ml., in 100 mM K_2HPO_4-KH_2PO_4, pH 7.2. The final concentrations of ascorbate and TMPD were 19 mM and 0.64 mM, respectively. Curve 2: CO was bubbled into the contents of the sample cuvette 4 minutes to produce a typical cytochrome o spectrum. B. Curve 1: As in A except succinate was present in a final concentration of 83 mM and an S mitochondrial fraction in a final concentration of 4.6 mg. protein per ml. was used. Curve 2: CO was bubbled into the contents of the sample cuvette to produce a typical cytochrome o spectrum. C. Curve 1: As in A except an S mitochondrial fraction in a final concentration of 19.9 mg. protein per ml. was reduced with dithionite and bubbled with CO. Curve 2: A 1:2 dilution of the contents of the sample and reference cuvettes.

two facts. First, as pointed out by Chance and Smith (17), the difference spectrum of cytochrome o resembles the spectrum of hemoglobin and its derivatives

and second, Keilin and Ryley (18) reported that aerated suspensions of Tetrahymena cells of the GL strain exhibited a weak oxyhemoglobin band at 582 mμ which was converted to carboxyhemoglobin (bands at 574 and 539 mμ) in the presence of CO. In consideration of these points, our tentative classification of the hemoprotein in mitochondrial preparations of Tetrahymena with maxima at 572, 537, and 418 mμ, and a trough at 557-558 mμ, as an o-type cytochrome is based on the following experimental evidence: (1) the triple-banded reduced plus CO minus reduced difference spectrum appears following the enzymatic reduction of the mitochondrial cytochromes by succinate, DPNH, or ascorbate-TMPD, and (2) a difference spectrum of a mitochondrial preparation bubbled with CO, but without added substrate, minus a similar mitochondrial sample also without added substrate, reveals only very weak peaks, at 563 and 531 mμ, which could be considered as belonging to carboxyhemoglobin; these peaks may, in fact, reflect some o pigment reduced by endogenous cytochrome-linked substrates known to be present in P_1. (A third small peak is observed at 597 mμ, probably the a_3-CO complex formed by reduction of cytochrome a_3 by endogenous substrates). Thus, contamination of the mitochondrial preparations with hemoglobin, if any, is minimal.

From preliminary experiments with the S strain, we estimate that about 70-85% of the total cellular o-type cytochrome is present in the mitochondrial fraction. The concentration of the o-type pigment in the mitochondrial fraction as determined by dithionite reduction ranges from 0.25-0.62 mμmole per mg. protein in the different strains of Tetrahymena studied (using a millimolar extinction coefficient for the α-peak of 5.67), but, it should be pointed out, these values do not take into account the impurity of the mitochondrial fractions and any contribution of carboxyhemoglobin to the difference spectra. If the mitochondrial fraction were pure, it is estimated that these concentrations would be approximately doubled.

While the ascorbate-TMPD → O_2 reaction is unaffected by HNO (3), this couple considerably reduces the o-type cytochrome (Fig. 2A), suggesting that the latter lies on the O_2-side of the HNO-sensitive site of the succinate and DPNH

oxidase systems (19). This is supported by the finding that little or none of the b- type cytochrome (561 mμ) is reduced by ascorbate-TMPD, which argues that the redox potential of cytochrome o is more positive than that of cytochrome b and possibly near that of cytochrome c. In several cases, indications are that cytochrome o itself is a b-type cytochrome (9).

Whether the o-type cytochrome participates in Tetrahymena electron transport is not yet known although this seems possible since it clearly can react with several primary dehydrogenases and probably other respiratory chain components. Cytochrome o may be a functional terminal oxidase in the ciliate under certain physiological conditions, or it may, by interacting with cytochromes a + a_3, participate in the terminal oxidase → O_2 reaction. Keilin has pointed out that no case has been reported of a ferroprotein that combines with CO but does not react with O_2 (20).

From the viewpoint of biochemical evolution and taxonomy, it is worth noting that cytochrome o is not present in animal cells, has not been reported in plant cells, but occurs rather widely among bacteria and has been found in several yeasts (21). An o-type pigment has been observed in the parasitic protozoon, Crithidia fasciculata (22).

No evidence has yet been found for the occurrence in T. pyriformis S or WH6 of another CO-binding pigment, P-450, present in mammalian tissues and certain bacteria; this agrees with the finding of others (16).

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