Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 502-508

PURIFICATION AND PARTIAL CHARACTERIZATION OF CYTOCHROME b_5 FROM *TETRAHYMENA PYRIFORMIS*

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Received February 11, 1982

SUMMARY: With the use of detergents and successive column chromatographies, *Tetrahymena* b-type cytochrome was purified from microsomes to a specific content of 36.0 nmol per mg of protein. The purified form showed a single band on SDS-polyacrylamide gel with molecular weight of 22,000. The spectral properties of the reduced b-type cytochrome, the α-peak of which is situated at 560 nm and asymmetric with a shoulder at 556 nm, was different from that of rat liver microsomal cytochrome b_5. However, it was reducible by NADH in the presence of NADH-cytochrome b_5 reductase purified from rat liver microsomes.
The results indicated that the microsomal b-type cytochrome should be designated as cytochrome b_5 of a ciliated protozoan, *Tetrahymena pyriformis*.

The ciliate *Tetrahymena pyriformis* exhibits a remarkable alteration in acyl chains of membrane phospholipids when exposed to a lower temperature (1). In earlier studies, we have reported that palmitoyl-CoA desaturase system which might be composed of flavoprotein, hemoprotein and terminal oxidase enzymes, plays a crucial role in the initial temperature acclimation (2,3).

Using low temperature spectroscopy, Iida *et al.* have recently demonstrated the different contents of hemoprotein *in situ* in *Tetrahymena* cell under various growth conditions (4). We found that unlike mammalian cells, the increase in *Tetrahymena* microsomal desaturase activities after temperature shift down (39.5°C → 15°C) might be due to the induction of the b-type cytochrome and flavoprotein synthesis as well as the level of the terminal oxidase, cyanide sensitive factor (CSF) (5). Up to now, whether *Tetrahymena* microsomal b-type hemoprotein is a typical cytochrome b_5 linked to the flavoprotein and the CSF as described in liver (6), remains to be ascertained. And only limited information has been obtained concerning the microsomal b-type cytochrome in ciliated

0006-291X/82/060502-07$01.00/0

protozoan *Tetrahymena* (4,7). In order to gain insight into the temperature acclimation mechanism, our initial attempt was to isolate microsomal b-type cytochrome which might be associated with the fatty acyl-CoA desaturase system.

In this communication, we presented first the isolation and some characterization of b-type cytochrome designated as cytochrome b_5 of *Tetrahymena pyriformis*.

MATERIALS AND METHODS

Growth of organisms and isolation of cytochrome b_5

The thermotolerant strain NT-I cells of *Tetrahymena pyriformis* in the early stationary phase (100 - 150 x 10^4 cells/ml) were grown at 39.5°C in an enriched proteose-peptone medium (8). Microsomal fraction was isolated from cell homogenates that were prepared with phosphate buffer (0.2 M K_2HPO_4, 0.2 M KH_2PO_4, 3 mM EDTA and 0.1 M NaCl, pH 7.4) as described previously (9). Collected microsomes were washed once by suspension in 0.1 M Tris-HCl buffer (pH 8.0) and centrifugation at 105,000 x g for 60 min, and the resulting pellet was then resuspended in 0.1 M Tris-HCl buffer (pH 8.0) and containing 20 % (w/v) glycerol plus 1 mM EDTA. Microsomal fraction (19.3 mg/ml) was solubilized with 3 % Triton X-100 and 1.5 % sodium cholate. After centrifugation (105,000 x g, 90 min), the supernatant fluid was diluted 3-fold with 5 mM Tris-HCl buffer (pH 8.0 and containing 1 mM EDTA) and applied to a DEAE-cellulose column (Whatman DE 52, 2.6 x 13 cm) which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.0 and containing 1 mM EDTA plus 0.5 % Triton X-100). Cytochrome b_5 was then eluted with 150 ml of a linear gradient of 0 to 0.2 M KCl in the same buffer. The cytochrome b_5 rich fractions were pooled, brought to 50 % of ammonium sulfate saturation, and centrifuged. The pink pellet obtained was suspended in 20 mM Tris-HCl buffer (pH 8.0 and containing 0.2 mM EDTA plus 1.0 % sodium cholate), and the solution was applied to a Sephadex G-100 column (2.6 x 85 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0 and containing 0.2 mM EDTA plus 1.0 % sodium cholate). The peak fractions of cytochrome b_5 were applied to a small DEAE-cellulose column (1.5 x 1.5 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0 and containing 0.2 mM EDTA plus 0.2 % Triton X-100). After washing the column with equilibration buffer, cytochrome b_5 was eluted with 0.1 M Tris-HCl buffer (pH 8.0 and containing 0.2 mM EDTA plus 0.2 % Triton X-100). The concentrated cytochrome b_5 solution was subsequently applied to a Sephadex G-100 column (1.6 x 80 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0 and containing 0.2 mM EDTA plus 1.0 % sodium cholate). The purity of the cytochrome b_5 obtained at each step was assessed by SDS-polyacrylamide gel electrophoresis. Cytochrome b_5 (10) and NADH-cytochrome b_5 reductase (11) were also purified from rat liver microsomes. Protein was determined by the method of Lowry *et al.* (12).

Assay of cytochrome b_5 and NADH-cytochrome b_5 reductase

Rat microsomal cytochrome b_5 was assayed according to the method of Omura and Sato (13). *Tetrahymena* cytochrome b_5 content was determined by measuring the reduced minus oxidized difference spectrum taking the extinction difference of the cytochrome between 425 and 410 nm as 216 $mM^{-1} \cdot cm^{-1}$ (Table II). The cytochrome was reduced by adding a few grains of solid sodium hydrosulfide. The amount of NADH-cytochrome b_5 reductase was determined by measuring its NADH -ferricyanide reductase activity (14).

Pyridine hemochromogen experiments

Heme in the purified preparation of cytochrome b_5 was analyzed as alkaline pyridine ferrohemochrome, and the heme content was determined from the absolute spectrum of pyridine ferrohemochrome at 557 as 34.7 $mM^{-1} \cdot cm^{-1}$ (13,15).

SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli (16). The separation gel contained 15 % acrylamide.

Table I. Purification of microsomal cytochrome b_5 from *Tetrahymena pyriformis*

Fraction	Protein (mg)	Cytochrome b_5		
		Total content (nmol)	Specific content (nmol/mg)	Yield (%)
Microsomes	965	332	0.34	100
Triton X-100 / sodium cholate extract	755	283	0.37	85.2
DEAE-cellulose	87	94	1.08	28.3
50 % $(NH_4)_2SO_4$	63	91	1.44	27.4
Sephadex G-100	9.1	45	4.95	13.5
DEAE-cellulose	2.6	29	11.2	8.7
Sephadex G-100	0.5	18	36.0	5.4

Aliquots of each fraction were assayed as described under "Materials and Methods".

RESULTS AND DISCUSSION

The process of purification is summarized in Table I. Six steps for microsomes were involved with a final purification of about 100-fold and with 5.3 % recovery. Fig. 1 shows the elution profile from the 1st DEAE-cellulose column that was loaded with *Tetrahymena* microsomes after Triton X-100 and sodium

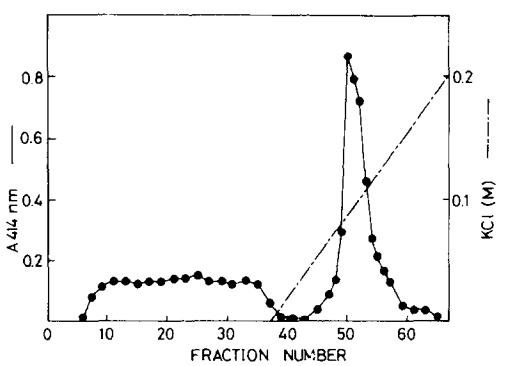

Fig. 1 First DEAE-cellulose column chromatography. Microsomes were treated with Triton X-100 and sodium cholate and then applied to a DEAE-cellulose column (2.6 x 13 cm). The column was washed with 20 mM Tris-HCl buffer containing 0.5 % Triton X-100 and 1.0 mM EDTA, pH 8.0. A linear gradient of 0 to 0.2 M KCl in the same buffer was applied to elute cytochrome b_5. Five-ml fractions were collected. Further details are described under "Materials and Methods".

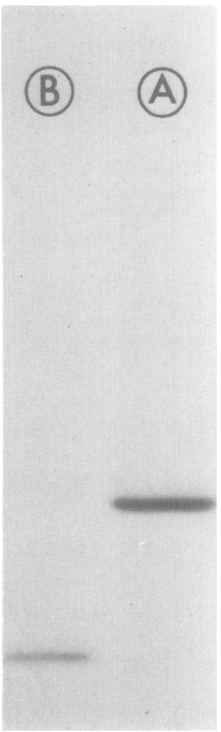

Fig. 2 Polyacrylamide gel electrophoresis of purified *Tetrahymena* (A) and rat liver (B) microsomal cytochrome b_5 in the presence of sodium dodecyl sulfate. The purified cytochrome b_5 (6 μg) and rat microsomal cytochrome b_5 (2 μg) were each applied to a 15 % polyacrylamide gel (pH 8.8) in the presence of 0.1 % SDS. Electrophoresis was performed as described by Laemmli (16) with migration from top to bottom.

cholate solubilization. The final product was free from NADH-ferricyanide reductase, NADPH-cytochrome c reductase and other proteins (Fig. 2). The purified preparation was stable in the oxidized state and could be stored for one month at -90°C without any changes in absorption spectrum. When the purified cytochrome from *Tetrahymena* was subjected together with that from rat liver for comparison, to sodium dodecyl sulfate gel electrophoresis, a single band was revealed (Fig. 2). The molecular weight was estimated to be approx. 22,000 based on mobility relative to standard proteins (Fig. 3). It should be noted that the molecular weight of *Tetrahymena* microsomal cytochrome obtained differs from either yeast (17) or liver (10) microsomal cytochrome b_5.

The absorption spectra of the oxidized and reduced forms of *Tetrahymena* cytochrome b_5 and the millimolar extinction coefficients of cytochrome b_5 at various absorption maxima are presented in Fig. 4 and Table II, respectively.

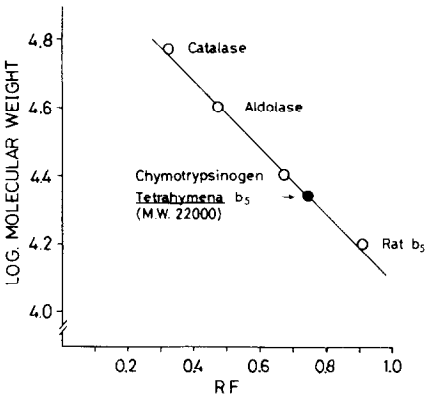

Fig. 3 Molecular weight determination of *Tetrahymena* cytochrome b_5 as a function of mobility in sodium dodecyl sulfate disc polyacrylamide gels. The standards employed were Catalase, Aldolase, Chymotrypsinogen and rat liver microsomal cytochrome b_5.

The absorption peaks were 414 nm in the oxidized form, and at 560, 528 and 425 nm in the dithionite-reduced form. Compared to the spectral property of yeast or rat liver microsomal cytochrome b_5 which was asymmetric α-peak at 556 nm with a shoulder at 560 nm (Fig. 4A), the α-peak of reduced *Tetrahymena* microsomal cytochrome b_5 is situated at 560 nm and asymmetric with a shoulder at 556 nm. The reduced peaks of *Tetrahymena* cytochrome b_5 suggest the presence of b-type cytochrome, and also the absorption peaks at 557, 524 and 418 nm in the pyridine ferrohemochrome spectrum indicate the presence of protoheme IX in the prosthetic group (data not shown). A value of one heme/mol of cytochrome b_5 was calculated based on the absorbance of ferropyridine hemochrome at 557 nm. This cytochrome b_5 does not bind CO.

Table II. Spectral properties of cytochrome b_5

	Absorption maxima				
	Reduced			Oxidized	Reduced-oxidized
	α	β	γ	γ	γ
λ, nm	560	528	425	414	425 - 410
ε mM*	27.8	14.8	221.5	140.3	216.0

* Values were calculated on the basis of protoheme content.

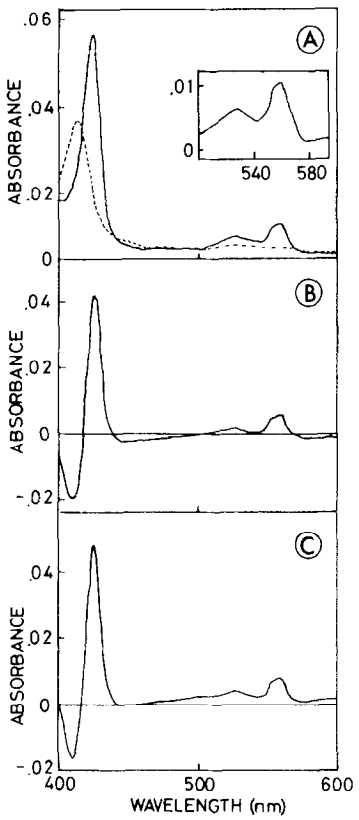

Fig. 4 Absorption spectra of purified *Tetrahymena* cytochrome b_5 (A). The reference cuvette contained 0.1 M potassium phosphate buffer at pH 7.4. --- Oxidized, —— reduced with $Na_2S_2O_4$.
Difference spectra of enzymatically reduced minus oxidized *Tetrahymena* cytochrome b_5. The both of the sample and reference cuvettes contain 0.3 nmol of purified *Tetrahymena* cytochrome b_5, 4.4 μmol of cytochrome b_5 reductase from rat liver microsomes in 1 ml 0.1 M potassium phosphate buffer, pH 7.4, and then 100 nmol of NADH (B) and a few grains of $Na_2S_2O_4$(C) was added to the content of the sample cuvette.

In addition, Fig. 4 B shows that the purified cytochrome can be reduced enzymatically with NADH in the presence of the purified NADH-cytochrome b_5 reductase from the liver microsomes. Under aerobic conditions, cytochrome b_5 was almost completely reduced enzymatically (Fig. 4 B), and complete reduction was observed upon the addition of dithionite (Fig. 4 C).

These results indicate that the microsomal b-type cytochrome should be designated as cytochrome b_5 of *Tetrahymena pyriformis*. The mammalian liver microsomal cytochrome b_5 which participates in many reactions of lipid metabolism such as cholesterol biosynthesis (18,19), plasmalogen biosynthesis (20), fatty acid desaturation (6,21,22) and elongation (23), has been widely studied.

Although the function(s) of *Tetrahymena* cytochrome b_5 remains to be determined, it can be expected that cytochrome b_5 will be found to be essential for lipid metabolism in *Tetrahymena* cells.

Acknowledgments

We are indebted to Dr. J.L. Gaylor of Experimental Station, DuPont for his critical reading. We wish to express thanks to Dr. Y. Yoshida of Mukogawa University for his useful discussions. This work was supported in part by a grant from the Ministry of Education, Culture and Science of Japan.

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