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Immunochemical evidence for participation of NADPH-cytochrome c reductase in microsomal fatty acyl-CoA desaturation of Tetrahymena cells lacking in cytochrome P-450

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SUMMARY: Rabbit antibody was prepared against NADPH-cytochrome c reductase of Tetrahymena microsomes. When examined by the Ouchterlony double diffusion test, anti-NADPH-cytochrome c reductase immunoglobulin formed a single precipitation line with Tetrahymena reductase but not rat liver one. The antibody inhibited the NADPH-cytochrome c reductase activity of Tetrahymena microsomes, but it did not affect either NADH-ferricyanide or NADH-cytochrome c reductase activity of Tetrahymena microsomes. The NADPH-dependent desaturation of stearoyl-CoA in Tetrahymena microsomes was inhibited by anti-reductase immunoglobuline, while the NADH-dependent desaturation was affected by neither anti-reductase nor control immunoglobuline.

It was suggested that the temperature associated-alteration of NADPH-cytochrome c reductase activities would be important for regulation of microsomal NADPH-dependent desaturase activities in Tetrahymena which contains no cytochrome P-450.

Tetrahymena pyriformis(NT-1) was proposed as a useful model system for studies of the temperature adaptation mechanism eukaryotic cells (1). Especially, we have reported that microsomal desaturation system would play a crucial role in the temperature acclimation (2-4). However, microsomal electron transport chains for desaturation of fatty acid are still unclear in Tetrahymena cells. The protozoan Tetrahymena pyriformis (5) as well as liver (6) or yeast (7) microsomes contain NADH- and NADPH-dependent fatty acid desaturation systems in microsomes. Although cytochrome P-450 was not detected by low-temperature spectroscopy (8), NAD(P)H-cytochrome c and NADH-ferricyanide reductase activities were observed to be present (4, 9, 10). As demonstrated previously, cytochrome b_{560ms} which is similar to but not identical with mammalian or yeast cytochrome b_5, was purified from Tetrahyema microsomes (11).

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In general, it is well known that NADPH-cytochrome c reductase which transfers electrons to cytochrome P-450, is important for the drug oxidations. Although we suggested that alterations in NADPH-cytochrome c reductase activity were found to be associated with low temperature acclimation(4), it is not clear whether or not NADPH cytochrome c reductase participates in microsomal fatty acyl-CoA desaturation in Tetrahymena cells without containing cytochrome P-450.

In this paper, we purified first NADPH-cytochrome c reductase and demonstrated the immunochemical evidence that NADPH-cytochrome c reductase is involved in stearoyl-CoA desaturation of Tetrahymena microsomes.

MATERIALS AND METHODS

Purification of NADPH-cytochrome c reductase from Tetrahymena microsomes

The thermotolerant strain NT-1 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 (12). The microsomal fraction was isolated from cell homogenate (13) that was prepared with 0.2 M phosphate buffer (pH 7.4), containing 3 mM EDTA, 0.1 M NaCl and 0.25 M sucrose (11). Detergent-solubilized NADPH-cytochrome c reductase from Tetrahymena microsomes was prepared by a modification of the method of Yasukochi and Masters (14). The microsomal fraction was suspended in 0.1 M Tris-HCl buffer (pH 8.0) containing 20 % (w/v) glycerol, 1 mM EDTA and 0.5 mM dithiothreitol (DTT), to make the microsomal suspension (85 ml, about 20 mg/ml). This suspension was mixed with 17 ml of 10 % sodium deoxycholate and 17 ml of 10 % Renex 690, stirred for 30 min and diluted 3-fold with 5 mM Tris-HCl buffer (pH 8.0 and containing 1 mM EDTA and 0.5 mM DTT). After centrifugation (105,000 x g, 90 min), the supernatant fraction was applied to a DEAE-cellulose column (4 x 15 cm) which had been previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 20 % glycerol, 0.5 % Renex 690, 0.1 % sodium deoxycholate, 0.5 mM EDTA and 0.5 mM DTT. The column was washed with the equilibrating buffer and the NADPH-cytochrome c reductase was then eluted with the same buffer containing 0.2 M KCl . The fractions with reductase activity were applied to a 2',5'-ADP Sepharose 4B column (1.5 x 3 cm, Pharmacia Fine Chemicals) previously equilibrated with 10 mM potassium phosphated buffer (pH 7.4) containing A: 20 % glycerol, 0.1 % Renex 690, 0.5 mM EDTA and 0.5 mM DTT. After the column was washed with 0.2 M potassium phosphate buffer (pH 7.4) containing A, the reductase was eluted with 10 mM potassium phosphate buffer (pH 7.4) containing A plus 3 mM NADP. To remove NADP and several minor proteins, the reductase was concentrated and passed through a Sephacryl-200 column (1.4 x 90 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing A. Thus a highly purified NADPH cytochrome c reductase was obtained. NADPH-cytochrome P-450 reductase was isolated from rat liver microsomes (14).

Preparation of antiserum against purified NADPH-cytochrome c reductase.

The purified enzyme (0.1 mg) in 1.0 ml of 50 mM potassium phosphate buffer (pH 7.4) was mixed with an equal volume of Freund's complete Adjuvant (Nakarai Chemicals) and injected subcutaneously to the animal. Two weeks later, 0.1 mg of enzyme which was mixed with an equal volume of Freund's complete Adjuvant, was injected subcutaneously to the same animal. One week later, blood was collected from ear veins of the immunized animal. The serum was brought to 25 % saturation with saturated ammonium sulfate solution, and the precipitate was discarded. The supernatant was brought to 50 % saturation, and the precipitate was collected by centrifugation and dissolved in 0.9 % NaCl buffered with 10 mM potassium phosphate (pH 7.4). The solution was again brought to 45 % saturation

with saturated ammonium sulfate solution and the precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.4) and dialyzed overnight against same buffer. Ouchterlony double diffusion analyses were carried out as described by Noshiro and Omura (15).

Assay of reductases

NAD(P)H-cytochrome c and NADH-ferricyanide reductase activities were measured at 25°C by changes in absorbance at 550 nm and 420 nm, respectively. Either NADH or NADPH (100 nmol), 20 nmol of cytochrome c (or 500 nmol of potassium ferricyanide) in 0.1 M potassium phosphate buffer (pH 7.4) were incubated in final volume of 1.0 ml. Reduction of cytochrome c and ferricyanide was recorded in a Hitachi 356 two-wave length double beam spectrophotometer, and the activities were calculated using respective extinction coefficients of 19.6 $mM^{-1} cm^{-1}$ (16) and 1.02 $mM^{-1} cm^{-1}$ (17). Protein was determined by the method of Lowry et al. using bovine serum albumin as standard (18).

Assay of stearoyl-CoA desaturase activity

Stearoyl-CoA desaturase activities of Tetrahymena microsomes were determined as previously described (5). The incubation mixture contained in a final volume of 0.5 ml, a suitable amount of microsomes (0.1 mg), 50 nmol of NADH (or NADPH), 0.1 M potassium phosphate buffer (pH 7.2) and 20 nmol of either [1-^{14}C] stearoyl-CoA (1 Ci/mol). The samples were preincubated for 1 min at 28°C prior to the addition of microsomes to initiate the reduction. The procedure for analysis of reaction products was essentially as previously described (5).

RESULTS AND DISCUSSION

NADPH-cytochrome c reductase which is able to transfer electrons to cytochrome P-450 has been purified from several different organisms (21 - 24). However, to our knowledge, this is the first report to describe the characterization of NADPH-cytochrome c reductase from a ciliated protozoan, Tetrahymena microsomes (Table I, II). The eluted enzyme represented a 1000-fold purification from the microsomal-bound state. The specific activity of purified reductase was 38.4 μmol cytochrome c reduced/min/mg in 0.1 M phosphate buffer (pH 7.4, 25°C). The molecular weight of the enzyme is approximately 70,000 as determined by

Table I Purification of microsomal NADPH-cytochrome c reductase from Tetrahymena pyriformis

Fraction	Protein (mg)	Specific activity (μmol/min/mg)	Total activity (μmol/min)	Yield (%)
Microsomes	1700	0.041	70	100
Solubilized microsomes	1633	0.042	68.6	98
DEAE-Cellulose	505.5	0.09	45.5	65
2',5'-ADP-Sepharose 4B	1.83	21.0	38.5	55
Sephacryl-200	0.51	38.4	19.6	28

Table II Characterizaton of purified NADPH-cytochrome c reductase from Tetrahymena microsomes

Molecular weight*	70,000
Prosthetic group**	FMN, FAD
Artificial acceptors	cytochrome c ferricyanide dichloroindophenol menadione

* Sodium dodecyl sulfate-polyacrylamide gel (10 %) electrophoresis was carried out according to the procedure of Laemmli (19).
** The fluorometric method of Faeder and Siegal (20) was used to determine specific amounts of FAD and FMN.

SDS-PAGE. The purified reductase which contains FAD and FMN, was able to transfer electron to various exogenous electron acceptors, such as dichloroindophenol, menadione and ferricyanide. Antibody was prepared by using as the antigens purified enzymes obtained from Tetrahymena microsomes. When examined by the Ouchterlony double diffusion test in an agar gel, anti-NADPH-cytochrome c reductase immunoglobulin (anti-reductase IG) formed, a single precipitation line with purified and crude reductase preparation from Tetrahymena microsomes (Fig. 1). These precipitation lines fused together. In contrast, anti-reductase IG formed no precipitation line with the purified NADPH cytochrome c reductase from rat liver microsomes.

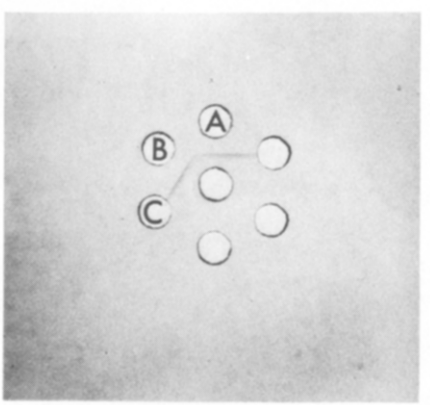

Fig. 1 Ouchterlony double diffusion analysis of NADPH-cytochrome c reductase. Center well contained anti-reductase IG. Tetrahymena NADPH-cytochrome c reductase: ADP-column eluate (A), Sephacryl-200 column eluate (B). Purified rat liver microsomal NADPH-cytochrome c reductase (C).

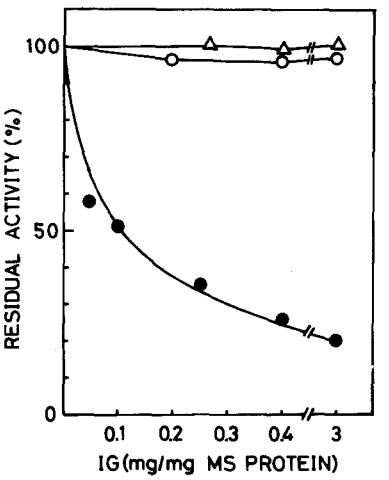

Fig. 2 Effect of anti-reductase IG on NAD(P)H cytochrome c and NADH-ferricyanide reductase activities in Tetrahymena microsomes. Tetrahymena microsomes were preincubated with anti-reductase IG for 10 min at 25°C before adding the necessary cofactors and substrates to initiate the reaction. The final concentrations of microsomes in the reacton mixture was 10 μg/ml (NADH-cyt. c red.), 20 μg/ml (NADPH-cyt. c red.) and 10 μg/ml (NADH-ferricyanide red.). (○) NADH-cyt. c red. (●) NADPH-cyt. c red. (△) NADH-ferricyanide red.

NAD(P)H-cytochrome c reductase and NADH-ferricyanide reductase activities were observed in Tetrahymena microsomes (4). NADPH-cytochrome c reductase activity of Tetrahymena microsomes was strongly inhibited by anti-reductase IG (Fig. 2). The inhibition of the NADPH-cytochrome c reductase activity by antibody was dependent on the ratio of the added to micosomal protein. More than 80 % of the NADPH-cytochrome c reductase activity was inhibited when the ratio was 3 mg of antibody per mg of microsomal protein. However, no inhibitory effect on the NADH-cytochrome c and ferricyanide reductase activities was observed with antibody. Consequently, like liver microsomal NADH-cytochrome c reductase(25), Tetrahymena microsomal NADH-cytochrome c reductase would require NADH-ferricyanide reductase and cytochrome b_{560ms} for activity. On the other hand, control IG had no inhibitory effect on NAD(P)H-cytochrome c and NADH-ferricyanide reductase activities (data not shown).

Tetrahymena microsomes were preincubated with varing amounts of control IG or anti-reductase IG. NADPH-dependent desaturase activity was strongly inhibited by the addition of anti-reductase IG (Fig. 3.A), while control IG exhibited slight increase of desaturase activity at the ratio of 0.3. On the contrary,

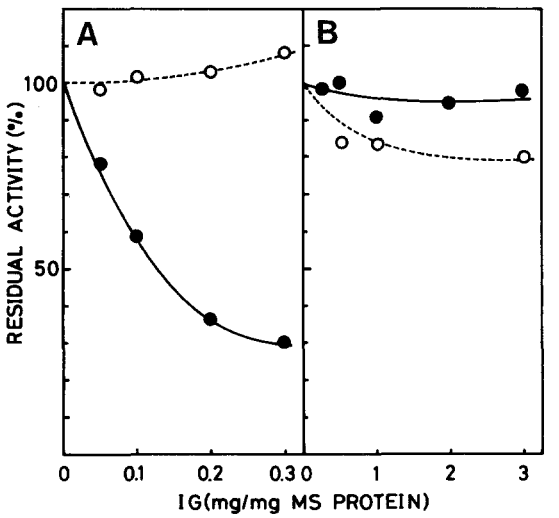

Fig. 3 Effects of anti-reductase IG and control IG on NADPH(A)- or NADH(B)-stearoyl-CoA desaturase activities in Tetrahymena microsomes. Tetrahymena microsomes were preincubated with anti-reductase IG and control IG for 10 min at 25°C before adding the necessary cofactors and substrate to initiate the reaction. The final concentration of microsomes in the reaction mixture was 0.1 mg/ml in all determination. (○) Control IG. (●) Anti-reductase IG.

NADH-dependent desaturations were affected by neither control nor anti-reductase IG (Fig. 3.B) (4). The present immunochemical study gave definite evidence for the participation of NADPH-cytochrome c reductase in NADPH-dependent desaturation reaction of Tetrahymena microsomes.

In mammalian cells, it is well documented that NADPH-cytochrome P-450 reductase (NADPH-cytochrome c reductase) is capable of reducing cytochrome b_5 at a sufficient rate (26). Since Tetrahymena microsomes contain no cytochrome P-450 which is reducible by NADPH, NADPH may provide preferentially electrons to cytochrome b_{560ms} and CSF by a link through the NADPH-cytochrome c reductase during low temperature acclimation.

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