Note

Purification and Characterization of Cytochrome b₅ from *Rhodotorula minuta*

Hideo Fukuda, Kiminori Nakamura, Makoto Iwata, Takahira Ogawa, and Takao Fujii

Department of Applied Microbial Technology, The Kumamoto Institute of Technology, Ikeda 4-22-1, Kumamoto 860, Japan

Received June 8, 1992

Isobutene, an unsaturated hydrocarbon with four carbon atoms, is one of the important starting materials in the petrochemical industry. We found that isobutene was formed from isovalerate in the yeast *Rhodotorula minuta* IFO 1102. Furthermore, we suggested that the isobutene-forming reaction is catalyzed by a microsomal cytochrome P-450 of *R. minuta* which we call cytochrome P-450-IFO. We have proposed a hypothetical model for isobutene formation, *i.e.*, two electrons are transferred from nicotinamide adenine dinucleotide phosphate (NADPH) to cytochrome P-450 via cytochrome P-450 reductase and/or cytochrome b₅, and then the reduced form of cytochrome P-450 catalyzes degenerative conversion of isovalerate to isobutene. Cytochrome b₅, which is a small membrane-bound heme protein, is reduced by cytochrome b₅ reductase or cytochrome P-450 reductase to an electron to steer-aryl-CoA desaturase and to cytochromes P-450. The role of cytochrome b₅ in cytochrome P-450-catalyzed reactions has been investigated in detail by using the purified proteins from mammalian cells. To date, however, there has been few reports about the purification of cytochrome b₅ from microorganisms.

Therefore, we attempted to purify cytochrome b₅ from *R. minuta*.

The content of cytochrome b₅ in the crude preparation or the purified preparation was estimated from the reduced versus oxidized spectra using an extinction coefficient increment of 185 cm⁻¹ mM⁻¹ between 424 and 409 nm or from the absorbance at 413 nm using an extinction coefficient of 113 cm⁻¹ mM⁻¹, respectively. Protein was measured by the method of Lowry et al. Most reagents were purchased from Wako Pure Chemical Industries Ltd. Several reagents obtained from other sources are indicated in parentheses.

*R. minuta* was grown in a chemically defined medium under the conditions previously described. The cells were harvested by centrifugation at 100,000 × g for 15 min at 4°C, and washed once with cold 20% glycerol solution. The washed cells were suspended in 40 mM phosphate buffer, pH 7.9 containing 20% glycerol and 1 mM ethylenediamine tetraacetic acid (EDTA) and then disrupted with a Dymo mill (Wilby A. Bachofen AG Maschinenfabrik, Basel, Switzerland). Cell debris was removed by centrifugation at 1500 × g for 20 min at 4°C. The resulting supernatant obtained was centrifuged at 160,000 × g for 1 h at 4°C to obtain microsomes. The microsomes were solubilized by adding 10 mM phosphate buffer, pH 8.0, containing 20% glycerol, 1 mM EDTA, 1.5% Triton X-100, and 0.5% deoxycholate. The solution was stirred for 1.5 h at 0°C, and then centrifuged at 160,000 × g for 1 h at 4°C to prepare supernatant. Cytochrome b₅ in the supernatant was purified as follows. All procedures were done at 4°C.

Cytochrome b₅ was salted out with 50% saturated (NH₄)₂SO₄ and then put on a first Sephadex G-100 (Pharmacia LKB Biotechnology, Uppsala, Sweden) column that had been previously equilibrated with 10 mM phosphate buffer, pH 7.5, containing 20% glycerol, 0.1% Emulgen 911, 0.1% deoxycholate, and 0.5 mM EDTA. Protein was eluted from the column with the same buffer. Fractions containing cytochrome b₅ were pooled and put on a DEAE-cellulose (Serva Feinbiochemica GmbH & Co., Heidelberg, FRG) column equilibrated with 10 mM phosphate buffer, pH 7.5, containing 20% glycerol and 0.5 mM EDTA. The column was washed with 100 mM phosphate buffer, pH 7.5, containing 20% glycerol, 0.1% Emulgen 911, 0.1% deoxycholate and 0.5 mM EDTA, and then eluted by a linear gradient of 0 to 1 M KCl in the same buffer. An eluted cytochrome b₅ sample was dialyzed against 10 mM phosphate buffer, pH 7.5, containing 0.1 M KCl.

| Table Summary of Typical Purification of Cytochrome b₅ from *Rhodotorula minuta* |
|-----------------|----------------|-----------------|----------------|----------------|
| Purification step | Protein (mg) | Total content (nmol) | Recovery (%) | Specific content (nmol/mg) | Fold |
| Microsomes | 666 | 61.1 | 100 | 0.092 | 1 |
| Solubilized supernatant | 365 | 61.0 | 99.8 | 0.167 | 1.82 |
| Ammonium sulfate precipitate | 225 | 82.4 | 135 | 0.366 | 3.98 |
| First Sephadex G-100 eluate | 31.5 | 14.3 | 23.4 | 0.453 | 4.92 |
| DEAE-Cellulose eluate | 2.88 | 13.4 | 21.9 | 4.65 | 50.5 |
| DE-52 eluate | 0.86 | 10.4 | 17.0 | 12.1 | 131 |
| Second Sephadex G-100 eluate | 0.18 | 5.10 | 8.40 | 29.3 | 318 |

About one liter of culture broth was used for the purification.

Fig. 1. Elution Profiles of Purified Cytochrome b₅ from Sephadex G-100 Column.

The void volume was 33.6 ml (fraction number 12). Volume of each fraction was 2.8 ml. A) Cytochrome b₅ dissolved in 10 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.1% deoxycholate was filtered on a Sephadex G-100 gel column (1.5 × 57 cm) equilibrated with the same buffer without deoxycholate. B) The same gel filtration was done as in A) except for the presence of 0.5% deoxycholate in the buffer.
20% glycerol, 0.1% deoxycholate, and 0.5 mM EDTA overnight. The dialyze was put on a DE-52 (Whatman BioSystems Ltd., Maidstone, Kent, UK) column equilibrated with 10 mM phosphate buffer, pH 7.5, containing 20% glycerol, 0.1% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate), 0.1% deoxycholate, and 0.5 mM EDTA. The column was washed with 50 mM phosphate buffer, pH 7.5, containing 20% glycerol, 0.1% CHAPS, 0.1% deoxycholate, and 0.5 mM EDTA, and eluted by a linear gradient of 0 to 1 M KCl in the same buffer. The cytochrome b₅ eluted was dialyzed against 10 mM phosphate buffer, pH 7.5, containing 20% glycerol, 0.1% deoxycholate, and 0.5 mM EDTA overnight, and concentrated by ultrafiltration with an YM-10 membrane (Amicon Corporation, Danvers, MA, U.S.A.). The concentrated cytochrome b₅ solution was put onto a second Sephadex G-100 column equilibrated with 20 mM phosphate buffer, pH 7.5, containing 20% glycerol, 0.5% deoxycholate, and 0.5 mM EDTA, and eluted with the same buffer. The Sephadex G-100 eluate was used as a purified enzyme.

As shown in Table, cytochrome b₅ was purified 318-fold as its specific activity of 29.3 nmol/mg protein was compared to that of the microsomes. The homogeneity of the final enzyme preparation was proved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of the enzyme was estimated to be 18,000 from the relative mobility on SDS-PAGE. The molecular weight was identical to, but the specific content was slightly smaller than those of cytochrome b₅ from Saccharomyces cerevisiae.⁹

When the purified enzyme was filtered on a Sephadex G-100 gel column, it was eluted with detergent-free buffer at the elution volume of 36 ml (fraction number 13) corresponding to a molecular weight of 140,000, and did with the buffer containing 0.5% deoxycholate at the elution volume of 56 ml (fraction number 20) corresponding to a molecular weight of 36,000 (see Fig. 1). These data showed that the cytochrome b₅ in detergent-free buffer aggregated as an octamer or a heptamer, and that deoxycholate caused the aggregate to be a dimer in contrast to the incomplete disaggregation of cytochrome b₅ from S. cerevisiae on the addition of detergent.¹⁰ It is thought that the aggregation occurs in aqueous media owing to the amphipathic nature of cytochrome b₅.¹³ Thus, it was confirmed that the cytochrome b₅ from R. minuta had an amphipathic nature.

The spectrophotometric properties of the purified enzyme is shown in Fig. 2. The enzyme in an oxidized state has an absorption peak at 556 nm, a β absorption peak at 526 nm, and a Soret absorption peak at 413 nm, and in a reduced state has a Soret absorption peak at 423 nm.

This is the first report about cytochrome b₅ from the genus Rhodotorula. We think that some characteristics of cytochrome b₅ different between R. minuta and S. cerevisiae must be present, but have not been found yet. However, the molecular weight of microsomal cytochrome b₅ from R. minuta, 18,000, is larger than that from liver microsomal cytochrome b₅, e.g., 16,700 for rabbit¹⁴ and 16,000 for calf.¹¹ Microsomal cytochrome b₅ from R. minuta may be somewhat structurally different from liver microsomal cytochrome b₅.

References

Fig. 2. Absorption Spectra of the Second Sephadex G-100 Eluate. Solid line, oxidized; dotted line, ditione-reduced.