EFFECT OF TRITON X-100 AND TRYPSIN ON NADPH-CYTOCHROME *C* REDUCTASE RECONSTITUTIVELY ACTIVE IN FATTY ACID ω-HYDROXYLATION

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1. Introduction

In earlier papers [1, 2], we reported that a fatty acid ω-hydroxylation system in porcine kidney cortex microsomes was resolved into 2 protein fractions called Fraction I and II, which contained a CO-binding hemoprotein, and NADPH-cytochrome *c* reductase, respectively. Fraction II was replaced by the corresponding fraction from porcine liver microsomes (liver Fraction II) or spinach ferredoxin-NADP reductase with ferredoxin. Recently, liver Fraction II has been extensively purified. The present paper describes that the purified preparation of liver Fraction II exists in the monodisperse form in the presence of an appropriate concentration of Triton X-100, and that it can be easily transformed into a reconstitutively inactive form, the molecular weight of which is similar to that of trypsin-extracted NADPH-cytochrome *c* reductase.

2. Methods and materials

2.1. *Purification of liver Fraction II*

Porcine liver microsomes were solubilized with Triton X-100, essentially in the same manner as described for porcine kidney cortex microsomes [2]. The resulting solution (2500 ml) was diluted with 2 vol of 0.075 M KCl and divided into 2 portions. Each was applied to a DEAE-cellulose column (5.6 cm × 30 cm), which had been equilibrated with 0.05 M KCl in 20 mM Tris-HCl buffer (pH 7.9) containing 0.1% Triton X-100, 0.1 mM dithiothreitol and 10% ethyleneglycol ("Buffer A"). Each column was washed with 600 ml of 0.05 M KCl in Buffer A followed by 1000 ml of 0.17 M KCl in 50 mM Tris-HCl buffer (pH 7.9) containing 0.1% Triton X-100, 0.1 mM dithiothreitol and 10% ethyleneglycol ("Buffer B"), then eluted with 0.3 M KCl in Buffer B, and active fractions ("crude Fraction II") were pooled. The enzyme solutions from both columns were combined, diluted with 0.5 vol of distilled water, and applied to a small DEAE-Sephadex A-50 column (3 cm × 4 cm), equilibrated with 0.25 M KCl in Buffer A. A yellow band which remained adsorbed at the top of the column was scraped off and then transferred to a large DEAE-Sephadex A-50 column (3.6 cm × 70 cm), equilibrated with 0.25 M KCl in Buffer A, and the column was eluted with a linear gradient of 1400 ml each of 0.25 M KCl in Buffer A and 0.4 M KCl in Buffer B. The enzyme was further rechromatographed on a DEAE-Sephadex A-50 column (3 cm × 50 cm), equilibrated with 0.25 M KCl in Buffer A. The column was washed with 500 ml of 0.285 M KCl in Buffer A followed by a linear gradient elution of 500 ml each of 0.285 M KCl in Buffer A and 0.37 M KCl in Buffer B. Active fractions (400 ml) were collected, concentrated with an ultrafiltration cell, and then layered over a 5–17% sucrose gradient containing 0.05 M KCl in Buffer B. It was centrifuged at 38,000 rpm for 15 hr at 2° on a Spinco model L2

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ultracentrifuge with a SW39L rotor. Finally, active fractions were subjected to preparative disc gel electrophoresis according to the technique of Maizel [3]. The apparatus was purchased from Toyo-Kagaku-Sangyo Co., Tokyo. Separating gel (6% acrylamide, pH 8.9) was 42 ml, and stacking gel (2.5% acrylamide, pH 6.9) was 24 ml. The upper buffer was 0.052 M Tris-glycine (pH 8.9), and the lower buffer and the elution buffer were 0.1 M Tris-HCl (pH 8.9). Commonly, these gels or buffers were supplied with 0.1% Triton X-100, 0.1 mM dithiothreitol and 10% ethyleneglycol. The initial potential was 350 V, and the current was 60 mA. Five-ml fractions were collected with a flow rate of 1.5 ml per min. Laurate ω-hydroxylation reconstitution activity with NADPH-cytochrome *c* reductase activity was eluted in a single peak 420 ml after the tracking dye, and the solution was concentrated with an ultrafiltration cell. The final preparation showed a specific activity of 8 .8 μmoles of cytochrome *c* reduced per min per mg of protein, and was purified approx. 350-fold over the crude Fraction II with respect to the reconstitution activity of laurate ω-hydroxylation.

2.2. *Preparation of Fraction I*

Preparation of Fraction I from porcine kidney cortex microsomes was described earlier [2]. A highly purified trypsin-extracted NADPH-cytochrome *c* reductase was prepared from rat liver microsomes by the method of Omura and Takesue [4].

The reconstitution activity of laurate ω-hydroxylation was determined using kidney Fraction I as described earlier [2]. NADPH-cytochrome *c* reductase activity was assayed by the method of Phillips and Langdon [5]. 5% Polyacrylamide gel electrophoresis in the presence of Triton X-100 was performed by a modification of the method of Dulaney and Touster [6]. NADPH-neotetrazolium reductase activity on the disc gel was located by staining as follows. After electrophoresis, the gel was bathed in a medium containing 50 μmoles of phosphate buffer (pH 7.5), 0.5 μmole of NADPH, 0.3 μmole of neotetrazolium chloride and water in a final volume of 1.0 ml, and incubated at 37° for 10–20 min with shaking. The activity was detected by the appearance of violet color due to reduced neotetrazolium.

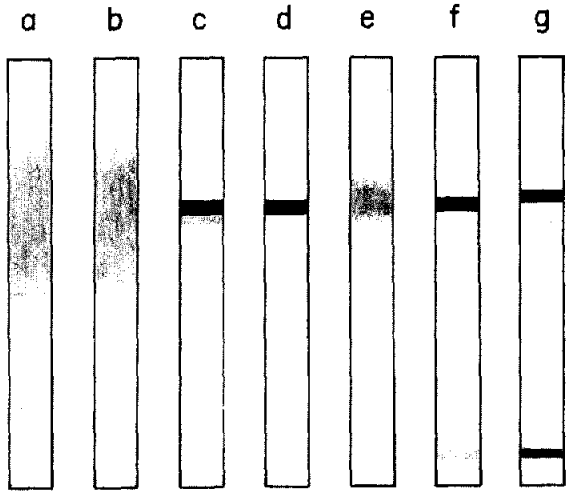

Fig. 1. Disc gel electrophoresis of Fraction II. The highly purified preparation of Fraction II (57 μg of protein) was subjected to gel electrophoresis in the presence of various concentrations of Triton X-100. Protein was stained with amidoblack (a and c), and neotetrazolium reductase activity was stained as described in the text (b, d, e, f and g). (a) and (b) without Triton X-100; (c) and (d) with 0.1% Triton X-100; (e) with 0.02% Triton X-100; (f) with 0.5% Triton X-100; (g) with 1% Triton X-100.

3. Results and discussion

Fig. 1 illustrates that the electrophoretic pattern of NADPH-neotetrazolium reductase activity of the highly purified preparation of liver Fraction II was strikingly affected by the variation of the concentration of Triton X-100 in the gel. When disc gel electrophoresis of Fraction II was carried out in the absence of Triton X-100, a polydisperse state was observed (fig. 1a and b). This would correspond to the aggregation of the enzyme protein. By contrast, Fraction II moved as one major protein band in the presence of 0.1% Triton X-100 (fig. 1c). This band was revealed to be enzymatically active by the staining for NADPH-neotetrazolium reductase activity (fig. 1d). Replacement of 0.1% Triton X-100 by 0.2% Tween-20 also gave a similar monodisperse species with a little greater mobility. With 0.02% Triton X-100 (fig. 1e), the enzyme still showed the polydisperse state to a lesser extent, thus indicating that an appropriate concentration of the detergent was required for the formation of a monodisperse state. Similar findings were recently

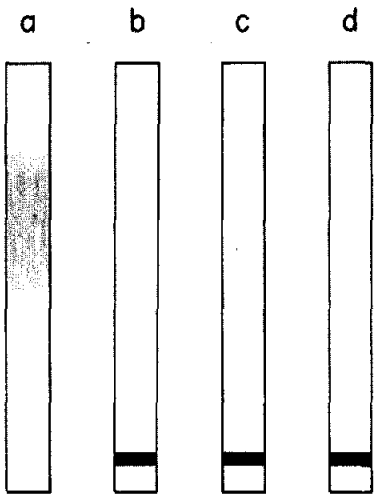

Fig. 2. Effect of trypsin or chymotrypsin on Fraction II. After Fraction II (70 μg of protein) was incubated with the protease (8 μg of protein) in a final volume of 50 μl at 4° for 20 min, the mixture was subjected to disc electrophoresis at 7° without Triton X-100. (a) Fraction II; (b) trypsin-treated Fraction II; (c) chymotrypsin-treated Fraction II; (d) trypsin-extracted NADPH-cytochrome *c* reductase from rat liver microsomes (90 μg of protein).

reported by Singh and Wasserman [7, 8] with cytochrome *f* and other membranous proteins. On the other hand, the presence of 0.5% Triton X-100 resulted in the appearance of a new discrete species with much greater mobility (fig. 1f). The formation of this fast-moving species progressed with increasing concentrations of Triton X-100 (fig. 1g).

The similar phenomenon was observed after treatment of Fraction II with trypsin prior to gel electrophoresis. When Fraction II was incubated with trypsin at 4° for 20 min, the fast-moving species with the same mobility as that observed in fig. 1 appeared (fig. 2b). Chymotrypsin gave the same effect as did trypsin (fig. 2c). Moreover, the formation of this species took place even upon a prolonged storage at −20°. In order to compare enzymic properties of both the slow-moving and fast-moving species, the gel was sliced off into pieces, and each piece was homogenized in 0.05 M Tris-HCl buffer (pH 7.9) containing 0.1 mM dithiothreitol and 10% ethyleneglycol and tested for enzymic activity. While the slow-moving species exhibited laurate ω-hydroxylation reconstitution activity as well as NADPH-cytochrome *c* reductase activity, the fast-moving species had only NADPH-cytochrome *c* reductase activity. No reconstitution activity was detected in the latter species. The mobility of this fast-moving species coincided with that of trypsin-extracted NADPH-cytochrome *c* reductase [4] (fig. 2d), the molecular weight of which was earlier described to be approx. 90,000 by other workers [4, 9, 10]. On the other hand, the slow-moving species was excluded on Sephadex G-100 but retarded on Sephadex G-200.

These results clearly show that liver Fraction II comprises the reconstitutively active NADPH-cytochrome *c* reductase with a molecular weight much larger than that of the classical water-soluble reductase. This high-molecular weight enzyme can exist as the mono-disperse form only in the presence of an appropriate concentration of non-ionic detergents.

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