Subcellular Distribution of Rat Liver NADPH-2,4-Dienoyl-CoA Reductase

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The subcellular distribution of 2,4-dienoyl-CoA reductase (EC. 1.3.1.34, DCR) in rat liver was studied biochemically and immunochemically after the induction of clofibrate. DCR activity was mainly detected in the mitochondrial fraction by sucrose density gradient centrifugation in the livers of both normal and clofibrate-treated rats. It was also shown that the polyclonal antibody against purified DCR detected the enzyme in the mitochondrial fraction. However, the antibodies, which were affinity purified using the purified mitochondrial DCR or were epitope-selected using the fusion-polypeptide expressed from the mitochondrial cDNA clone (λ gt11-RDR181), were cross-reacted with the peroxisomal DCR. These results suggest that peroxisomal DCR is immunochemically indistinguishable from mitochondrial DCR.

Key words β -oxidation; 2,4-dienoyl-CoA reductase; unsaturated fatty acid; mitochondrial enzyme; peroxisomal enzyme; rat liver

The β -oxidation of saturated fatty acids to acetyl-CoA occurs and provides substantial energy in living cells under aerobic conditions. It has been accepted that the β -oxidation of unsaturated fatty acids occurs essentially by the same mechanism.¹⁾ However, unsaturated fatty acids lead to intermediates which are not encountered during the oxidation of saturated fatty acids. Therefore, it has been considered that unsaturated fatty acids require the two auxiliary enzyme activities, Δ^3, Δ^2 -enoyl-CoA isomerase (EC. 5.3.3.8) and D-3-hydroxyacyl-CoA epimerase (EC. 5.1.2.3) (now called enoyl-CoA hydratase (EC. 4.2.1.17) and also enoyl-CoA hydratase 2 (EC. 4.2.1.99)), in addition to the essential enzymes active in the β -oxidation cycles of saturated fatty acids.²⁾

In 1978, Kunau and Dommes proposed an alternative pathway for the β -oxidation of linoleic acid.³⁾ According to their report, in bovine liver, after enzymic dehydrogenation of *cis*-4-decenoyl-CoA to *cis*-4, *trans*-2-decadienoyl-CoA, this dienoyl-CoA can be reduced to *trans*-3-decenoyl-CoA in the presence of NADPH by the novel enzyme, 2,4-dienoyl-CoA reductase (EC. 1.3.1.34, DCR). The activity of DCR has also been detected in *Escherichia coli*,⁴⁾ rat liver^{5,6)} and rat heart,⁷⁾ suggesting that this widely distributed enzyme commonly participates in the degradation of fatty acids. In rat liver, this reductase activity is located in both the mitochondria and the peroxisomes, and is induced by clofibrate treatment.^{1,2)} However, it has not been clarified whether the reductase in the two organelles were molecularly different from each other or the same.

We attempted to demonstrate the distribution of DCR in both mitochondria and peroxisomes, and to demonstrate whether or not the DCRs in two organella were the same. This paper discusses the subcellular distribution based on enzyme activity analyses and immunochemical analyses using the polyclonal antibody against purified DCR.⁸⁾

MATERIALS AND METHODS

Materials *cis*-4, *trans*-2-Decadienoic acid was synthesized by the method of Richards and Weller⁹⁾ with a slight modification. The CoA ester was prepared by the method described previously.¹⁰⁾ Fixed *Staphylococcus aureus* cell suspension (10%, w/v) was purchased from Sigma Chemical Company. A protein assay kit and molecular weight marker proteins for sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories. Other chemicals were all from Wako Pure Chemical Industry.

Animals Male Wistar Imamichi rats were obtained from the Mice Center of Tohoku University and fed *ad libitum* a diet with or without 0.3% (w/w) clofibrate for 4 weeks.

Subcellular Fractionation of Rat Liver Livers were homogenized and fractionated according to the method of de Duve *et al.*¹¹⁾ Heavy and light mitochondrial fractions were not separated unless necessary. The mitochondrial fraction or the light mitochondrial fraction was subjected to sucrose density gradient centrifugation according to the method of Osumi and Hashimoto.¹²⁾ The sucrose density gradient solution included 10 mM glycylglycine-NaOH buffer (pH 7.2). Centrifugation was carried out at 23000 rpm for 2 h at 4°C in a Hitachi SRP-28A rotor. After centrifugation, 1.5 ml fractions were collected from the bottom to the top of the tube, and were stored at -80°C.

Enzyme Assay The activity of DCR was assayed by the method of Hiltunen *et al.*¹³⁾ using *cis*-4, *trans*-2-decadienoyl-CoA as a substrate. The activity of catalase¹⁴⁾ and cytochrome c oxidase¹⁵⁾ was assayed as described previously.

Preparation of 2,4-Dienoyl-CoA Reductase and Its Antibody DCR was purified from the livers of rats treated with clofibrate, as described previously.⁸⁾ The purified enzyme was dialyzed against 50 mM potassium phosphate, pH 7.5, containing 5 mM 2-mercaptoethanol and 40% (v/v)

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glycerol, and the sample was kept at -20°C . The stocked enzyme was dialyzed against phosphate buffered saline and concentrated. This concentrated enzyme (1 mg/ml) was emulsified with an equal volume of Freund's complete or incomplete adjuvant, and immunized into a New Zealand white rabbit every two or three weeks. Two weeks after the last injection, which was more than 3 months after the first injection, the blood of the rabbit was taken from the carotid artery. The antibody was partially purified by ammonium sulfate fractionation followed by dialysis against phosphate buffered saline.

Selection of Antibodies Specific for the 2,4-Dienoyl-CoA Reductase and the Fusion Polypeptide from the cDNA Clone of 2,4-Dienoyl-CoA Reductase Specific antibodies for DCR were selected by nitrocellulose membrane transferred with the purified DCR.¹⁶⁾ A specific antibody for expressed polypeptides from the cDNA clone of DCR were selected by the method of Weinberger *et al.*¹⁷⁾ Briefly, nitrocellulose membranes containing protein from λ gt11, clone RDR181 (a cDNA clone of rat DCR¹⁸⁾) were used to select the antibody. *E. coli* Y1090, as the host cell, was used to produce proteins. Both selected antibodies were used directly for immunoblot analysis, or were concentrated and used for immunoprecipitation analysis.

Immunoblot Analysis SDS-PAGE in 12% acrylamide slab gel was performed by the method of Laemmli.¹⁹⁾ After electrophoresis, proteins were electrophoretically transferred to the nitrocellulose membrane. After the transfer, the membrane was first incubated in phosphate buffered saline containing 1% bovine serum albumin (BSA) for 30 min at room temperature, followed by a 4 h-incubation of the crude antibody or selected antibody against DCR. After being washed with phosphate buffered saline, the membrane was incubated with horseradish peroxidase conjugated goat anti-rabbit immunoglobulins for 2 h at room temperature. The membrane was then washed and

stained using 3,3'-diaminobenzidine tetrahydrochloride as the substrate.

Immunoprecipitation Procedure The peroxisomal fraction (fraction number 3) or the mitochondrial fraction (fraction number 7) described in Fig. 3 was mixed with antibody of a known concentration, and incubated at 4°C for 2 h. Peroxisomal and mitochondrial fractions containing similar levels of DCR activities were prepared. The incubation mixture contained 50 mM potassium phosphate, pH 7.4, 1% BSA, 1 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100 in a total volume of 100 μl . After the incubation, 50 μl of 10% *S. aureus* suspension was added to each incubation mixture, and the new mixture was shaken at 4°C for 1 h. Each mixture was then centrifuged at 14000 rpm for 5 min at 4°C , and 100 μl of the suspension was used for the assay of DCR.

RESULTS

Subcellular Distribution of the Activity of 2,4-Dienoyl-CoA Reductase in Rat Liver Rat liver homogenates were fractionated by differential centrifugation (Fig. 1). The distribution pattern of the DCR was similar to the pattern of cytochrome c oxidase,⁵⁾ known as the marker enzyme of mitochondria. Figure 2 shows the distribution of several enzyme activities after the sucrose density gradient centrifugation of the light mitochondrial fraction. The activity of catalase, known as the marker enzyme of peroxisomes, was mainly detected in fraction 3, and cytochrome c oxidase was mainly detected in the fraction 8. The activity of DCR was detected in both fractions 3 and 8. These results were similar to those of previous reports,⁸⁾ although it is noteworthy that most DCR activity was found in the heavy mitochondrial fraction in the rat liver. Next, we performed sucrose density gradient centrifugation for the mitochondrial fraction, which had

Fig. 1. Subcellular Distribution of Enzyme Activities after Differential Centrifugation

The sum of protein contents of all fractions was set at 100%. (a), Cytochrome c oxidase; (b), catalase; (c), 2,4-dienoyl-CoA reductase; (N), nuclei; (H), heavy mitochondria; (L), light mitochondria; (M), microsomes; (S), soluble proteins; upper panel, data of normal rat; lower panel, data of rat treated with clofibrate.

Fig. 2. Distribution of Enzyme Activities after Sucrose Density Gradient Centrifugation of the Light Mitochondrial Fraction

(a), Protein; (b), cytochrome c oxidase; (c), catalase; (d), 2,4-dienoyl-CoA reductase; upper panel, data of normal rat; lower panel, data of rat treated with clofibrate. Fractions were taken from the bottom of the centrifugation tubes. The frequency represents the $(\text{value of parameter fractions}) \times (\text{sum of parameters in all fractions})^{-1} \times 100$.

Fig. 3. Distribution of Enzyme Activities after Sucrose Density Gradient Centrifugation of the Mitochondrial Fraction

(a), Protein; (b), cytochrome c oxidase; (c), catalase; (d), 2,4-dienoyl-CoA reductase; upper panel, data of normal rat; lower panel, data of rat treated with clofibrate. Fractions were taken from the bottom of the centrifugation tubes. The frequency represents the $(\text{value of parameter fractions}) \times (\text{sum of parameters in all fractions})^{-1} \times 100$.

both light mitochondrial and heavy mitochondrial fractions (Fig. 3). This fraction contained 80–90% of the total activity of DCR in the liver homogenate. Most of

the activity was observed in fractions 7 and 8, as in the case of cytochrome c oxidase. Although some of the activity of catalase was found in these fractions, the

distribution pattern of the DCR was not similar to that of catalase. Thus, DCR was mainly detected in the mitochondria. Moreover, similar results were obtained for materials from the rat treated with clofibrate (Figs. 1–3). These results suggested that the mitochondrial DCR was mainly induced by clofibrate treatment in the rat liver.

Western Blot Analysis of 2,4-Dienoyl-CoA Reductase

The affinity selected antibody against purified DCR was used for immunoblot analysis. As shown in Fig. 4, the purified antibody only reacted with a single 35 kDa polypeptide among proteins in the liver homogenates of normal rats or rats treated with clofibrate. The molecular weight of immuno-reacted polypeptide in the homogenates was the same as that of purified DCR. These results indicated the high specificity of the selected antibody for the DCR. A equal volume of the sucrose gradient subfraction, as shown in Fig. 3, was separated by SDS-PAGE and transferred to the nitrocellulose membrane. Polypeptide which reacted with the antibody against DCR was mainly located in fractions 7 and 8, which were considered to be mitochondrial fractions. Similar data were obtained in the experiments using both normal and clofibrate-treated rats. These immunochemical experiments suggested that purified DCR was the mitochondrial enzyme.

To determine whether the selected antibody cross-reacted with the peroxisomal DCR, the light mitochondrial fraction was further analyzed, since this fraction contained numerous peroxisomes. After sucrose density gradient centrifugation, peroxisomal and mitochondrial fractions were collected. The activity of each fraction and the dot-blot immunotitration analysis of these fractions are shown in Figs. 5a and 5c, respectively. The ratio of DCR activity of mitochondrial values to peroxisomal values was about three times higher in the control rat (NM/NP) and about 12 times higher in the clofibrate treated rat (CM/CP). The dot-blot immunotitration analysis showed that the ratio of the mitochondrial immuno-reacted constant to peroxisomal enzyme was about two (NM/NP)

in the control rat and about eight (CM/CP) in the clofibrate-treated rat. To further demonstrate the cross-reactivity of the selected antibody with peroxisomal DCR, equal activities of DCR in both mitochondrial and peroxisomal fractions were separated by SDS-PAGE and transferred to the nitrocellulose membrane. As shown in Fig. 5b, the selected antibody reacted with the same intensity with about a 33 kDa polypeptide of each fraction. In the control rat experiment the intensities were weak, but it was thought that the transfer efficiency was low because of the low proportion in the amounts of DCR in the control rat liver. Equal activity of the purified DCR to that of each fraction was analyzed at the same time

Fig. 4. Western Blot Analysis of the Fraction from Sucrose Density Centrifugation of Mitochondrial Fraction by Using the Antibody Specific for 2,4-Dienoyl-CoA Reductase

The mitochondrial fraction contains both heavy and light mitochondrial fractions. (N) and (C) represent the liver homogenates of normal and of treated clofibrate rats, respectively; (E) is the purified 2,4-dienoyl-CoA reductase. Equal activities of these homogenates or the purified enzyme was subjected to SDS-PAGE and transferred to the nitrocellulose membrane. Each number at the top of the panel corresponds to the number of fractions described in Fig. 3. An equal volume of each fraction was subjected to SDS-PAGE and transferred to the membrane. The partially purified antibody, specific for 2,4-dienoyl-CoA reductase, was used to detect the proteins which immunoreacted. Staining procedures were described in Materials and Methods. The molecular standards used were as follows: phosphorylase a (93000), bovine serum albumin (68000), ovalbumin (43000), carbonic anhydrase (31000), and soybean trypsin inhibitor (21400).

Fig. 5. Western Blot and Dot Blot Analysis of the Peroxisomal 2,4-dienoyl-CoA Reductase

(E), purified 2,4-dienoyl-CoA reductase; (CM), mitochondrial fraction of clofibrate treated rats; (CP), peroxisomal fraction of rats treated with clofibrate; (NM), mitochondrial fraction of normal rats; (NP), peroxisomal fraction from normal rats. The values in the table (a) and the activities of fractions used for dot blot analysis (c). The values of the activities are represented as nmol/min/ml. For western blot analysis (b), each sample with the same activity was subjected to SDS-PAGE and transferred to the membrane. The partially purified antibody specific for the purified 2,4-dienoyl-CoA reductase was used to detect proteins which immunoreacted. Staining procedures were described in Materials and Methods.

Fig. 6. Immunoprecipitation Analysis of Mitochondrial and Peroxisomal Activity of 2,4-Dienoyl-CoA Reductase from Sucrose Density Centrifugation of Mitochondrial Fraction

Mitochondrial fractions were taken from the fraction 3 described in Fig. 3. The each fraction was incubated with verified amounts of the antibody. The immunoprecipitation procedures are described in Materials and Methods. Symbols of circle and triangle representing, the antibodies fractionated by ammonium sulfate, were used (a and b). Closed circle and triangle, experiment 1; opened circle and opened triangle, experiment 2. Symbols of opened square, the antibodies specified by the purified 2,4-dienoyl-CoA reductase (c). Symbols of closed square, the antibodies specified by the expressed fusion protein from the clone λ gt11-RDR181 were used (c).

(Fig. 5b, lane E). The selected antibody was also reacted with this purified DCR in the same intensity. These results suggest that the mitochondrial DCR and peroxisomal DCR have the same molecular weight and similar immuno-responsibility.

Immunoprecipitation of Activities of Mitochondrial and Peroxisomal 2,4-Dienoyl-CoA Reductase By using the immunoprecipitation method, the reactivities of the three grades of polyclonal antibodies (anti-DCR, antibody selected by purified DCR and selected by λ gt11-RDR181 expressed polypeptide) with the DCR of both peroxisomal and mitochondrial fractions were studied. Both fractions were obtained by sucrose density gradient fractionation of the light mitochondrial fraction. Samples containing equal activity of DCR were tested. Non-immunized antibodies did not precipitate either mitochondrial or peroxisomal DCR (data not shown). The antibody against DCR, prepared by fractionation with ammonium sulfate, immunoprecipitated the activities of DCR in both the mitochondrial and peroxisomal fractions (Figs. 6a and 6b). Moreover, the immuno-titration curves of both fractions were indistinguishable. These results suggest that DCR in the mitochondria and peroxisomes have very similar immunoreactivities in our antibody preparation. Both antibody preparations, selected by the purified DCR and fusion-polypeptide expressed from rat liver mitochondrial DCR cDNA clone, immunoprecipitated the peroxisomal DCR (Fig. 6c).

DISCUSSION

It has been demonstrated that the degradation capacity of polyunsaturated fatty acids in peroxisomes was the same as or larger than the degradation capacity in mitochondria,^{20,21)} and that DCR was essential for both the mitochondrial and the peroxisomal β -oxidation of all unsaturated fatty acids processing double bonds at even numbered carbon atoms.^{7,22)} In this study, we used decadienoyl-CoA as a substrate, because it has been shown that purified DCR catalyzes the reduction of decadienoyl-

CoA at the highest velocity among dienoyl-CoAs having six to sixteen carbon atoms. And we demonstrated that most DCR activity exists in the mitochondrial fraction of the rat liver (Fig. 3). Moreover, using the polyclonal antibody directed to the purified DCR, the enzyme was detected mostly in the mitochondrial fraction, and the mitochondrial enzyme was mainly induced by clofibrate treatment rather than the peroxisomal one (Fig. 3). There is a possibility that different reductase(s) may function in mitochondria or in peroxisomes for the degradation of very long chain unsaturated fatty acids. Hiltunen *et al.* reported the existence of another DCR isoenzyme in mitochondria.²³⁾ The very long chain fatty acyl-CoA synthetase is different from the acyl-CoA synthetase purified previously.^{24,25)} The second possible reason may be that a small amount of peroxisomal DCR is sufficient for peroxisomal degradation of polyunsaturated fatty acids in the normal state. Many polyunsaturated fatty acids can be potent inhibitors of mitochondrial β -oxidation.²⁶⁾ The potential of this inhibitory effect was demonstrated to be caused by limiting the activity of mitochondrial DCR.^{3,27)} Moreover, it is reported that 2,4-dienoyl-CoAs may be degraded by peroxisomal bi-functional enzyme more than by mitochondrial 2-enoyl-CoA hydratase,²⁸⁾ and that the bifunctional enzyme includes the activity of 3-enoyl-CoA isomerase as an additional function,²⁹⁾ which catalyzes the isomerization of 3-enoyl-CoAs by DCR to 2-enoyl-CoAs.

As mentioned above, the present results suggest that purified DCR is a mitochondrial enzyme. However, subfractionation of light mitochondria (Fig. 2 and ref. 5) demonstrated the presence of the activity of DCR in peroxisomes. The activity of DCR observed in peroxisomes was much larger than the value which was expected by the contamination of mitochondria-distributed enzyme, in comparison with the contamination of cytochrome c oxidase activity (for the mitochondrial marker enzyme) in peroxisomal fractions of both normal and clofibrate treated rats. Moreover, in the same immuno-responsibility for mitochondrial DCR, some grades of antibodies we

prepared reacted with peroxisomal DCR (Figs. 5 and 6). Immunocytochemical analysis revealed that the antibody against DCR, prepared by us, also reacted with the peroxisomal enzyme.^{16,30)} It is noteworthy that the antibody selected by the expressed fusion-polypeptide from the cDNA clone completely immunoprecipitated peroxisomal DCR. These results suggest that immunologically and genetically similar enzymes are present in both mitochondria and peroxisomes.

It has already been demonstrated that the peroxisomal and mitochondrial pathways of saturated fatty acid degradation were identical, but the enzymes were all different.^{31,32)} However, the peroxisomal DCR was immunologically indistinguishable from the mitochondrial DCR in this study. Accordingly, the acyl-CoA synthetase distributed in the three organella (mitochondria, peroxisomes and endoplasmic reticulum) also cannot be biochemically and immunochemically differentiated from one another. Moreover, serine-pyruvate aminotransferase (SPT) is located in both the mitochondria and peroxisomes in rat liver. The peroxisomal SPT cannot be differentiated from the mitochondrial SPT by biochemical³³⁾ or immunochemical³⁴⁾ methods. On the other hand, the mitochondrial SPT is selectively induced by glucagon treatment,³⁵⁾ and the peroxisomal SPT is selectively induced by di-(2-ethylhexyl) phthalate (DEHP) treatment.³⁶⁾ The mitochondrial DCR in the rat liver is, however, induced selectively by clofibrate, DEHP¹⁶⁾ and growth hormone.³⁷⁾

If the same DCR is present in both organella, the precursor of the enzyme may have two different target signals, and under some conditions the mitochondrial DCR may be selectively induced. However, the protein transport mechanism of DCR for both organella remains to be elucidated. Therefore, a more precise investigation is necessary not only to clarify the transport mechanism but also to search for further cDNA clones and the gene for DCR.

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