Identification of a Substrate-binding Site in a Peroxisomal β-Oxidation Enzyme by Photoaffinity Labeling with a Novel Palmitoyl Derivative

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Peroxisomes play an essential role in a number of important metabolic pathways including β-oxidation of fatty acids and their derivatives. Therefore, peroxisomes possess various β-oxidation enzymes and specialized fatty acid transport systems. However, the molecular mechanisms of these proteins, especially in terms of substrate binding, are still unknown. In this study, to identify the substrate-binding sites of these proteins, we synthesized a photoreactive palmitic acid analogue bearing a diazirine moiety as a photophore, and performed photoaffinity labeling of purified rat liver peroxisomes. As a result, an 80-kDa peroxisomal protein was specifically labeled by the photoaffinity ligand, and the labeling efficiency competitively decreased in the presence of palmitoyl-CoA. Mass spectrometric analysis identified the 80-kDa protein as peroxisomal multifunctional enzyme type 2 (MFE2), one of the peroxisomal β-oxidation enzymes. Recombinant rat MFE2 was also labeled by the photoaffinity ligand, and mass spectrometric analysis revealed that a fragment of rat MFE2 (residues Trp249 to Arg251) was labeled by the ligand. MFE2 mutants bearing these residues, MFE2(W249A) and MFE2(R251A), exhibited decreased labeling efficiency. Furthermore, MFE2(W249G), which corresponds to one of the disease-causing mutations in human MFE2, also exhibited a decreased efficiency. Based on the crystal structure of rat MFE2, these residues are located on the top of a hydrophobic cavity leading to an active site of MFE2. These data suggest that MFE2 anchors its substrate around the region from Trp249 to Arg251 and positions the substrate along the hydrophobic cavity in the proper direction toward the catalytic center.

Peroxisomes are organelles bound by a single membrane, which are present in almost all eukaryotic cells. Peroxisomes are involved in a variety of important metabolic processes including the β-oxidation of fatty acids, synthesis of plasmalogens, and bile acids (1). A specialized set of enzymes responsible for the peroxisomal functions are compartmentalized in the organelle, and the deficiency of peroxisomal enzymes causes severe metabolic disease such as acyl-CoA oxidase deficiency and d-bifunctional protein deficiency (2–4). Fibroblasts from patients with these deficiencies exhibit the disturbed peroxisomal β-oxidation of very long-chain fatty acids (VLCFAs), and VLCFAs are accumulated in the plasma and tissues of these patients (5). Analyses of these peroxisomal diseases reflect the importance of the peroxisomal β-oxidation in organisms along with disclosing the enzymatic organization of the peroxisomal β-oxidation system. Peroxisomes are involved in the β-oxidation of VLCFAs, branched chain fatty acids such as pristanic acid, and bile acid intermediates such as dihydroxycholestanolic acid and trihydroxycholestanolic acid, which are incompatible with mitochondrial β-oxidation (6). Peroxisomal β-oxidation of fatty acids proceeds via a four-step pathway as in mitochondria, and multiple enzymes are involved in each step of the pathway. The first step of peroxisomal β-oxidation is the desaturation of acyl-CoA to the corresponding 2E-enoyl-CoA. In mammals, acyl-CoA oxidase 1 (ACOX1) oxidizes straight chain acyl-CoAs, whereas trihydroxycholestanoyl-CoA oxidase (ACOX2) and pristanoyl-CoA oxidase (ACOX3) oxidize both straight and 2-methyl-branched acyl-CoAs (7, 8). The second and third reactions are catalyzed by two multifunctional enzymes (MFEs) by stereochemically distinct pathways. MFE1 hydrates 2E-enoyl-CoA to 3S-hydroxyacyl-CoA and subsequently dehydrogenates the 3S-hydroxyacyl-CoA to 3-ketoacyl-CoAs, which is a naturally occurring pathway in the β-oxidation of straight chain fatty acids (9, 10). MFE2, the other peroxisomal multifunctional enzyme, which also possesses 2E-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, converts the 2E-enoyl-CoA into the corresponding 3-ketoacyl-CoA via a 3R-hydroxyacyl-CoA intermediate.

The abbreviations used are: VLCFA, very long-chain fatty acid; ACOX, acyl-CoA oxidase; H2, 2-enoyl-CoA hydratase; HD, 3R-hydroxyacyl-CoA dehydrogenase; LCFA, long-chain fatty acid; MFE1 and MFE2, multifunctional enzyme type 1 and type 2; PMP70, 70-kDa peroxisomal membrane protein.

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Photoaffinity Labeling of a Peroxisomal β-Oxidation Enzyme

Photoaffinity labeling of a peroxisomal β-oxidation enzyme with the novel photoreactive fatty acid analogue to identify fatty acid binding proteins and analyze the substrate binding of the peroxisomal β-oxidation system and peroxisomal fatty acid transport system has been made relatively clear. However, the precise molecular mechanisms of these proteins, especially in terms of substrate recognition, are poorly understood. In this study, we synthesized a photoreactive palmitic acid analogue bearing a diazirine moiety (Fig. 1) to afford compound 2 (0.63 g, 85%) as a pale yellow oil; 1H NMR (CDCl3) δ 7.16 (d, 1H, J = 7.7 Hz), 6.75 (d, 1H, J = 7.7 Hz), 6.60 (s, 1H), 5.59 (s, 1H), 5.36 (d, 1H, J = 3.4, 9.8 Hz), 3.92 (m, 1H), 3.50 – 3.62 (m, 9H), 3.36 (d, 1H, J = 3.4, 13.2 Hz), 3.32 (br q, 2H, J = 5.1 Hz), 3.01 (d, 1H, J = 9.8, 13.2 Hz), 1.45 (s, 9H), 1.44 (s, 9H). 3-[2-[2-(2-tert-Butyloxycarbonylaminoothoxy)ethoxy]ethyl]oxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl-L-alanine tert-Butyl Ester (3)—An 80% ethanol solution of NH4OH/HCl (1 m, 5 ml) was added to a solution of compound 2 (0.66 g, 0.81 mmol) in CH2Cl2 (5 ml). The reaction mixture was stirred at 45 °C for 1 h. After removal of the solvent, the residue was dissolved in CH2Cl2 and purified by column chromatography on silica gel (hexane:EtOAc = 1 : 1) to afford compound 3 (0.44 g, 92%) as a pale yellow oil; 1H NMR (CDCl3) δ 7.16 (d, 1H, J = 7.7 Hz), 6.75 (d, 1H, J = 7.7 Hz), 6.60 (s, 1H), 5.36 (d, 1H, J = 3.4, 9.8 Hz), 4.12 (d, 1H, J = 3.4, 5.1, 9.8 Hz), 4.02 (br s, 1H), 3.89 (br s, 1H), 3.83 (d, 1H, J = 3.4, 5.1, 11.1 Hz), 3.77 – 3.80 (m, 1H), 3.69 (m, 1H), 3.63 – 3.66 (m, 2H), 3.55 – 3.60 (m, 1H), 3.49 – 3.54 (m, 1H), 3.33 (br s, 2H), 3.27 (br s, 2H), 2.91 (d, 1H, J = 8.3, 13.9 Hz), 1.42 (s, 9H), 1.34 (s, 9H).

N-Palmitoyl-3-[2-[2-(2-tert-butyloxycarbonylaminoethoxy)ethyl]oxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl-L-alanine tert-Butyl Ester (4)—A CH2Cl2 solution (1 ml) of compound 3 (200 mg, 0.35 mmol) was added to a CH2Cl2 solution (3 ml) of palmitic acid (90 mg, 0.35 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (74 mg, 0.38 mmol), and Et3N (39 mg, 0.38 mmol). The reaction mixture was stirred overnight at room temperature. After removal of the solvent, the residue was purified by column chromatography on silica gel (hexane:EtOAc = 1 : 1) to afford compound 4 (223 mg, 79%) as a pale yellow solid; 1H NMR (CDCl3) δ 7.16 (d, 1H, J = 7.7 Hz), 6.73 (d, 1H, J = 7.7 Hz), 6.59 (s, 1H), 6.37 (d, 2H, J = 8.1 Hz), 5.12 (br s, 1H), 4.71 – 4.77 (m, 1H), 4.08 – 4.18 (m, 2H), 3.88 – 3.96 (m, 2H), 3.71 – 3.79 (m, 2H), 3.67 (t, 2H, J = 4.7 Hz), 3.55 (t, 2H, J = 5.1 Hz), 3.31 (q, 2H, J = 5.1 Hz), 3.07 (dd, 1H, J = 9.6, 13.5 Hz), 2.98 (dd, 1H, J = 5.8, 13.5 Hz), 2.04 – 2.14 (m, 2H), 1.40 – 1.50 (m, 11H), 1.38 (s, 9H), 1.12 – 1.32 (m, 26H), 0.88 (t, 3H, J = 6.8 Hz).

N-Palmitoyl-3-[2-[2-(2-aminoethoxy)ethyl]oxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenylalanine (5)—Deprotection of compound 4 (0.20 g, 0.25 mmol) was performed with 50% TFA/CHCl3 (4 ml) at room temperature.
After stirring for 3 h, the solvent was removed. The product was extracted with CHCl₃ three times from aqueous NaHCO₃ solution. The organic solution was combined, washed twice with brine, and then dried over MgSO₄ to afford compound 5 (0.16 g quantity) as a pale yellow solid. Removal of the two tert-butyl groups was confirmed by NMR and the product was used without further purification; ¹H NMR (CDCl₃) δ 7.21 (d, 1H, J = 7.8 Hz), 6.81 (br s, 1H), 6.69 (d, 1H, J = 7.8 Hz), 6.51 (s, 1H), 4.52 (br s, 1H), 4.07–4.16 (m, 2H), 3.89–3.95 (m, 1H), 3.75–3.86 (m, 2H), 3.65–3.69 (m, 2H), 3.41–3.47 (m, 1H), 3.33–3.39 (m, 1H), 3.19 (dd, 1H, J = 5.1, 13.7 Hz), 2.92 (dd, 1H, J = 5.1, 12.8 Hz), 2.73 (dd, 1H, J = 6.4, 12.8 Hz), 2.18 (t, 2H, J = 6.8 Hz), 2.09 (t, 2H, J = 7.7 Hz), 1.55–1.70 (m, 4H), 1.37–1.45 (m, 4H), 1.18–1.32 (m, 24H), 1.09–1.16 (m, 2H), 0.88 (t, 3H, J = 7.1 Hz).

Preparation of Rat Liver Peroxisomes

The livers of male Wister rats were fractionated by the methods of de Duve et al. (33). The livers were homogenized in 4 volumes of SVEH (0.25 M sucrose, 1 mM EDTA, 0.1% ethanol, and 5 mM Hepes-KOH, pH 7.4), and the supernatant (post-nuclear supernatant) was recovered by centrifugation at 1,000 × g for 10 min at 4 °C. The heavy and light mitochondrial fraction was sedimented from the post-nuclear supernatant fraction by centrifugation at 20,000 × g for 22 min. About 0.7 ml of the light mitochondrial fraction was applied onto a 10-ml linear Nycodenz gradient (density span from 1.15–1.25 g/ml in SVEH), and centrifuged 193,000 × g for 2 h at 4 °C. Fractions of ~1.0 ml were collected in preweighed microtubes, and the density of each fraction was determined by refractometry. The peroxisomal fraction was identified by the density and distribution of catalase (33).

MALDI-TOF Analysis of the Labeled Protein

After photoaffinity labeling of purified MFE2-His (100 μg of protein), the reaction product was applied onto SoftLink Soft Release Avidin Resin equilibrated with the binding buffer. After incubation of the suspensions for 2 h at 4 °C, the beads were collected by centrifugation and washed five times with 250 μl of the binding buffer. Labeled proteins were eluted by 2 × sample buffer for SDS-PAGE (4% (w/v) SDS, 19.8% (v/v) sucrose, 0.08 M Tris-HCl, pH 6.8, 20 mM DTT, and 0.002% (w/v) bromphenol blue), and separated on a 5–10% SDS-polyacrylamide gradient gel. After electrophoresis, labeled proteins were transferred onto a PVDF membrane, stained with Coomassie Brilliant Blue, destained, and then the bands corresponding to the labeled proteins were excised from the membrane. The membrane fragments were incubated for 1 h at 27 °C with reduction buffer (0.5 M Tris, pH 8.5, 8 M guanidinium HCl, 0.3% (w/v) EDTA, and 5% acetonitrile) containing 10 mg/ml of DTT and then for 20 min in the same buffer containing 30 mg/ml of iodoacetamide. The S-carboxymethylated sample was soaked in 100 μl of digestion buffer (50 mM NH₄HCO₃, pH 8.0, and 70% acetonitrile), and digested with 2 μg of sequencing-grade modified trypsin for 12 h at 27 °C. The digestion was terminated by the addition of PMSF to 10 μM of the final concentration and the sample was concentrated in a SpeedVac concentrator (Savant, Thermo Scientific, USA) after the digestion was completed.
Farmingdale, NY). The resulting residue was resuspended in 10 μl of 0.1% TFA. The tryptic peptide mixture was desalted with Zip Tip (Millipore, Billerica, MA) and eluted in 2 μl of 33.3% acetonitrile with 0.06% TFA. The recovered tryptic peptides (1 μl) were mixed with 1 μl of a 10 mg/ml solution of N-hydroxysuccinimide (NHS)-biotin and analyzed by MALDI-TOF mass spectrometry on a Bruker MALDI-TOF AutoFlex mass spectrometer (Bruker-Daltonics, Billerica, MA).

**Other Methods**

Protein was assayed as described previously (29). Western blot analysis was performed using primary antibodies and a secondary antibody, donkey anti-rabbit or antimouse IgG antibody-conjugated horseradish peroxidase (GE Healthcare). Antigen-antibody complex was visualized with ECL+ Western blotting detection reagent (GE Healthcare).

**RESULTS**

**Chemistry**—To identify and characterize long-chain fatty acid (LCFA)-binding proteins in peroxisomes, we synthesized a novel photoreactive LCFA probe 1 (Fig. 1A). The synthetic probe consists of a LCFA, a diazirine-based photophore, and a biotin moiety with a hydrophilic linker. The (3-trifluoromethyl)phenyl diazirine is one of the most promising photophores. By photolysis, it produces a carbene intermediate, and immediately makes a covalent bond with spatially close molecules. A biotin moiety was directly anchored to the photophore via a hydrophilic linker as a tag for the detection and isolation of labeled proteins utilizing biotin-avidin interaction, and is also useful for identifying the labeled protein and its ligand-interacting site. For preparation of a photoreactive fatty acid analogue, a phenylalanine analogue has been employed to introduce a diazirine photophore with an accompanying carboxylate at the end of palmitic acid (16:0). Preparation of the photoreactive phenylalanine derivative was achieved in excellent yield according to the previous method (35). The coupling reaction with palmitic acid and the subsequent biotinylation to give the corresponding photoreactive fatty acid probe 1 is outlined in Fig. 1B.

**Photoaffinity Labeling of Rat Liver Peroxisomes**—Using the photoreactive LCFA analogue as a probe, we performed photoaffinity labeling experiments on purified rat liver peroxisomes. Purified rat liver peroxisomes were incubated with the photoreactive fatty acid analogue, and the labeled proteins were separated on a 7–15% SDS-polyacrylamide gradient gel, and detected by streptavidin-HRP. As shown in Fig. 2, an 80-kDa protein was specifically labeled by the photoaffinity probe upon activation with UV irradiation. The efficiency of the labeling of the 80-kDa protein increased with the irradiation time. In addition to the 80-kDa protein, two bands with molecular masses of 110 and 70 kDa, respectively, were also detected by streptavidin-HRP. These two bands were detected even in the non-irradiated sample, suggesting that these two proteins were not photolabeled products, but nonspecifically reacted with streptavidin-HRP.
Next, to characterize the binding specificity of the probe to the 80-kDa protein, we examined the competitive effects of palmitoyl-CoA, palmitic acid, and CoA on the photoaffinity labeling of the 80-kDa protein. As shown in Fig. 3A, the probe photoincorporation within the 80-kDa protein was suppressed by the presence of palmitoyl-CoA in a concentration-dependent manner. On the other hand, the labeling was not inhibited even in the presence of an excess amount of palmitic acid or CoA (Fig. 3B and C). These data indicate that the 80-kDa protein has a potent capacity to interact with palmitoyl-CoA, and the photoreactive LCFA probe could bind to the 80-kDa protein in the region responsible for its palmitoyl-CoA-binding site.

Identification of the 80-kDa Protein—In preliminary experiments, the 80-kDa protein labeled by the photoaffinity probe was susceptible to extraction with sodium carbonate, and was protected from digestion with trypsin in the absence of Triton X-100, but degraded by trypsin in the presence of Triton X-100 (data not shown). These behaviors were not similar to those of PMP70, a peroxisomal membrane protein, but resembled those of catalase, a peroxisomal matrix protein, implying that the 80-kDa protein is located in the peroxisomal matrix. To identify the 80-kDa protein, peroxisomes were solubilized with 0.1% Triton X-100 after photoaffinity labeling, and the labeled proteins were isolated by streptavidin-agarose resin. As shown in Fig. 4, the 80-kDa protein was successively purified. The purified probe-incorporated 80-kDa protein was digested with trypsin, and the resulting tryptic peptide mixture was analyzed by MALDI-TOF mass spectrometry. As a result, several specific tryptic peptide fragments of the labeled protein were obtained, and the MASCOT search engine revealed that almost all of the major fragments correspond to MFE2-specific tryptic fragments (supplemental Fig. S1). In addition to these fragments, a characteristic peak was observed at m/z 1345.81. We assigned the fragment as a photoadduct fragment (details are shown below).

Together with MFE2, a 65-kDa protein was also purified by streptavidin-agarose resin (Fig. 4). The 65-kDa protein was indeed labeled by the LCFA probe and the labeling of the 65-kDa protein was competitively inhibited by the presence of palmitoyl-CoA with almost the same extent as that of MFE2 (Fig. 3). Accompanying with intact MFE2, the 65-kDa protein was also detected in the purified rat liver peroxisomes by immunoblotting using anti-MFE2 antibody (data not shown). These data indicate that MFE2 is partially degraded to the 65-kDa protein and the 65-kDa region of MFE2 still possesses a potent capacity to interact with palmitoyl-CoA and the photoreactive LCFA probe.

MFE2, also called multifunctional protein 2 or δ-bifunctional protein, is a 79-kDa enzyme that possesses a typical peroxisomal matrix protein targeting signal (COOH-terminal Ala-Lys-Leu). MFE2 consists of a 3R-hydroxyacyl-CoA dehydrogenase.

**FIGURE 3.** Photoaffinity labeling of rat liver peroxisomes in the presence of palmitoyl-CoA, palmitic acid, and CoA. Purified rat liver peroxisomes were photoaffinity labeled in the presence of increasing amounts of palmitoyl-CoA (A), palmitic acid (B), and CoA (C), respectively. After UV irradiation, labeled proteins were separated on a 7–15% SDS-polyacrylamide gradient gel, and detected by streptavidin-HRP (upper panels). Western blot analysis for PMP70 is used as a loading control (lower panels). The arrowheads and asterisks indicate the 80-kDa protein and the 65-kDa protein labeled by the photoaffinity probe, respectively.

**FIGURE 4.** Purification of the photolabeled 80-kDa protein. Purified rat liver peroxisomes (1 mg of protein) were labeled by the photoreactive LCFA probe. After solubilization with 0.1% Triton X-100, labeled proteins were purified by SoftLink Soft Release Avidin Resin. Purified proteins were separated on a 5–10% SDS-polyacrylamide gradient gel, and stained with silver staining. The arrowhead and asterisk indicates the 80- and 65-kDa proteins labeled by the photoaffinity probe, respectively.
(HD) domain, a 2E-enoyl-CoA hydratase 2 (H2) domain, and a sterol carrier protein 2-like domain, and is known to catalyze the second and third steps of the peroxisomal β-oxidation of fatty acids and their derivatives (36).

Purification and Photoaffinity Labeling of MFE2—COOH-terminal His6-tagged rat MFE2 (MFE2-His) was expressed in Escherichia coli and purified with Talon metal affinity resin. MFE2-His was expressed upon isopropyl-β-D-thiogalactopyranoside induction as a major protein and exclusively recovered in a soluble form. MFE2-His purified from the soluble fraction was >80% pure as judged by SDS-PAGE (supplemental Fig. S1A). It is suggested that rat MFE2 functions as a homodimer. Therefore, we analyzed the oligomeric state of purified MFE2 using sucrose density gradient centrifugation. The molecular mass of the purified MFE2-His was estimated to be 155 kDa on sucrose density gradient centrifugation, which almost corresponded to a dimeric form of MFE2-His (79.4 kDa) (supplemental Fig. S2B). The purified MFE2-His was subjected to photoaffinity labeling with the photoreactive LCFA probe. As shown in Fig. 5, MFE2-His was efficiently labeled by the probe. On the other hand, purified His6-tagged Pex19p (His-Pex19p), a cytosolic protein involved in the targeting of peroxisomal membrane proteins (37), was not labeled by the probe under the same conditions. These findings indicate that the photoaffinity ligand indeed incorporates into MFE2 with high specificity.

Identification of the Substrate-binding Site of MFE2—As mentioned above, the photoreactive LCFA probe bound to MFE2 at its palmitoyl-CoA-binding site. Therefore, we tried to identify the individual amino acid residues labeled by the probe within MFE2 to characterize the substrate-binding site of MFE2. MFE2-His was incubated with the probe and the labeled MFE2-His was purified by avidin resin. After digestion with trypsin, the resulting peptide mixture was analyzed by MALDI-TOF mass spectrometry. As shown in Fig. 6, various tryptic fragments with observed molecular masses that were compatible with the calculated molecular mass of MFE2 tryptic fragments were identified in samples prepared from both labeled and unlabeled MFE2. However, a peak at m/z 1345.39 was detected only in the sample prepared from labeled MFE2. The fragment was also detected in the cross-linked MFE2 purified from rat liver peroxisomes as described above (supplemental Fig. S1A). The molecular mass of the fragment did not agree with any of the theoretical MFE2 fragments digested with trypsin, instead, it matched exactly with the mass of the tryptic peptide of MFE2 (residues Trp249-Glu250-Arg251; m/z 489.23) modified with the probe (m/z 1345.69 = 489.23 + 856.46). On the other hand, the tryptic fragment corresponding to MFE2 sequence Trp249-Glu250-Arg251 (m/z 489.24) was absent from the labeled MFE2-His, but observed in the control experiment using tryptic digestion of the unlabeled MFE2-His. To confirm that the amino acid region (Trp249-Glu250-Arg251) within MFE2 is involved in ligand binding, we purified the MFE2 mutants by replacing these amino acids with Ala, and analyzed the ligand binding efficiency. As shown in Fig. 7, wild type MFE2 was labeled by the photoaffinity probe. However, MFE2(W249A) and MFE2(R251A) exhibited a decreased labeling efficiency. The labeling efficiencies of MFE2(W249A) and MFE2(R251A) with the probe decreased to ~30% compared with that of the wild type MFE2 (Fig. 7C). These three amino acid residues lie within the HD domain of MFE2, and a disease-causing mutation has been identified in this region (38). The mutant, MFE2(W249G), also exhibited a decreased labeling efficiency, whereas MFE2(N158D), another disease-causing mutant in which the mutated residue is situated outside of the labeled region (38), exhibited only a slightly affected labeling efficiency for the photoreactive LCFA probe under the same conditions. The purified MFE2 HD domain was also labeled by the photoaffinity ligand, and MFE2 HD(W249A) exhibited a decreased labeling efficiency. However, the ligand was incorporated in the purified HD domain very weakly compared with full-length MFE2 (data not shown). The ternary composition of the three domains of MFE2 is required for the efficient substrate binding of MFE2. These data indicate that the amino acid region within MFE2 (Trp249-Glu250-Arg251) is important for ligand binding.

Characterization of the Substrate Binding Mode of MFE2—As described above, the photophore of the photoreactive LCFA probe cross-linked with MFE2 at the three amino acid regions of the MFE2 HD domain (Trp249, Glu250, Arg251). The crystal
structure of the HD domain of rat MFE2 HD has been solved by Haapalainen et al. (34). Based on the three-dimensional structure, these residues lie on the β strand located on top of the dimerization interface of the subunits (Fig. 8A). The location is apart from the catalytic triad of MFE2 HD domain (Ser151, Tyr164, Lys168). However, these residues also locate at the tip of the hydrophobic cavity leading to the active site (Fig. 8B). Therefore, we speculated that MFE2 could anchor the photophore of the LCFA derivative ligand on the top of the hydrophobic cavity (Trp249-Glu250-Arg251) and bury the hydrophobic probe along with the hydrophobic cavity directing the fatty-acyl tail of the ligand toward the active site. To challenge this hypothetical substrate binding mode of MFE2 HD, we replaced the hydrophobic residues (Ile180, Ile288) and the aromatic residue (Tyr156) forming the hydrophobic cavity of the MFE2 HD domain, and examined the photoaffinity labeling of these mutant proteins. As shown in Fig. 9, wild type MFE2 was labeled by the photoaffinity probe. However, MFE2(I180N), MFE2(I288N), and MFE2(Y156S) were impaired in the interaction with the photoreactive LCFA probe. These data indicate that the hydrophobic cavity leading to the active site of the MFE2 HD domain can be assigned as one of the substrate-binding sites of MFE2.

**DISCUSSION**

Peroxisomes play important roles in fatty acid metabolism. Therefore, they contain various enzymes involved in fatty acid metabolism and transport. However, because of the highly hydrophobic nature of the substrates, the precise mechanisms of their substrate recognition and binding has been poorly understood. In this study, we adopted a novel photochemical approach. We synthesized a photoreactive palmitic acid analogue bearing a diazirine moiety as a photophore, and characterized the molecular mechanisms especially in the substrate binding of peroxisomal ß-oxidation enzymes by photoaffinity labeling using a novel photoreactive LCFA probe.

At first, we identified MFE2 as a ligand-binding protein based on the following observations. 1) The probe used in this study
was specifically incorporated into an 80-kDa peroxisomal matrix protein upon activation with UV irradiation (Fig. 2), and the MALDI-TOF mass analysis identified the 80-kDa protein as peroxisomal MFE2 (supplemental Fig. S1). 2) Recombinant MFE2-His was specifically labeled by the photoreactive LCFA probe. The labeled proteins were separated on a 7–15% SDS-polyacrylamide gradient gel, and detected by streptavidin-HRP. C, the amount of probe-incorporated MFE2 shown in B was quantified with a LAS4000 luminoanalyzer (Fuji Film, Tokyo, Japan) and the relative labeling percentage was expressed as a ratio of the labeling percentage of each mutant MFE2 with that of wild type MFE2.

indicate that MFE2 could bind LCFA derivatives as substrates. Why was only MFE2 specifically labeled by the photoreactive LCFA probe among the peroxisomal proteins supposed to interact with fatty acid derivatives such as AOX1? We do not know the exact reason at present. However, we presume a possible explanation as follows. The photoprobe used in this study is composed of LCFA moiety, a diazirine-based photophore, and a biotin moiety with a hydrophilic linker. The structure of the photoprobe is different from that of CoA, LCFA, and also LCFA-CoA. However, amphipathic characteristics of the photoprobe seem to resemble somehow that of LCFA-CoA. In addition, as mentioned above MFE2 shows broad substrate specificity to hydrophobic molecules. MFE2 can also accept medium chain fatty acyl-CoA, 17β-estradiol, and 5-androstene-3β,17β-diol (12, 16, 19, 44–46). Therefore, MFE2 could have a potent capacity to interact with the photoprobe at its substrate-binding site. On the other hand, acyl-CoA-binding proteins such as AOX1 did not bind the photoprobe because they probably recognize acyl-CoA more in a rigorous manner. To improve the limited utility of this ligand as LCFA-CoA probe, we are now trying to modify the structure and the position of the photophore and detection tag not to affect the nature of LCFA-CoA.

MFE2 is a matrix protein existing inside of peroxisomes. Therefore, the photoreactive LCFA probe must be imported into peroxisomes. We assessed the transport of the photoreactive LCFA probe across the peroxisomal membrane by limited trypsin digestion. The ligand-incorporated MFE2 was protected from trypsin digestion after photoaffinity labeling. However, photoaffinity labeling of MFE2 did not occur when ligand was incubated with peroxisomes pretreated with trypsin. Under this condition, peroxisomal membrane proteins were degraded but peroxisomal matrix proteins were not (data not shown). These data indicate that the ligand is transported into peroxisomes by peroxisomal membrane proteins. However, at present, we have not identified the peroxisomal membrane proteins that interact with this ligand.

Next, we characterized the substrate binding mode of MFE2. We suggest that MFE2 anchors its substrate around the region consisting of Trp249 to Arg251 and buries the substrate along the hydrophobic cavity in the proper direction toward the catalytic center of the MFE2 HD domain based on the following observations. 1) MFE2 was labeled by the photoreactive LCFA probe, and the labeling was competitively inhibited in the presence of palmityl-CoA (Fig. 3). 2) MALDI-TOF mass analysis identified three amino acid residues of the MFE2 HD domain (Trp249-Glu250-Arg251) as ligand adduct fragment (supplemental Fig. S1A and Fig. 6), and the MFE2 mutants bearing these residues (W249A, W249G, R251A) exhibited a decreased binding efficiency (Fig. 7). 3) Based on the three-dimensional structure of rat MFE2 HD, these residues are located apart from the catalytic center, but located on the tip of the hydrophobic cavity leading to the active site (Fig. 8). 4) The MFE2 mutants disrupting the aromatic and hydrophobic residues forming the hydrophobic cavity of the MFE2 HD domain (Y156S, I180N, I288N) were impaired in the interaction with the photoreactive LCFA probe (Fig. 9). The HD domain of MFE2 belongs to the short-chain alcohol dehydrogenase/reductase (SDR) super-
family, containing a typical Rossmann-fold scaffold for NAD\(^+\) binding and a Ser-Tyr-Lys triad for catalysis (47). The hydrophobic cavity leading to the active site also appears to be a common feature among members of the short-chain alcohol dehydrogenase/reductase family. Tanaka N. et al. (48) first reported a ternary structure of \(E.\) coli \(\beta\)-hydroxysteroid dehydrogenase, an short-chain alcohol dehydrogenase/reductase protein, complexed with NAD\(^+\) and glycochenodeoxycholic acid as a substrate. Based on the ternary structure, the substrate was deposited in the hydrophobic cavity. In addition, 17\(\beta\)-estradiol and \(R\)-phenylethanol were shown to locate in the hydrophobic cavities of human 17\(\beta\)-hydroxysteroid dehydrogenase 1 and \(Lactobacillus\) brevis \(R\)-specific alcohol dehydrogenase, respectively (49, 50). These observations suggest that the hydrophobic cavity leading to the active site is important for substrate binding of short-chain alcohol dehydrogenase/reductase proteins. In the case of MFE2 HD, Ylianttila et al. (51) recently performed a docking study of \(3R\)-hydroxydecanoyl-CoA to a heterodimer of yeast \(Candida\) tropicalis MFE2 HD-A and MFE2 HD-B. They hypothesized the residues of the COOH-terminal domain of MFE2 HD-B form hydrogen bonds with the phosphate groups of the CoA, and the fatty acid tail of the substrate could be guided into the hydrophobic pocket. Our studies also showed the diazirine photophore covalently attached to 3 amino acid residues in the COOH-terminal portion of MFE2 HD, and suggest that the fatty acid tail penetrates into the hydrophobic pocket of the other MFE2 HD (Fig. 9).

The three amino acid region (Trp\(^{249}\)Glu\(^{250}\)Arg\(^{251}\)), which we identified as a photoprobe anchoring site, is located on the dimer interface of the MFE2 HD domains. Among them, Trp\(^{249}\) of both monomers is positioned in close proximity and the side chains of the two tryptophan residues lie parallel with each other. Therefore, Trp\(^{249}\) is suggested to be important for homodimerization of the domains. Ferdinandusse et al. (38) identified a W249G mutation in a patient with MFE2 deficiency, and they considered the mutation to be responsible for dimerization based on structural analysis. In this study, MFE2(W249A) and MFE2(W249G) indeed affected the ligand binding efficiency (Fig. 7). However, these mutants as well wild type MFE2 exhibited a molecular mass corresponding to a dimer on sucrose density gradient centrifugation (data not shown). These data indicate that the tryptophan residue could be responsible for substrate binding and not solely for dimerization.

As for the photoaffinity studies on peroxisomal proteins, it was useful to identify the fatty acid-binding proteins. Mange et al. (52) previously found that acyl-CoA oxidase was

![Image](https://via.placeholder.com/150)
specifically labeled by 11-diazirinophenoxy-undecanoyl-CoA in the lysed peroxisomes of C. tropicalis. Rajasekharan et al. (53) reported that acyl-CoA oxidase was labeled in vitro with 12-azido-oleoyl-CoA and 12-(4-azidosalicyl)-aminodecanoyl-CoA. However, the precise mechanisms of their substrate binding were poorly understood.

In this study, we have identified a peroxisomal β-oxidation enzyme by photoaffinity labeling using a novel photoreactive LCFA probe. Furthermore, we aimed to develop a photochemical study to characterize the substrate binding mode of the protein. This technique would be useful to identify and characterize the fatty acid transporting proteins and metabolizing enzymes with some modifications.

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