**SUPPLEMENTAL INFORMATION**

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

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**EXPERIMENTAL PROCEDURES**

***MALDI-TOF analysis of the labeled protein*** - After photoaffinity labeling of rat liver peroxisomes, the reaction product was diluted with 5 volumes of SVEH, and peroxisomes were reisolated by centrifugation at 20,000 x g for 20 min. Peroxisomes were then solubilized with 0.1% Triton X-100, and labeled proteins in the resulting supernatants were purified with SoftLink SoftRelease Avidin Resin. The labeled proteins were separated on a 5-10% SDS-polyacrylamide gradient gel, transferred onto a PVDF membrane. The bands corresponding to labeled proteins were excised from the membrane, and the probe-incorporated proteins were digested with trypsin on the PVDF membrane. The resulting tryptic peptide mixtures were analyzed by MALDI-TOF mass spectrometry on a Bruker MALDI-TOF AutoFlex mass spectrometer (Bruker-Daltonics, Billerica, MA). Positive-ion mass spectra of peptide maps were collected in the reflection mode and the peaks obtained from the MALDI spectra were used to identify proteins using the MASCOT search engine (Matrixscience, London, UK).

***Purification of MFE2-His*** - Overexpression and purification of recombinant MFE2 was performed as described by Haapalainen *et al*. with some modifications (1). The plasmid, pET21a/MFE2 was transfected to competent BL21(DE3) *E. coli* cells (Novagen, Madison, WI) according to standard procedures. The *E. coli* cells harboring pET21a/MFE2 were grown at 37 $^{o}$C in LB media containing 0.1 mg/ml ampicillin. At a cell density of 0.9 ($OD_{600}$), protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 20 h at 20 $^{o}$C. The cells were harvested by centrifugation at 4,000 x g for 20 min, resuspended in 35 ml of the lysis buffer (25 mM Tris-HCl, pH 7.8, 300 mM NaCl, and 5 mM imidazole) and disrupted 20 times for 20 sec in an ice bath by an Astrason XL-2020 ultrasonic processor (Misonix Inc., Farmingdale, NY). The lysate was centrifuged at 20,000 x g for 30 min and COOH-terminal $His_6$ tagged MFE2 (MFE2-His) in the supernatant was immediately applied to

10 ml of TALON Metal Affinity Resin (Clontech, Palo Alto, CA) equilibrated with the lysis buffer. After extensive washing, the MFE2-His was eluted with the lysis buffer containing 150 mM imidazole. After exchanging the elution buffer with binding buffer (25 mM Tris-HCl, pH 7.8, 300 mM NaCl, 5 mM EDTA, and 1 mM DTT) using a MicroSpin G-25 column (Amersham Biosciences, Piscataway, NJ), the purified MFE2-His was stored at -80 $^{o}$C until use.

***Sucrose density gradient analysis*** - Purified MFE2-His (100 μg protein) was subjected to equilibrium density centrifugation in a 10 ml linear sucrose gradient (5-30% (w/v) sucrose in binding buffer) in a NVT65 rotor (Beckman, Fullerton, CA). The gradient rested on 0.5 ml of 50% (w/v) sucrose in binding buffer. Centrifugation was carried out at 165,000 x g for 4 h at 4 $^{o}$C. Fractions of approximately 1.0 ml were collected from the bottom of the tube and the density of each fraction was determined by refractometry.

**REFERENCES**

1. Haapalainen, A.M., Koski, M.K., Qin, Y.-M., Hiltunen, J.K., and Glumoff, T. (2003) *Structure* **11**, 87-97

**SUPPLEMENTAL FIGURES**

**FIGURES LEGENDS**

Fig. 1. Identification of the probe-incorporated 80-kDa protein. *A*) MALDI-TOF mass spectrometry of tryptic peptide fragments obtained from purified 80-kDa protein after photoaffinity labeling. *B*) Matched peptide sequences corresponding to the major peaks labeled in *A*. *C*) Schematic drawing of the assembly of the three enzymatic domains in the MFE2 dimer. HD; 3*R*-hydroxyacyl-CoA dehydrogenase, H2; 2*E*-enoyl-CoA hydratase, SCP; sterol carrier protein 2-like domain.

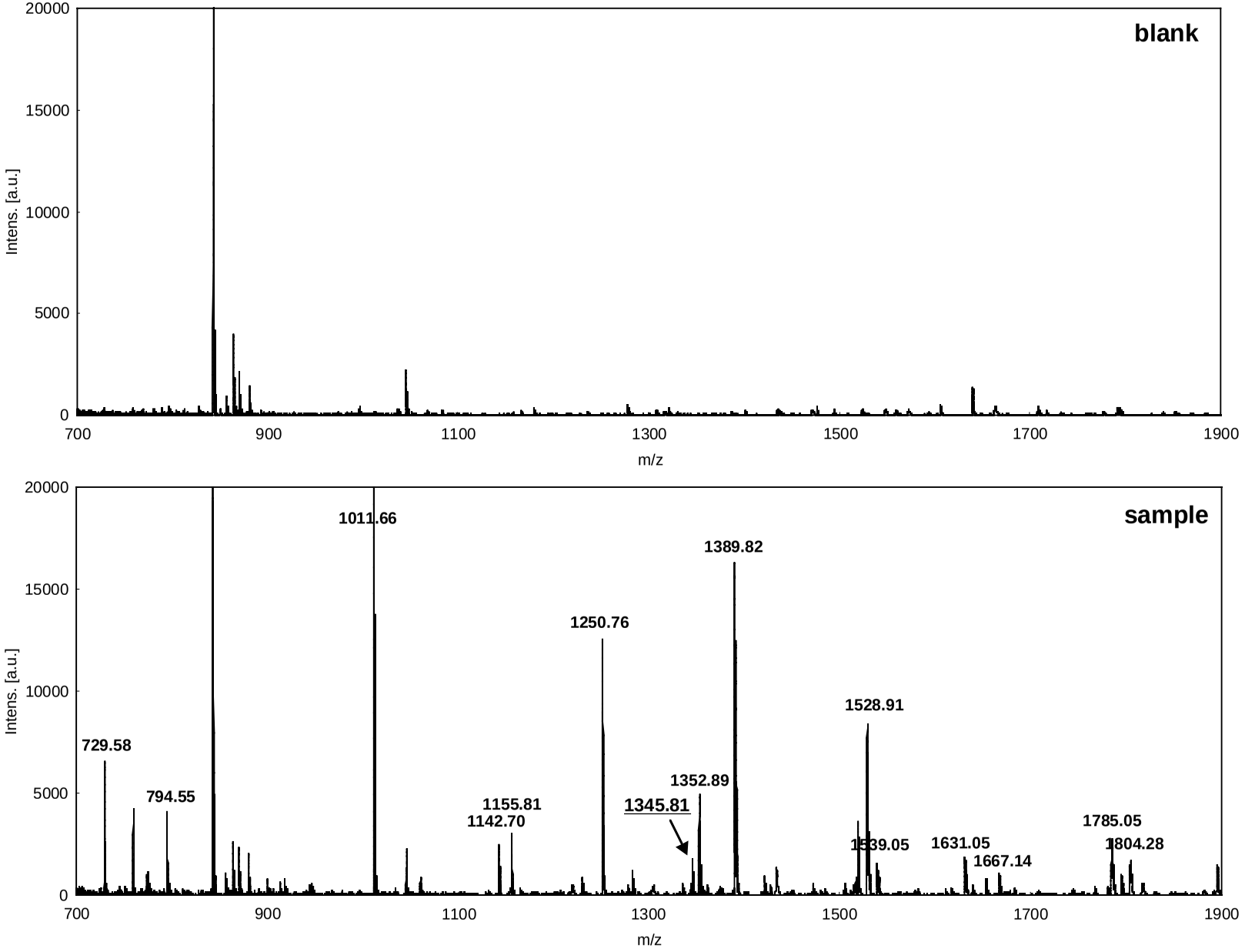
Fig. 2. Overexpression and purification of MFE2-His. *A*) MFE2-His was expressed and purified as described under "*Experimental Procedures*". Lane 1, total bacterial proteins before IPTG induction; Lane 2, total bacterial proteins after IPTG induction; Lane 3, soluble proteins after sonication; Lane 4, fusion proteins (10 μg) purified by TALON Metal Affinity Resin. *B*) Sucrose gradient centrifugation analysis of purified MFE2-His. Purified MFE2-His (100 μg protein) was loaded on the sucrose gradient (See "*Experimental Procedures*"). After centrifugation, 11 fractions were collected from the bottom of the tube. Aliquots of each

fraction were subjected to SDS-PAGE and analyzed by Western blotting with anti-His antibody. The molecular mass of the purified MFE2-His was calculated by referring to the molecular weights of the standard proteins, carbonic anhydrase (29 kDa), bovine serum albumin (66.2 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and apoferritin (443 kDa), respectively.

**Supplemental Table 1. Oligonucleotide primer sequences used for the generation of mutant MFE2 constructs**

| Conctruct name | Oligonucleotide Primer (from 5' to 3') |
|---|---|
| MFE2(W249A) | GATTGGAAAATTGCGCGCGGAGAGGACCCTGGG |
| | CCCAGGGTCCTCTCCGCGCGCAATTTTCCAATC |
| MFE2(R251A) | GAAAATTGCGCTGGGAGGCGACCCTGGGAGCC |
| | GGCTCCCAGGGTCGCCTCCCAGCGCAATTTTC |
| MFE2(W249G) | GATTGGAAAATTGCGCGGGGAGAGGACCCTGG |
| | CCAGGGTCCTCTCCCCGCGCAATTTTCCAATC |
| MFE2(N158D) | GCTTCTGGAATATACAGCGACTTTGGCCAGGCAAATTATAG |
| | CTATAATTTGCCTGGCCAAAGTCGCTGTATATTCCAGAAGC |
| MFE2(Y156S) | CAGCTTCTGGAATATCCAGCAACTTTGGCCAGGC |
| | GCCTGGCCAAAGTTGCTGGATATTCCAGAAGCTG |
| MFE2(I180N) | CGCCAATACTCTCGTGAATGAAGGCAGGAAGAAC |
| | GTTCTTCCTGCCTTCATTCACGAGAGTATTGGCG |
| MFE2(I288N) | GCAAGCCGAAGAGCAATCAAGAGTCCACAGG |
| | CCTGTGGACTCTTGATTGCTCTTCGGCTTGC |

**(A)**

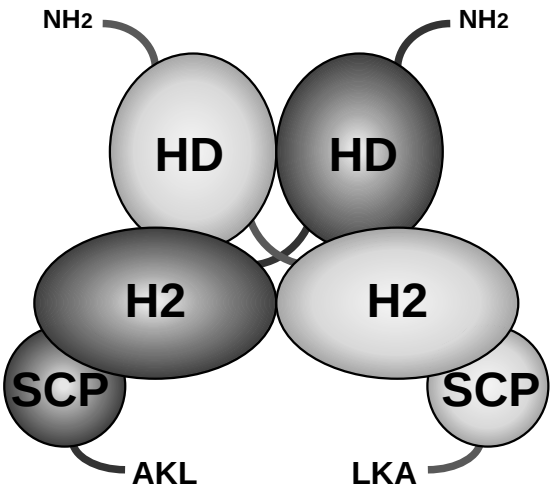



**Supplemental Fig. 1A. Y. Kashiwayama et al.**

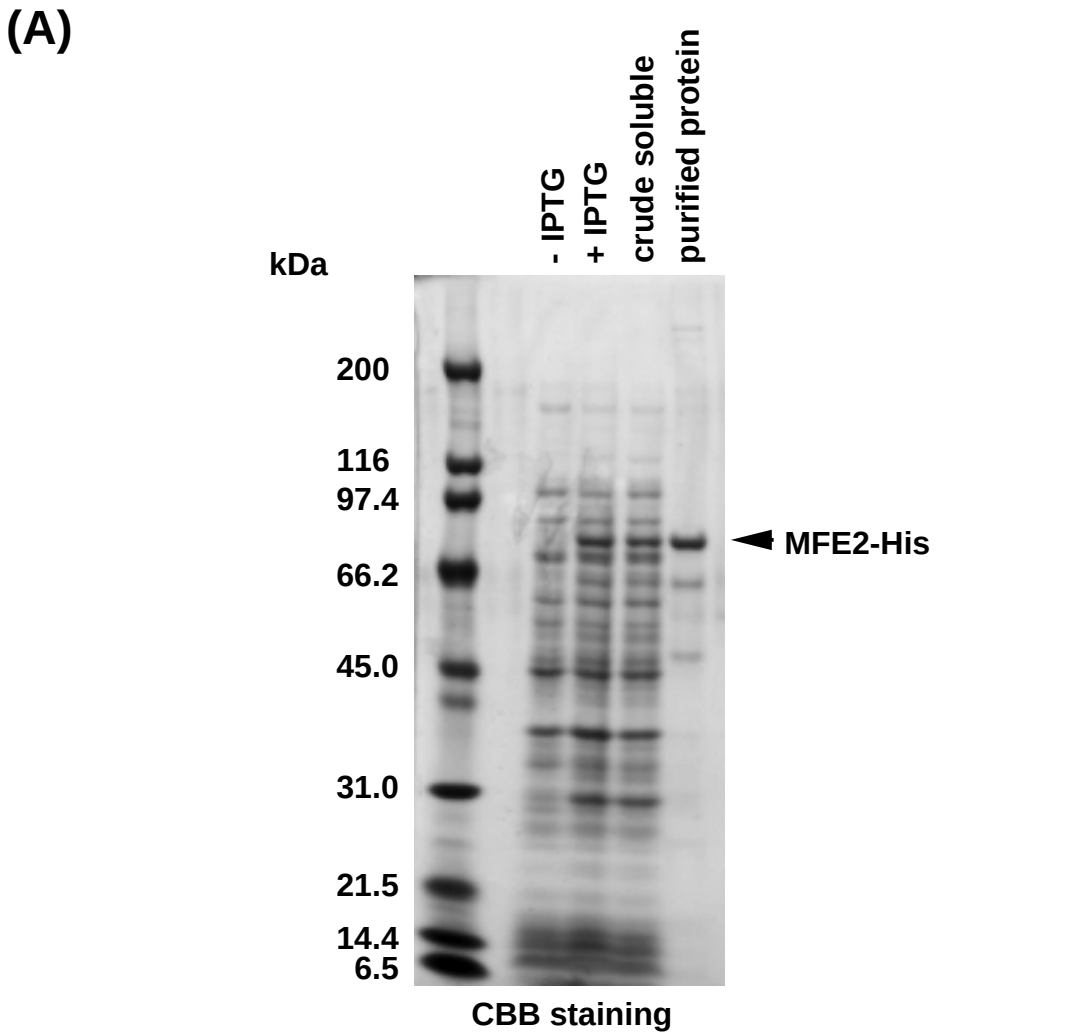
**(B)**

| Mass | Start End | Sequence |
|---|---|---|
| 729.58 | 252 - 258 | R.TLGAIVR.K |
| 794.55 | 126 - 132 | R.GSFQVTR.A |
| 1011.66 | 24 - 32 | R.AYALAFAER.G |
| 1142.70 | 261 - 270 | R.NQPMTPEAVR.D |
| 1155.81 | 11 - 32 | R.VVLVTGAGGGLGR.A |
| 1250.76 | 702 - 712 | R.LNPQNAFFSGR.L |
| 1352.89 | 494 - 505 | R.DTTSLNQAALYR.L |
| 1389.82 | 111 - 121 | R.ISDEDWDIIQR.V |
| 1528.91 | 542 - 554 | R.HVLQQFADNDVSR.F |
| 1539.05 | 169 - 183 | R.LGLLGLANTLVIEGR.K |
| 1631.05 | 7 - 23 | R.FDGRVVLVTGAGGGLGR.A |
| 1667.14 | 169 - 184 | R.LGLLGLANTLVIEGRK.N |
| 1785.05 | 261 - 275 | R.NPQMTPEAVRDNWVK.I |
| 1804.28 | 542 - 556 | R.HVLQQFADNDVSRFK.A |

**(C)**




**Supplemental Fig. 1BC. Y. Kashiwayama et al.**




**Supplemental Fig. 2. Y. Kashiwayama et al.**