

**Characterization and Inactivation
Studies of Enzymes Involved in Fatty
Acid Oxidation**

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參與脂肪酸代謝的酶的特征和失活研究

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Abstract

Numerous diseases have been reported in relation to fatty acids, such as cardiovascular disease, cancer, diabetes, etc. The regulation of fatty acid oxidation has been reported as a potential method treating non-insulin dependent diabetes mellitus (NIDDM) and inhibitors of enzymes involved in the metabolism of fatty acids have been synthesized and studied as potential medicines. Mitochondrial trifunctional protein (MTP), 3-ketoacyl-CoA thiolase (KT), and 2-enoyl-CoA hydratase 2 (ECH 2) are three key enzymes involved in the β -oxidation of fatty acid. Glutaryl-CoA dehydrogenase (GCD) and isobutyryl-CoA dehydrogenase (IBD) catalyze the oxidation of branched chain fatty acids from the catabolism of amino acids.

MTP catalyzes the last three steps of the β -oxidation of long-chain fatty acids. The 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities reside on the α -subunit, whereas the 3-ketoacyl-CoA thiolase activity is located on the β -subunit. This enzyme complex is bound to the mitochondrial inner membrane. Both the α and the β subunit were overexpressed and purified separately with nickel-metal affinity column to apparent homogeneity. The pMIS3.0E:: β plasmid was then transformed into competent cells containing pMIS3.0E:: α plasmid, and the MTP containing both the α and the β subunit was overexpressed and purified as a protein complex. FPLC analysis indicates that the MTP contains two α subunits and two β subunits. Kinetic studies of the α subunit, the β subunit, and the MTP $\alpha_2\beta_2$ protein complex were carried out. The results show that all three enzymatic activities including enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and acyl-CoA thiolase activities,

increased when the α and the β subunit form $\alpha_2\beta_2$ complex. The MTP $\alpha_2\beta_2$ complex prefers longer chain substrate in both binding capacity and catalytic rate. (Methylenecyclopropyl)formyl-CoA (MCPF-CoA) was found to be a mechanism-based irreversible inhibitor of the α subunit, while trimetazidine and oct-2-yn-4-enoyl-CoA were found to be two mechanism-based irreversible inhibitors of the β subunit. The mechanistic studies of the inactivation of the α and the β subunit by above three inhibitors were carried out. Glu151, Cys105 and Cys424 were found to be labeled by MCPF-CoA, trimetazidine and oct-2-yn-4-enoyl-CoA, respectively.

KT catalyzes the last step reaction of the β -oxidation cycle, which involves thiolytic cleavage of 3-ketoacyl-CoA substrate by free coenzyme A. We found that the enzyme has intrinsic isomerase activity, which was confirmed using incubation followed with HPLC analysis. The isomerase activity of the enzyme was thoroughly characterized through studies of kinetics, substrate specificity, pH dependence, and enzyme inhibition. Cys382 was identified as the catalytic residue for both thiolase and isomerase activities of the enzyme. In addition, we found that Cys92 was covalently labeled by oct-2-ynoyl-CoA. This result clearly demonstrated that oct-2-ynoyl-CoA is an irreversible inhibitor of the thiolase. This study of selective inactivation of KT by 2-alkynoyl-CoA via its intrinsic isomerase activity provides an example for rationally developing mechanism-based inhibitors based on a side activity of the enzyme, and may become a supplemental method for better treatment of cardiovascular disease and cancer.

ECH 2 is the middle part of the mammalian peroxisomal multifunctional enzyme type 2 (MFE-2), which catalyzes the second reaction of the fatty acid β -oxidation. We cloned the gene of rat ECH 2 to a bacterial expression vector pLM1 with six continuous histidine codons attached to the N-terminus of the gene. Cloned gene of ECH 2 was overexpressed in *Escherichia coli* and purified. MCPF-CoA,

oct-3-ynoyl-CoA and oct-2-yn-4-enoyl-CoA were identified as three new irreversible inhibitors of ECH 2 and Glu47 of ECH 2 was covalently labeled by these inhibitors. Comparative inhibition studies of ECH 1 and ECH 2 were carried out. This result indicates ECH1 and ECH2 have certain difference in active site geometry. Oct-3-ynoyl-CoA may selectively inactivate the β -oxidation in peroxisomes without significant effect on the β -oxidation in mitochondria.

GCD and IBD are two enzymes involved in oxidation of branched chain fatty acids, which are in the pathways for the catabolism of lysine and valine, respectively. We cloned the genes of rat GCD and IBD in a bacterial expression vector pET28a. Cloned genes of GCD and IBD were overexpressed in *Escherichia coli* and purified. We found that oct-4-en-2-ynoyl-CoA and oct-2-ynoyl-CoA are two irreversible inhibitors of GCD, but these two compounds have no inhibition on IBD. Glu419 was found to be labeled by oct-4-en-2-ynoyl-CoA and oct-2-ynoyl-CoA. In addition, we also noted that oct-3-ynoyl-CoA and oct-2-en-4-yn-CoA are two competitive inhibitors of GCD. We also found that GCD has intrinsic isomerase activity, which was confirmed using incubation followed with HPLC analysis. IBD did not show this intrinsic isomerase activity. Glu370 was identified as the catalytic residue for both dehydrogenase and isomerase activities of the enzyme. Study for straight chain substrate specificity of rat GCD and IBD was also carried out. The results indicate that the straight chain substrate pattern of GCD was broader than that of IBD.

Moreover, based on above results, oct-2-yn-4-enoyl-CoA was identified as the first multifunctional irreversible enzyme inhibitor of fatty acid oxidation, which can inactivate long-chain fatty acid metabolism in both mitochondria and peroxisomes.

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List of Abbreviations

Å	Angstrom
AACoA	acetoacetyl-CoA
AcAcCoA	acetoacetyl-CoA
ABC	ATP-binding cassette
ACADSB	gene of short/branched chain acyl-CoA dehydrogenase
ACBP	acyl-CoA-binding protein
ACD/ACAD	acyl-CoA dehydrogenase
ACS	acyl-CoA synthetase
AD	Alzheimer's disease
ADP	adenosine diphosphate
AMP	Adenosine monophosphate
AOX	alcohol oxidase
ATP	adenosine triphosphate
B-factor	temperature factor
bp	base pair(s)
BSA	bovine serum albumin
4-CBA	4-chlorobenzoyl coenzyme A
CD	circular dichroism
cDNA	complementary deoxyribonucleic acid
Cn	carnitine
CoA	coenzyme A

CPT	carnitine palmitoyltransferase
C-terminus	carboxyl terminus
DCIP	2,6-dichlorophenol indophenol
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECH 2	2-enoyl-CoA hydratase 2
ECI	Δ^3 - Δ^2 -enoyl-CoA isomerase
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetate
ER	endoplasmic reticulum
ETF	oxidized electron transfer flavoprotein
ETFH	reduced electron transfer flavoprotein
FA	fatty acids
FABP	fatty acid binding protein
FAD	flavin adenine dinucleotide (oxidized form)
FPLC	fast protein liquid chromatography
GBP	gastrin-binding protein
GCAD/GCD	glutaryl-CoA dehydrogenase
GC-MS	gas chromatography/mass spectrometry
GFP	green fluorescent protein
HADHA	the gene of the α -subunit of MTP
HADHB	the gene of the β -subunit of MTP

HADHSC	the gene of L-3-hydroxyacyl-CoA dehydrogenase
4-HBA	4-hydroxybenzoyl coenzyme A
HAD	L-3-hydroxyacyl-CoA dehydrogenase
HPLC	high-performance liquid chromatography
IBD	isobutyryl-CoA dehydrogenase
IPTG	isopropyl β -D-1-thiogalactopyranoside
IVA	isovaleric academia
IVD	isovaleryl-CoA dehydrogenase
kb	kilobase(s)
KT / KAT	3-ketoacyl-CoA thiolase
kDa	Kilodalton
LCAD	long-chain acyl-CoA dehydrogenase
LCHAD	long-chain 3-hydroxyacyl-CoA dehydrogenase
2MBCD	2-methylbutyryl-CoA dehydrogenase
MCAD	medium chain acyl-CoA dehydrogenase
MCPA-CoA	methylenecyclopropylacetyl-CoA
MFE-1, -2	multifunctional enzyme type 1, type 2
MIM	mitochondrial inner membrane
MISTIC	membrane-integrating sequence for translation of integral membrane protein constructs
mmBCFAs	monomethyl branched-chain fatty acids
MS/MS	tandem mass spectrometry

MTP / TFP	mitochondrial trifunctional protein
NAD ⁺	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NCS	noncrystallographic symmetry
NSAID	nonsteroidal anti-inflammatory drug
nt	nucleotide(s)
N-terminus	amino terminus
ORE	oleate response element
ORF	open reading frame
PCR	polymerase chain reaction
PD	Parkinson's disease
PDB	protein data bank
pFOX	partial fatty acid oxidation
PHHI	persistent hyperinsulinemic hypoglycemia of infancy
PMS	phenazine methosulfate
PPi	pyrophosphate
PUFA	poly unsaturated fatty acid
RC	respiratory chain
RNA	ribonucleic acid
SBCAD	short/branched chain acyl-CoA dehydrogenase
SCAD	short chain acyl-CoA dehydrogenase
SCEH	short chain enoyl-CoA hydratase

SCHAD	short chain 3-hydroxyacyl-CoA dehydrogenase
SDR	short-chain alcohol dehydrogenase/reductase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UTR	untranslated region
UV	ultraviolet
VLCAD	very long-chain acyl-CoA dehydrogenase

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