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REDESIGN OF CARNITINE ACETYLTRANSFERASE SPECIFICITY BY PROTEIN ENGINEERING

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REDESIGN OF CARNITINE ACETYLTRANSFERASE SPECIFICITY BY PROTEIN ENGINEERING

Memoria presentada por Antonio Felipe García Cordente, Licenciado en Química y Bioquímica por la Universidad de Barcelona, para optar al grado de Doctor por la Universidad de Barcelona.

Esta tesis ha sido realizada bajo la dirección de la Dra. Dolors Serra y el Dr. Fausto García Hegardt.

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PRESENTATION

In eukaryotes, L-carnitine is involved in energy metabolism, where it facilitates β-oxidation of fatty acids. Carnitine acetyltransferases (CrAT) catalyze the reversible conversion of acetyl-CoA and carnitine to acetylcarnitine and free CoA. There are three carnitine acyltransferase families, which differ in their acyl-chain length selectivity: carnitine palmitoyltransferases (CPTs), CPT I and CPT II, catalyze long-chain fatty acids, carnitine octanoyltransferase (COT) prefers medium-chain fatty acids, and carnitine acetyltransferase (CrAT) uses short-chain acyl-CoAs. These enzymes contain about 600 amino acid residues and share 30% amino acid sequence identity. Little was known about the molecular basis for the different substrate preference in this family of proteins; only Cronin (1998) studied the molecular determinants of carnitine/choline discrimination in choline acetyltransferase (ChAT), the fourth member of the acyltransferase family. However, the recent report of the 3-D structures of CrAT and COT has provided valuable insights into the molecular basis of substrate specificity and catalytic activity in the acyltransferase family (Jogl 2003, Jogl 2005).

In this study, we attempted to identify the amino acid residues responsible for acyl-CoA specificity in the acyltransferase family through structure-based mutagenesis studies on rat CrAT and COT proteins. As a result, we identified an amino acid residue (Met^{564} in rat CrAT) that is critical to fatty acyl chain-length specificity. A CrAT protein carrying the M564G mutation behaved as if its natural substrates were medium-chain acyl-CoAs, similar to COT. The kinetic constants of the mutant CrAT were modified in favour of longer acyl-CoAs as substrates. In the reverse case, mutation of the orthologous glycine (Gly^{553}) to methionine in COT decreased activity towards its natural substrates, medium-chain acyl-CoAs, and increased activity towards short-chain acyl-CoAs. A second putative amino acid involved in acyl-CoA specificity was identified (Asp^{356} in rat CrAT) and the double CrAT mutant D356A/M564G behaved as a pseudo-CPT in terms of substrate specificity. Three-dimensional models revealed a deeper hydrophobic cavity for the binding of acyl groups in both CrAT M564G and D356A/M564G mutants in the same position as the shallow cavity in the wt enzyme. This hydrophobic pocket is accessible to longer acyl groups; this is consistent with the preference of both mutants for medium and long-chain acyl-CoAs. Furthermore, we

studied the effect of C75-CoA, a potent and competitive inhibitor of CPT I, on CrAT activity. It has been demonstrated that C75-CoA occupies the same pocket in CPT I as palmitoyl-CoA, suggesting an inhibitory mechanism based on mutual exclusion. To determine whether this inhibitor would fit in the open hydrophobic pocket formed in CrAT mutants M564G and D356A/M564G, we carried out competitive inhibition assays. Our experiments showed that while C75-CoA is a potent inhibitor of CrAT mutants M564G and D356A/M564G, it has no effect on wt CrAT.

Choline acetyltransferase (ChAT) catalyzes a similar reaction to CrAT, with the difference that in ChAT the acetyl group from acetyl-CoA is transferred to choline instead of carnitine. Cronin (1998) successfully redesigned ChAT to use carnitine instead of its natural substrate choline. In the present study, our aim was to achieve the opposite, that is, to redesign rat CrAT specificity from carnitine to choline. We prepared a mutant CrAT that incorporates four amino acid substitutions (A106M/T465V/T467N/R518N), and the resulting mutant shifted the catalytic discrimination between L-carnitine and choline in favour of the latter substrate.

The food industry is interested in the production of esters for use as flavouring compounds; for example, esters are responsible for the fruity character of fermented alcoholic beverages such as beer and wine. The ability to create large quantities of an ester or to genetically alter a host to produce a stronger or altered ester scent would have many industrial applications. Esters are produced in an enzyme-catalyzed reaction between a higher alcohol and an acyl-CoA molecule. Since CrAT is responsible for the modulation of the acyl-CoA/CoA ratio, we hypothesized that overexpression of this enzyme could modify ester production in yeast. Therefore, we overexpressed CrAT in yeast and analysed its effect on ester production during alcoholic fermentation. Compared with control cells, overexpression of CrAT caused a significant reduction in the production of some esters, including the important flavour components ethyl acetate and 3-methyl-butyl acetate (isoamyl acetate).

In conclusion, the amino acid substitutions in rat CrAT and COT in this study reveal several residues that are involved in acyl-CoA and carnitine/choline substrate recognition and provide insight into the molecular requirements for their correct

positioning in order to achieve efficient catalysis. These results not only help us to understand the structure-function relationship within the acyltransferase family, but may also facilitate studies on obesity, non-insulin dependent diabetes (NIDDM), and patients with defective β-oxidation. Moreover, our results open the possibility of biotechnological applications of the enzymes of the carnitine acyltransferase family in the wine industry.

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Toni

A mi padre

ABBREVIATIONS

ABBREVIATIONS

3-D	three-dimensional
AATase	Alcohol acetyltransferase
ABD-F	7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide
ACC	Acetyl-CoA carboxylase
ACh	acetylcholine
ACS	Acyl-CoA synthetase
AGP2	yeast plasmalemmal carnitine transporter
amp^r	ampicillin-resistance
Atf	Alcohol acetyltransferase
ATP	adenosine 5´-triphosphate
bd.	bidistilled water
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CACT	Carnitine:acylcarnitine translocase
CAT	Chloramphenicol acetyltransferase
CAT2	yeast peroxisomal/mitochondrial carnitine acetyltransferase
cDNA	complementary DNA
ChAT	Choline acetyltransferase
CIT2	Citrate synthase
CM	complete minimal
Cn	carnitine
CoA	Coenzyme A
COT	Carnitine octanoyltransferase
cpm	counts per minute
CrAT	Carnitine acetyltransferase
CRC1	yeast carnitine acetylcarnitine translocase
CPT	Carnitine palmitoyltransferase
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTPs	2'-deoxynucleosides 5'-triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
E2pCD	catalytic domain of dihydrolipoyl transacetylase
ECF	enhanced chemifluorescence
EDTA	ethylenediamine-tetraacetic acid
Eht	Ethanol hexanoyl transferase
ER	endoplasmic reticulum
FA-CoA	fatty acyl-CoA
FAS	Fatty acid synthase
F.I.	fluorescence intensity
for	forward
GAL1	galactose inducible promoter
GC/MS	gas chromatography/mass spectrometry
GlmU	N-acetylglucosamine-1-phosphate uridyltransferase
GSH	glutathione (reduced form)
GST	glutathione S-transferase
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HS	headspace solid
I	inhibitor
IC_{50}	concentration of drug needed to inhibit 50%
IPTG	isopropyl β-D-thiogalactoside
K_{cat}	catalytic rate constant
K_i	inhibition constant
K_{inact}	inactivation constant
K_m	Michaelis constant
K_s	dissociation constant
kDa	kiloDalton
LCAS	long-chain acyl-CoA synthetase
LCFA	long-chain fatty acid
LCFA-CoA	long-chain acyl-CoA
mA	milliamps

MCFA-CoA	medium-chain acyl-CoA
MIM	mitochondrial inner membrane
min	minute
mit	mitochondrial
MOM	mitochondrial outer membrane
MOPS	3-[N-morpholino] propane sulfonic acid
MTS	mitochondrial targeting signal
MW	molecular weight
NIDDM	non-insulin dependent diabetes mellitus
nm	nanometers
nt	nucleotides
NZW	New Zealand White
OCTN2	plasmalemmal carnitine transporter
OD	optical density
o/n	overnight
ORF	open reading frame
p	protein
PAGE	polyacrylamide gel electrophoresis
panK	Pantothenate kinase
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline tween
PCR	polymerase chain reaction
PDB	Protein Data Bank
PDH	Pyruvate dehydrogenase
PEG	polyethylene glycol
per	peroxisomal
PGK	Phosphoglycerate kinase
PMSF	phenylmethylsulfonyl fluoride
PTS	peroxisomal targeting signal
QM	quadruple mutant
rev	reverse
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute

r.t.	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
S.A.	specific activity
s.c.	subcutaneous
SCFA-CoA	short-chain acyl-CoA
S.D.	standard deviation
SDS	sodium dodecyl sulphate
sec.	seconds
SIDA	stable isotope dilution analysis
sp.	species
SPME	solid phase microextraction
S.R.	specific radioactivity
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	N,N,N'N'-tetramethyl-ethylenediamine
tm	transmembrane
T_m	melting temperature
TM	triple mutant
Tris	tris(hydroxymethyl) aminomethane
U	Units
URA	uracil
UV	ultraviolet
V	volts
V_{max}	maximum enzyme velocity
wt	wild-type
w/v	weight/volume
YAT1	yeast outer mitochondrial membrane carnitine acetyltransferase
YAT2	yeast cytosolic carnitine acetyltransferase
YNB	yeast nitrogen base
YPD	yeast peptone dextrose medium

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