First publ. in: Flavins and flavoproteins 1999 : proceedings of the Thirteenth International Symposium, Konstanz, Germany, August 29 - September 4, 1999, pp. 523-526

Substrate chain length specificity of acyl-CoA dehydrogenases: Studies on different mutants.

B. Küchler, A. Nandy, A. Ghany and S. Ghisla
Faculty of Biology, University of Konstanz, PO Box 5560-M644, D-78434 Konstanz, Germany

Introduction

Acyl-CoA dehydrogenases belong to a class of flavoproteins that catalyse the desaturation of acyl-CoA substrates. The members of this family that are involved in mammalian mitochondrial ß-oxidation are classified according to their chain length specificity. Thus, the four presently known members are short-, medium-, long and very long chain acyl-CoA dehydrogenases (SCADH, MCADH, LCADH, and VLCADH). A marked difference within this group concerns the location of the catalytically essential Glu that initiates catalysis. In LCADH it is found at pos. 255,

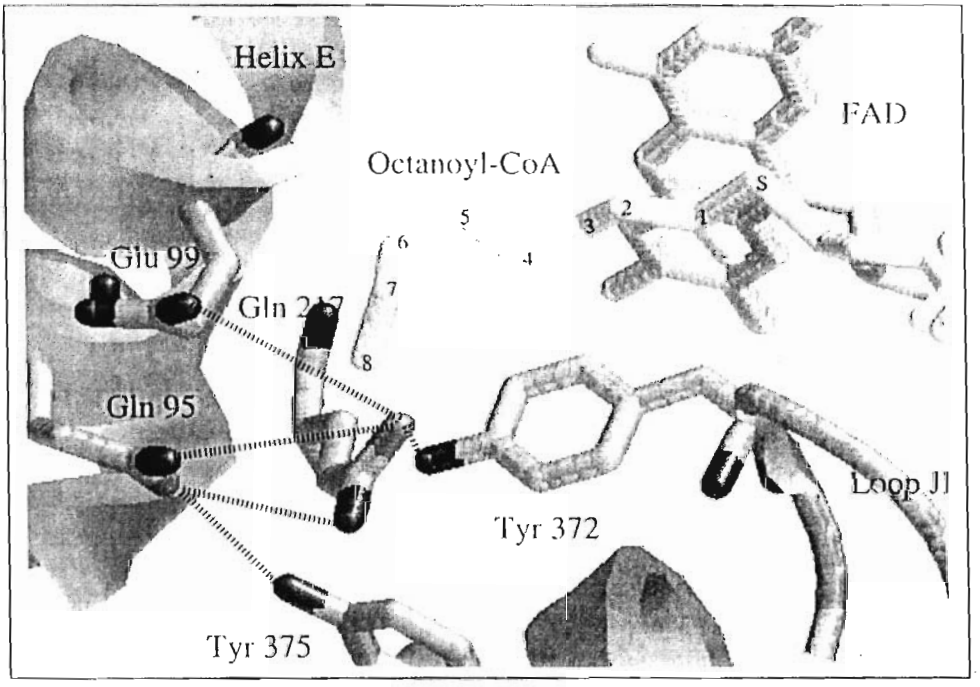

Figure 1: Part of the hydrogen network on the bottom of the active center of MCADH. The polarity of the bottom of the active center of MCADH is in comparison to other ACDH´s significantly higher. Substrates longer than C_{12}-CoA seem to be negative influenced in binding (based on data of (1).

whereas in the other three members it is at pos. 376 (MCADH numbering). In a double mutant the active center arrangement of LCADH was mimicked in MCADH (E376G/T255E-MCADH, or MLCADH (2)). In this enzyme maximal activity is with C_{12}-CoA (MCADH: C_8-CoA), and corresponds to that of LCADH. A comparison of the 3D structures of MCADH and LCADH uncovers further differences at the bottom of the active center cavities, which might play a role in substrate specificity: In MCADH this region has a high polarity characterised by a network of hydrogen bonds compared to LCADH (Fig. 1). In view of this we have constructed MCADH mutants affecting this network. Thus E99 has been mutated to Gly, the AA found in VLCADH. E99G-MCADH is active and shows two activity maxima, one corresponding to C_8-CoA and a second to C_{12}-CoA/C_{14}-CoA. With Q95G-MCADH the activity maximum is shifted from C_8-CoA to C_{10}-CoA. The effects observed with these two mutants have thus been combined with that implemented in MLCADH. The resulting mutant protein (E99G/T255E/E376G-MCADH) is essentially devoid of activity with substrates $<C_{10}$-CoA, however, its activity/chain length profile is similar to that of VLCADH.

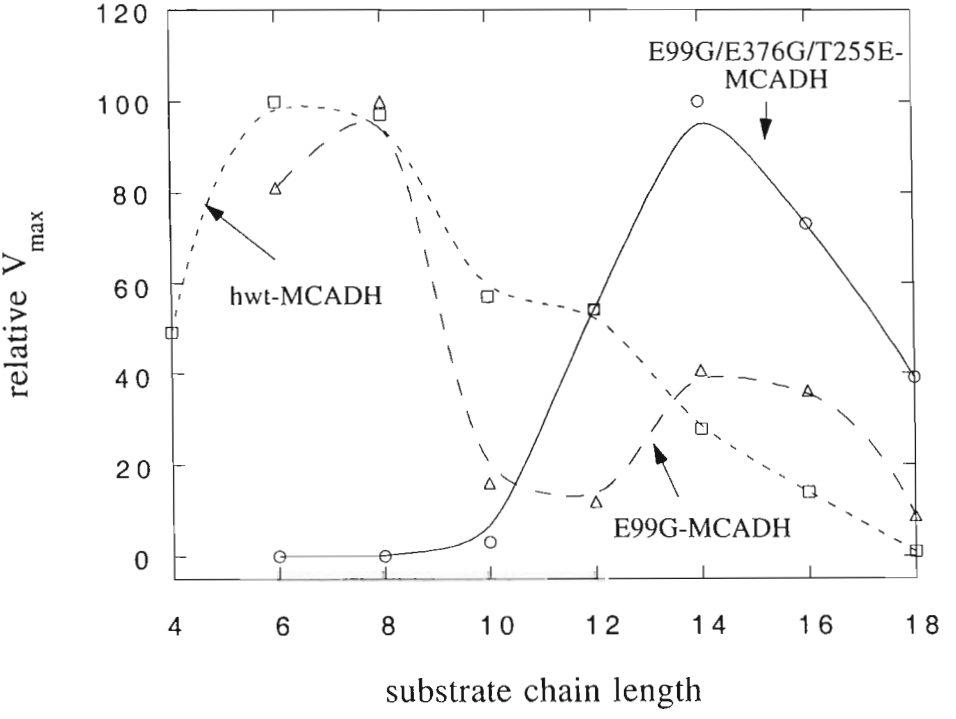

Figure 2: Comparison of relative V_{max} of hwt-MCADH, E99G-MCADH and E99G/E376G/T255E-MCADH. Values are normalised for better comparison. All measurements were done with the ferricenium assay (3).

Results

E99G-MCADH and Q95G-MCADH were constructed by site directed mutagenesis using the plasmid pTrchwt-MCADH (2). After transformation into E.*coli* TG1 the enzymes were overexpressed and purified in a stable form. The mutants are both active, all activities were determined with the ferricenium assay (3). The results are listed in Table 2. E99G-MCADH and Q95G-MCADH both show a wider substrate spectrum compared to hwt-MCADH.

Table 2: Catalytic parameter of hwt-MCADH, E99G and Q95G-MCADH in dependence of the substrate chain length specificity. Red_{max} is the extent of enzyme flavin reduction observed upon anaerobic incubation with the substrate indicated.

Enzyme	Parameter Units, Conditions	Substrate chain length C6	C8	C10	C12	C14	C16	C18
hwt-MCADH	v_{max} (min^{-1})	1010	980	580	550	280	140	
	K_m (_M)	15	3,4	2,5	2,5	2,3	1,6	
	V_{max}/K_m	67	288	232	220	122	88	
	$Red._{max.}$		91	90	88		53	
	k_{red} (min^{-1})		>20000	>20000	5400	420		
	$V_{reox-O2}$ (min^{-1})	0,028	0,004	0,004	0,01	0,021	0,02	
E99G-MCADH	v_{max} (min^{-1})	560	690	110	280	280	250	60
	K_m (_M)	7,6	7,6	3,3	2,5	2,3	2,8	12,3
	V_{max}/K_m	70	90	35	30	120	90	5
	$Red._{max.}$	88	98	97	92	91	82	37
	$V_{reox-O2}$ (min^{-1})	0,155	0.056	0,025	0,018	0,005	0,12	0,13
Q95G-MCADH	v_{max} (min^{-1})	1200	1250	1900	820	790	200	
	K_m (_M)		10	6,5	3,3	3,2	1,6	
	V_{max}/K_m		115	310	245	245	120	
	$Red._{max.}$		94	95	94	93	69	23
	$V_{reox-O2}$ (min^{-1})		0,0042					

Compared to hwt-MCADH the activity profile of E99G-MCADH shows a ≈90% increased activity with C_{16}-CoA (see Fig 2, Table 2), With its best substrate (C_8-CoA) E99G-MCADH has about 70% the activity of the best substrate of hwt-MCADH (C_8-CoA). The main effect of the E99G-mutation is thus not a shift of the activity maximum to longer substrate chains, but the generation of a second maximum around C_{14}-CoA / C_{16}-CoA (Fig. 2). The V_{max}/K_m - maximum of E99G-MCADH, on the other hand, is shifted from C_8-CoA (hwt-MCADH) to C_{14}-CoA (Table 2). The rate of enzyme flavin reduction for E99G-MCADH with C_{16}-CoA is

about 160% of that measured with hwt-MCADH. However, the rate of reoxidation with O_2 upon reduction with C_{16}-CoA is about 4 fold lowered in comparison to hwt-MCADH. Q95G-MCADH has a V_{max}, which is shifted one C_2-unit to C_{10}-CoA. The activity maximum and the V_{max}/K_m values are drastically increased over the whole substrate spectrum. This effect originates in a lowering of the apparent pK of the V/pH-profile from pK=8.7 for hwt-MCADH to pK = 7.3 for Q95G-MCADH. E99G/E376G/T255E-MCADH was created by cloning the bottom of the active site of E99G-MCADH into the E376G/T255E-MCADH. Upon expression in E.coli TG1 the protein could be purified only up to ≈20%. The instability is due to loss of FAD. The relative activity profile has a characteristic similar to that of VLCADH.

Discussion

Modifications of the "bottom" of the active site of hwt-MCADH that widen and depolarise this cavity induce a shift of the substrate specificity towards "longer chain lenghts". The extension of this concept by implementation of the active site functional group arrangement of LCADH into E99G-MCADH (as in MLCADH (2)), leads to an enzyme with a "VLCADH-similar" substrate chain length spectrum. We conclude that the factors determining the chain length specificity of acyl-CoA dehydrogenases are manifold and concatenated. They affect both binding/accommodation of substrate and the chemistry underlying catalysis. Thus the volume of the cavity obviously plays a primary role. The polarity, as influenced by the presence of hydrogen bonds and of specific polar groups, and as reflected by shifts of apparent activity pK's affects indirectly catalysis. The flexibility of the active site affects the capacity to accommodate the substrate "tail" as well as the accessibility/reactivity of oxygen. The position of the essential active site carboxylate respective to substrate influences directly the rate of the chemical step of dehydrogenation.

References

1. Kim J.J.P., M. Wang, Paschke, R. (1993) Crystal structures of medium-chain acyl-CoA dehydrogenase from pig liver mitochondria with and without substrate. Proc. Natl. Acad. Sci. USA. **90**, 7523-7527

2. Nandy, A., Kieweg, F., Kräutle, F., Vock, P., Küchler, B., Bross, B., Kim, J.,P., Rasched, Ghisla, S.: Medium/Long Chain Chimeric Human Acyl-CoA Dehydrogenase: Medium Chain Enzyme with the Active Center Base Arrangement of Long Chain Acyl-CoA Dehydrogenase. Biochemistry **35,** 12402-12411.

3. Lehman, T. C., Thorpe, C. (1990): Alternate electron acceptors for medium-chain acyl-CoA dehydrogenase: use of ferricenium salts. Biochemistry **29**, 10594-602