Biochemical characterization of purified, human recombinant Lys304→Glu medium-chain acyl-CoA dehydrogenase containing the common disease-causing mutation and comparison with the normal enzyme

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Recombinant, normal human medium-chain acyl-CoA dehydrogenase (MCADH) and the common, human disease-causing K304E mutant ([Glu304]MCADH) protein were expressed in *Escherichia coli* using an optimized system, and the enzymes were purified to apparent homogeneity. The crucial factor leading to the production of active [Glu304]MCADH protein is the expression in *E. coli* cells at reduced temperature (28°C). Expression in the same system at 37°C results in very low amounts of active mutant protein. Several catalytic and physicochemical parameters of these two proteins have been determined and were compared to those of purified pig kidney MCADH. Although [Glu304]MCADH has approximately the same rate of substrate reduction with dodecanoyl-CoA and the same V_{max} as human MCADH with the best substrate for the latter, octanoyl-CoA, the K_m in the mutant MCADH is fourfold higher, which generates a correspondingly lower catalytic efficiency. Importantly, V_{max} obtained using the natural acceptor, electron transfer flavoprotein, is only a third that for human MCADH. The V_{max}/K_m versus chain-length profile of the mutant shows a maximum with dodecanoyl-CoA which differs markedly from that of human MCADH, which has maximal efficiency with octanoyl-CoA. The substrate specificity of the mutant is broader with a less pronounced activity peak resembling long-chain acyl-CoA dehydrogenase. The purified mutant enzyme exhibits a reduced thermal stability compared to human wild-type MCADH. The major difference between the two proteins expressed in *E. coli* is the more pronounced lability of the K304E mutant in crude extracts, which suggests a higher susceptibility to attack by endogenous proteases. Differences between tetrameric [Glu304]MCADH which survives the first step(s) of purification and corresponding MCADH are minor. The overall differences in properties of [Glu304]MCADH together with its impaired folding and tetramer assembly may contribute to the generation of the abnormalities observed in patients homozygous for the K304E mutation.

Keywords: acyl-CoA dehydrogenase; genetic defect; fatty acid oxidation; stability; specificity.

Acyl-CoA dehydrogenases catalyze the initial and rate-limiting step of the mitochondrial β-oxidation by carrying out the α,β-dehydrogenation of fatty acid CoA thioesters [1]. The best studied among the family of seven related mammalian dehydrogenases [2] is medium-chain acyl CoA dehydrogenase (MCADH), which has a rather broad substrate chain length specificity centered around octanoyl-CoA (C_8-CoA). Several recent papers have dealt with purification of MCADH from various sources [3−7], its catalytic properties [4, 5, 8, 9], cloning of the MCADH cDNA [10], and determination of the three-dimensional structure [11]. MCADH has special relevance because MCADH deficiency is the most frequently observed genetic defect in mitochondrial fatty acid β-oxidation [12, 13]. About 70−90% of the alleles causing MCADH deficiency carry the A985→G point mutation in their cDNA, which results in the substitution of Lys with Glu at position 304 of the mature enzyme [14−18]. The homozygote frequency of the G985 mutation in caucasians in north-western Europe and the USA is in the range of 1 in 10000 to 1 in 40000 [18−20]. [Glu304]-MCADH is present in patient cells at concentrations dramatically lower than those of native MCADH [14, 21−24]. Expression studies of [Glu304]MCADH cDNA in both prokaryotic and eucaryotic systems revealed that the mutant variant is capable of forming active enzyme [24−27] with a specific activity in the range of the wild-type enzyme [26, 27]. The major reason for the decreased level of mutant enzyme observed in patient tissues has been attributed to impaired folding and tetramer assembly that presumably results in premature degradation of accumulating deviant folding/assembly intermediates [26, 28−30].

The mutation site in [Glu304]MCADH is located in helix H, at the interface between the subunits of the homotetramer and is

not directly involved with catalysis, FAD binding, or substrate binding [11]. Recently, we created specific mutations in the vicinity of position K304 to distinguish between the mutational effects on folding and subunit assembly. Expression studies with these variants support the concept of both impaired folding and tetramer assembly as the pathogenic mechanism [30]. These experiments suggested also that the assembled mutant enzyme displays decreased thermostability. The relevance of the decreased thermostability was not clear because detailed studies of the enzymatic and biochemical properties of [Glu304]MCADH in a well-defined system had not been done due to the decreased stability and consequent failure to isolate of [Glu304]MCADH mutant enzyme for these analyses. Furthermore, subsequent to our first report on the expression and isolation of MCADH [6, 17] evidence has been forthcoming that the human MCADH, although overall very similar to its well studied pig counterpart, also exhibits specific differences [7, 31].

To overcome these limitations of previous studies, we improved the prokaryotic expression system reported originally [6] and optimized the purification procedure to isolate significant amounts of pure [Glu304]MCADH for biochemical studies. These improvements resulted in an approximately 12-fold higher yield of MCADH and of stable mutants [32] compared to a system recently published by Peterson et al. [7] and in quantities of K304E mutant which did permit its biochemical characterisation. Herein, we report some specific biochemical properties of MCADH allowing direct comparison with the mutant enzyme. These properties are complementary to those described recently by Peterson et al. [7] who focused primarily on kinetic experiments and on the reaction with octanoyl-CoA. The comparison of [Glu304]MCADH with MCADH allows a more detailed evaluation of the contribution of the various effects of the K304E mutation to the impairment of β-oxidation observed in patients.

MATERIALS AND METHODS

Instrumentation. Visible and ultraviolet spectra were recorded with a Kontron Uvikon 930 spectrophotometer, and fluorescence spectra were obtained with a Kontron SFM 25 instrument. Single-turnover experiments were performed with a stopped-flow spectrophotometer which has a 2.0-cm path-length cell [33, 34] and which is equipped with a diode array detector (Spectroscopy Instruments GmbH) and interfaced with a MacIntosh IIcx computer for data acquisition (POSMA 2.3 k software, SI). Rapid reactions were routinely recorded in the range 300−650 nm using the normal scan mode with a scan time of 10 ms/spectrum and with a resolution of 2 pixels/nm. For reactions proceeding with a $k_{obs} > 90$ s^{-1}, a fast access routine was used, which has an acquisition time of 0.7 ms/data point at 450 nm. For these experiments, the BG24A filter was removed to yield sufficient light intensity. For the determination of kinetic constants, the arithmetic mean of at least three traces was formed, and the resultant was fitted using the A program (Dr D. P. Ballou, University of Michigan, Ann Arbor). Other fit routines were done with KaleidaGraph for MacIntosh from Synergy Software. Unless otherwise stated, all measurements were made at 25°C in 50 mM KP$_i$ at pH 7.8. The concentrations mentioned in the context of stopped-flow experiments are those of the reagents after mixing, i.e. 1:1 dilution, from the initially prepared solutions.

Materials and enzymes. CoA derivatives, ampicillin, protocatechuate, phenazine methosulfate (PMS) and 2,6-dichloroindophenol (Cl$_2$Ind) were from Sigma. Tryptone, yeast-extract, isopropyl β-D-thiogalactopyranoside, Tricine, acrylamide/bis-

acrylamide and Tris from Roth. Sephadex G-25®, Q-Sepharose FF® and Superdex® pg 200 (XK 26/60) were from Pharmacia. Hydroxyapatite fast flow gel was from Fluka. CoA-persulfide was prepared as described by Williamson et al. [35]. Ferricenium hexafluorophosphate was prepared according to Lehman et al. [36]. 3-Methyl lumiflavin and lumiflavin 3-acetate were made according to Hemmerich [37]. Unless otherwise stated, all buffers contained 0.3 mM EDTA. Additionally, all buffers employed during the MCADH purification contained 1 μM FAD at pH 7.8. ETF from pig kidney was purified according to Gorelick et al. [38] with the exception of the use of Q-Sepharose instead of DEAE-cellulose (DE-52). Although ETF does not adsorb to the DE-52 column, it binds weakly to Q-Sepharose (column, 50×450 mm) from which it can be eluted with a NaCl gradient (0−400 mM NaCl in 450 min with a flow of 4.5 ml/min). Pig kidney MCADH was purified as described by Thorpe et al. [4], but using Q-Sepharose and hydroxyapatite and an additional octyl-Sepharose step [39]. Protocatechuate dioxygenase was a kind gift from Dr D. P. Ballou (University of Michigan, Ann Arbor).

Absorption coefficients. Absorption coefficients (see Table 2) were determined with the SDS and trichloroacetic acid methods [40]. FAD: $\varepsilon_{450} = 11.3$ mM^{-1}cm^{-1} [41]; butyryl-, hexanoyl-, octanoyl- and palmitoyl-CoA: $\varepsilon_{260} = 15.4$ mM^{-1} cm^{-1} [42].

Enzyme assays. The following assays were used (see Table 3): (a) ETF under anaerobic conditions [43], but using 6 μM ETF; (b) PMS (1.4 mM)/Cl$_2$Ind (35 μM) [4] in 0.7 ml 100 mM KP$_i$ pH 7.6; (c) ETF (10.5 μM)/Cl$_2$Ind [38]; (d) ferricenium in 50 mM KP$_i$ pH 7.6, but without N-ethylmaleimide [36]. Unless otherwise stated, all assays were carried out at 25°C.

Thermal effects and stability. The Arrhenius activation energy was estimated in the range 5−62°C and in 100 mM KP$_i$ pH 7.6, by addition of equal amounts of MCADH or [Glu304]MCADH to buffer at the correct temperature containing a saturating concentration of octanoyl-CoA (50 μM). Thermal stability was studied by diluting enzyme into such buffer solutions, which were kept at the indicated temperature for 15 min before the assay. For the study of time versus temperature effects on activity (Fig. 6, below), extracts of *Escherichia coli* cells were incubated at various temperatures, and aliquots were assayed at incremental times using the ferricenium assay.

Plasmids and expression of human wild-type and [Glu304]MCADH. *E. coli* cells (strain TG1) were transformed with human wild-type and [Glu304]MCADH-encoding plasmids pTrc MCADH [32] and pTrc [Glu304]MCADH in which the *Eco*RI−*Hin*dIII fragment coding for the C-terminal MCADH was replaced by the *Eco*RI−*Bam*HI fragment of p985 [26] carrying the K304E mutation, first subcloned into *Eco*RI/*Bam*HI-digested pUC 8.2 which supplied the needed *Hin*dIII site. Cells were grown in 40 l (Bioengineering fermentor) dYT medium (16 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl). The expression was performed under conditions as described by Nandy et al. [32] at a growth temperature of 28°C and the cells harvested with a Cepa continuous centrifuge. The wet cell paste (250−300 g) was stored at −20°C.

General procedure for the purification of recombinant MCADHs from *E. coli* cells. *E. coli* cell paste (wet, about 120 g) was thawed and suspended in 0.25 M Tris/HCl pH 7.8. The cell suspension was sonicated three times for 5 min (50% pulsed), and the resulting slurry (about 1 l) was centrifuged for 30 min at 15 000 g. The pooled supernatants were fractionated between 40−80% ammonium sulfate, and the pellet obtained after centrifugation for 30 min at 15 000 g was dissolved, dialyzed against 50 mM Tris/HCl, and then loaded on a Q-Sepharose (fast flow) column (30×400 mm, equilibrated with 50 mM

Table 1. Summary of the purification of human MCADH and of [Glu304]MCADH. The purification of MCADH was from 123 g, that of [Glu304]MCADH from 107 g E. coli cells. Data in square brackes are for [Glu304]MCADH. The mass of MCADH was estimated from the activity or from the FAD absorption at 445 nm upon hydroxyapatite chromatography. Activities were measured with the ferricenium assay.

Step	Protein	Volume	Total protein	MCADH	Specific activity	Total activity	Purification factor	Yield
	mg/ml	ml	mg		U/mg	U		%
Cell extract	11.2 [17]	900 [650]	10000 [11050]	655 [160]	1.6 [0.27]	16300 [3000]	1 [1]	100 [100]
40−80% $(NH_4)_2SO_4$ cut and dialysis	11 [16]	490 [430]	5400 [7100]	515 [55]	2.4 [0.11]	12800 [950]	1.5 [−]	78 [34]
Q-Sepharose, 80% $(NH_4)_2SO_4$ and dialysis	30 [15]	70 [70]	2100 [1100]	290 [41]	3.4 [0.65]	7200 [710]	2.1 [2.4]	44 [26]
Hydroxyapatite	1.9 [0.2]	120 [170]	230 [34]	230 [34]	24.9 [17.5]	5700 [610]	15.5 [65]	35 [21]

Tris/HCl, flow rate 4 ml/min). MCADH binds as a green/yellow band at the top. After elution of unbound protein, a gradient (0−300 and 300−600 mM NaCl in 300 min and 60 min, respectively) was applied, which leads to elution of a green/yellow band at 150 mM NaCl containing the MCADH activity. The color and ionic strength of elution are conveniently used to locate MCADH protein in the case of inactive mutants. The pooled fractions having activity were concentrated by precipitation with 80% ammonium sulfate, and the dissolved pellet was dialyzed against 5 mM KP_i pH 7.8. At this point, the protein solution had an $A_{272/444}$ ratio of 30 and was applied to a hydroxyapatite column (30×220 mm, equilibrated with 5 mM KP_i and flow rate of 1.5 ml/min) which was washed extensively with 100 mM KP_i. MCADH was eluted at 300 mM KP_i using a linear gradient of 100−500 mM KP_i over 300 min. Elution of MCADH usually occurs in two, poorly resolved bands corresponding to the green and yellow forms, respectively, of MCADH. At this stage, the yellow form of MCADH has an $A_{272/444}$ ratio of about 6 and purity of >90%. Final purification was by gel filtration on a HiLoad 26/60 Superdex 200-pg in 100 mM KP_i from which MCADH elutes with an apparent molecular mass of 155 kDa. The isolated MCADH shows a single band on SDS/PAGE with an apparent molecular mass of 42 kDa. It should be noted that a mixture of green and yellow forms of MCADH were usually obtained during purification and that the relative concentration of the two depends on the type of mutant being purified and on the conditions of cell growth and disruption. MCADH preparations were degreened by an adaptation of the method of Williamson et al. [35]: to about 1 ml of a concentrated solution of MCADH (> 100 µM protein), 200 µl of a saturated solution of dithionite in 50 mM KP_i, pH 7.8, was added, causing loss of color of the solution. After 2 min, the enzyme was separated from small molecules by filtration over a Sephadex G-25 column (5 ml, equilibrated with 50 mM KP_i, pH 7.8). The enzyme reoxidizes during elution and elutes in the yellow form. Addition of CoA-persulfide leads to the green enzyme form.

Miscellaneous methods. Static spectral experiments were performed at 25°C in 50 mM KP_i, pH 7.8, containing 0.3 mM EDTA, when not otherwise indicated. Anaerobiosis was achieved by flushing and evacuation cycles using O_2-free argon in the presence of an oxygen scavenging system (glucose oxidase/glucose/catalase or protocatechuate dioxygenase/protocatechuate) either directly in the solution or in a sidearm of the cuvette. Protein was determined with the Pierce Coomassie dye-binding reagent (Bradford assay) according to the manufacturer's instructions.

RESULTS

Expression of human recombinant wild-type MCADH and of [Glu304]MCADH. With our previously described E. coli expression system, yields of about 0.9 mg pure MCADH/l cell culture were obtained [6]. Peterson et al. [7] improved this system by using a pET11a-MCAD, E. coli BL21 (DE3)pLysS and reported a yield of about 150 mg pure MCADH from 80-l fermentor cultures. We had previously explored the same system and obtained comparable results for MCADH and stable mutants. In our hands, however, that expression system was inadequate for a workable purification of the relatively unstable [Glu304]-MCADH. Evaluation of various expression vectors and conditions revealed that our best results were obtained with the expression of MCADH under the control of the strong Trc promoter of the expression vector pTrc99C at growth temperatures of 28°C [32]. This minimizes the problem of aggregation of the recombinant protein and the formation of inclusion bodies. The yield of purified MCADH and of most stable mutants was up to 900 mg from 40-l fermentor runs, with 5−10% of soluble protein as MCADH in the crude extracts, providing about 25-fold improvement compared to our original system [6] and 12-fold greater yield than Peterson et al. [7]. Expression at low culture temperature was the only method allowing us to obtain soluble, active proteins with some of the labile mutants, particularly [Glu304]MCADH. Growth temperatures <28° have no further beneficial effect on the yield. The point should be stressed that for [Glu304]MCADH, and at 37°C, essentially no activity in crude extracts could be detected.

Purification of human recombinant MCADH proteins. The procedure used for the purification of MCADH and of most mutants is derived from the original one by Thorpe et al. [4] which could be simplified extensively because of the high content of MCADH in crude E. coli extracts. Thus, two chromatographic steps allow the purification of up to 300 mg for some MCADH mutants from about 120 g bacterial paste with an apparent purity >95% (Table 1). The major improvement was achieved by the use of Q-Sepharose instead of other anionic-exchange resins and of commercially available hydroxyapatite (Fluka) in an FPLC system. For analytical purposes, a final Pharmacia HIload 26/60 Superdex 200 pg step yields enzyme with an apparent >98% purity. Following expression in E. coli cells, the different MCADH proteins occur as mixtures of their yellow and green forms which correspond to free MCADH and enzyme complexed with CoA-persulfide, respectively [35, 44].

Table 2. Summary of physicochemical properties of human MCADH, [Glu304]MCADH and of pig kidney MCADH. ε_{448} for the reduced enzyme was measured upon reduction with C_8-CoA.

Enzyme	E_{ox}			E_{red} ε_{448}	Bleaching with C_n-CoA, $n =$			Redox potential
	λ_{max}	ε_{272}, ε_{448}	$\varepsilon_{\lambda max1}/\lambda_{\lambda max2}$ $\varepsilon_{\lambda max2}/\varepsilon_{\lambda max3}$		4	8	16	
	nm	mM^{-1} cm^{-1}		mM^{-1} cm^{-1}	%			mV
Human MCADH	272, 373, 444	85, 14.9	5.7, 0.6	2.5	73,	85,	49	-114[c]
[Glu304]MCADH	275, 371, 445	110, 15.1	7.3, 0.74	3.0	74,	80,	70	-115[c]
Pig kidney MCADH	272, 373, 446[a]	76, 15.4[b]	5.7, 0.65[a]	3.2[a]	70,	79,	37[a]	-145[d]

[a] Thorpe et al. (1979).
[b] Lau et al. (1986).
[c] Mancini-Samuelson et al. (1996).
[d] Johnson et al. (1995).
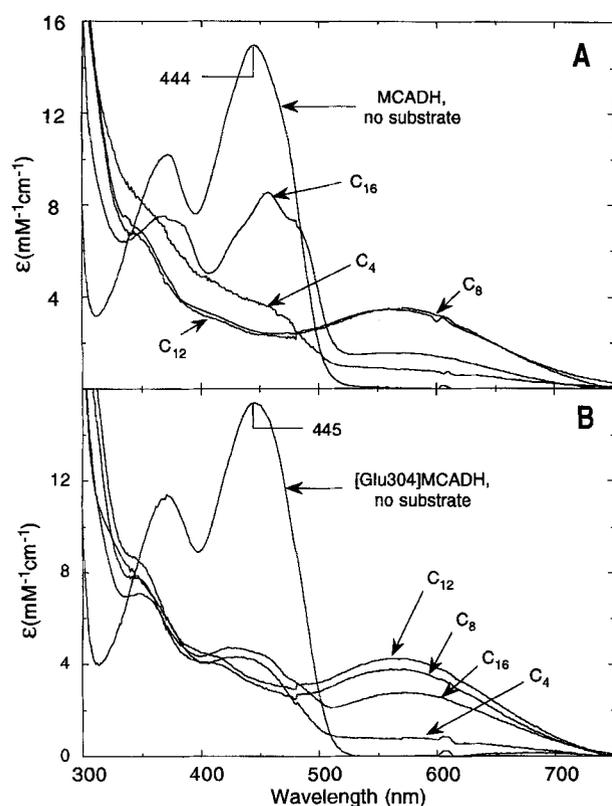
Fig. 1. Absorption spectra of MCADH and of [Glu304]MCADH and reaction with substrates of different chain length. (A) The initial spectrum is that of MCADH, 18 µM in 100 mM KP_i, pH 8.0, at 25°C. The enzyme was reacted with C_4-CoA (2.4), C_8-CoA (29), C_{12}-CoA (5), or C_{16}-CoA (16 equivalents), and the spectra were recorded upon equilibration of the system. (B) Spectrum of [Glu304]MCADH, 12 µM in 100 mM KP_i, pH 7.6, at 25°C. The enzyme was reacted similarly with C_4-CoA (5), C_8-CoA (30), C_{12}-CoA (5) or C_{16}-CoA (15 equivalents).

Physical properties. The visible and ultraviolet absorption spectrum of human MCADH is very similar, but not identical, to those of MCADHs from other sources (Fig. 1, Table 2) [1, 3, 4, 45, 46]. In particular, the ratio $\varepsilon_{272}/\varepsilon_{444}$ (Table 2) is lower for MCADH (5.7) than that reported for MCADH purified from human liver (6.7) [5], indicating a higher degree of purity, which is not surprising in view of the different origins of the materials used for the purification. The differences in ε_{max} of the visible

and 272-nm bands of human wild-type compared to pig kidney MCADH, although small, are real (Table 2). Gel filtration of both human wild type and [Glu304]MCADH are compatible with an estimated molecular mass of 155 kDa and with the proteins existing as tetramers because the monomer molecular mass calculated from the cDNA derived sequence is 43.6 kDa.

The reaction with substrates. The effects induced by addition of substrates of varying chain lengths on the spectrum of oxidized MCADH are shown in Fig. 1 A. With one equivalent of C_8-CoA, about 90% of the spectral changes at 444 nm are observed which are maximally attainable at saturation (not shown). With the [Glu304]MCADH mutant, five equivalents C_8-CoA lead to about 80% reduction. The charge transfer band of reduced MCADH complexed with product species is significantly more intense ($\varepsilon_{572} = 3.2$ mM^{-1} cm^{-1}) compared to that of pig kidney MCADH ($\varepsilon_{572} = 2.5$ mM^{-1} cm^{-1}) [4]. The same holds for the bands observed with C_4-CoA and C_{16}-CoA, implying a substantially higher thermodynamic stabilization of the reduced enzyme product complex with MCADH than with pig kidney MCADH. In line with this, bleaching at 444 nm is more extensive with MCADH at the same molar excess of C_8-CoA (85% versus 79%) [1, 3, 4]. The same trend is observed with excess C_4-CoA and C_{16}-CoA; on the other hand, upon addition of one equivalent of C_4-CoA to MCADH, reduction is only 11% compared to 31% with pig kidney MCADH [4] probably reflecting weaker binding ($E_{ox} + S \Leftrightarrow E_{ox} \sim S$) with the human enzyme. The extent of reduction with excess C_4-CoA is about 70% for MCADH, pig kidney MCADH and [Glu304]MCADH, but the [Glu304]MCADH mutant shows approximately 20% more bleaching with C_{16}-CoA. It is interesting to note that the redox potential of MCADH [47] is substantially higher than that of pig kidney MCADH, in line with the greater degree of bleaching observed with, for example, C_8-CoA. With the mutant [Glu304]MCADH, the redox potential is almost identical to the wild type, -115 mV, compared to -114 mV for human MCADH. The spectral data concerning the three forms of MCADH are listed in Table 2.

Catalytic properties and substrate specificity. The catalytic parameters of MCADH and [Glu304]MCADH under turnover conditions and using four different assays/acceptors systems are reported in Table 3, where the data for the pig enzyme are also listed for comparison. Fig. 2 depicts and compares the dependence of the catalytic efficiency (V_{max}/K_m) on the substrate chain length. Indeed the two proteins differ markedly in their prefer-

Table 3. Catalytic properties of human MCADH, [Glu304]MCADH and comparison with pig kidney MCADH. Data were obtained at 25°C in 50 mM KP_i pH 7.6. The values for human MCADH are shown for comparison, and were adapted from Nandy et al. [32]. The data scatter (error) for the values obtained with the ferricenium and Cl_2Ind-based assays is $\pm 5\%$ and $\pm 10\%$ respectively. Values in brackets are the fixed concentrations of C_n-CoA substrate (choosen as $\gg K_m$) at which the data were obtained.

| Enzymes	Substrates	Parameters with electron acceptors					
		ferricenium		ETF V_{max}	ETF/Cl_2Ind V	PMS/Cl_2Ind	
		V_{max}	K_m			V_{max}	K_m
		min^{-1} (µM)	µM	min^{-1} (µM)			µM
Human MCADH	C_4-CoA	500	175		340 (700)	130	173 ±60
	C_6-CoA	1010	15		330 (100)	100	0.7± 0.3
	C_8-CoA	980	3.4	1500 (60)	350 (100)	150	<1
	C_{10}-CoA	580	2.5		260 (100)		
	C_{12}-CoA	550	2.5		280 (100)		
	C_{14}-CoA	280	2.3		165 (100)		
	C_{16}-CoA	140	1.6		130 (100)		
[Glu304]MCADH	C_4-CoA	540	120				
	C_6-CoA	760	7.5				
	C_8-CoA	970	12	580 (60)			
	C_{10}-CoA	530	7.7				
	C_{12}-CoA	570	3.3				
	C_{14}-CoA	500	5.2				
	C_{16}-CoA	175	2.0				
	C_{18}-CoA	40					
Pig kidney MCADH	C_4-CoA	380 (750)	90		300 (700)	75	50
	C_6-CoA	1250 (70)	9		240 (100)	130	≈1
	C_8-CoA	1100 (85)	3.5	680	300 (100)	180	<1
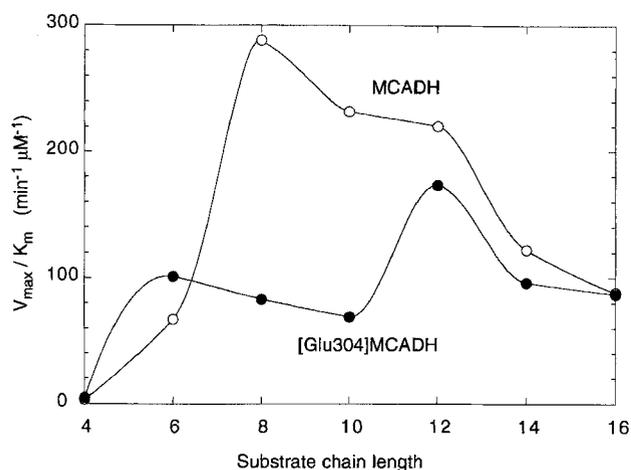
Fig. 2. Chain-length dependence of V_{max}/K_m for [Glu304]MCADH and comparison with MCADH. The data were obtained using the ferricenium assay [36] as described in Materials and Methods. See Table 3 for the values of V_{max} and K_m.
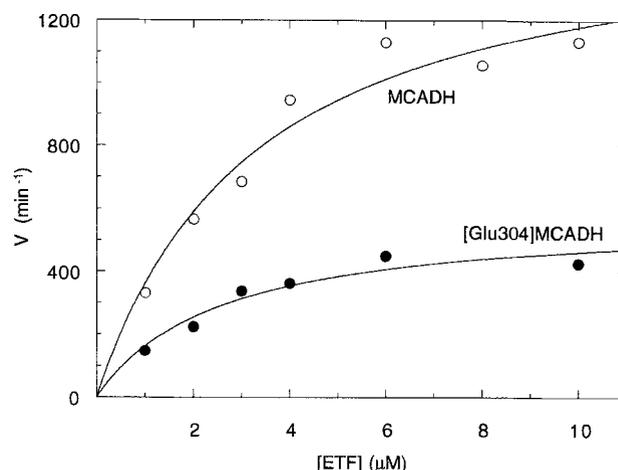
Fig. 3. Dependence of MCADH and [Glu304]MCADH activity on the ETF concentration. The velocities (V) were obtained from the rates of monophasic fluorescence decrease of pig kidney ETF (λ_{ex} = 435 nm, λ_{em} = 490 nm) using ≈ 1 nm MCADH (o) and [Glu304]MCADH (●) at the indicated concentrations of ETF. The assay conditions were 20 mM Tris/HCl pH 8.0; [C_8-CoA] = 60 µM. The decrease of ETF fluorescence corresponds to its reduction to the semiquinone form (cf. Materials and Methods for further details).

ences, the mutant having long-chain character. In view of the known problems inherent in the measurements of acyl-CoA dehydrogenase activities [48], the data listed in Table 3 and shown in Fig. 2 were obtained in parallel measurements whenever possible. We have also used the ETF fluorescence assay (ETF purified from pig kidney [43]) for the comparison of human wild-type and [Glu304]MCADH because this assay most probably best reflects the *in vivo* conditions. This system displays Michaelis-Menten behavior (Fig. 3), and it is apparent that [Glu304]-MCADH has about 40% the activity of MCADH, although the interaction constant with ETF is comparable. Pig kidney

MCADH has about 50% the activity of MCADH in the same assay (not shown). Preliminary measurements using human recombinant ETF indicate analogous values suggesting that human and pig ETF are similarly efficient in their electron acceptor capacities towards MCADH. Using the ferricenium assay, we found virtually the same V_{max} for human wild-type and [Glu304]MCADH with C_8-CoA to C_{12}-CoA. Notable differences for the mutant were found only with C_6-CoA and with C_{14}-CoA
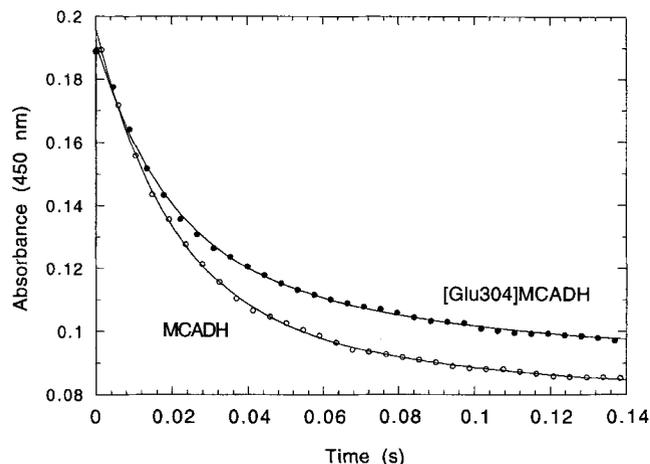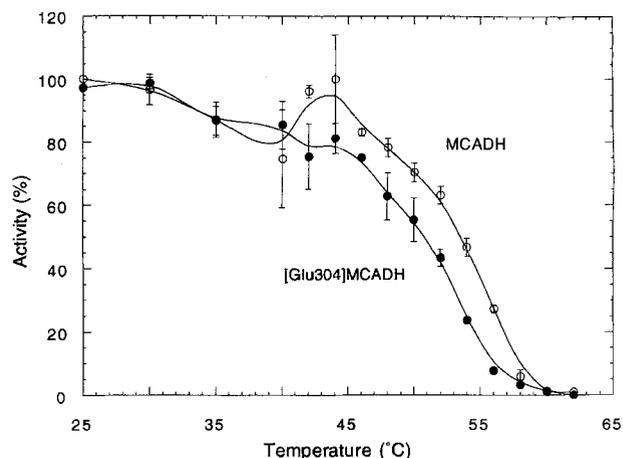
Fig. 4. Time course of the reaction of MCADH (○) and [Glu304]-MCADH (●) with C$_{12}$-CoA. The enzymes, 6.5 μM in 100 mM KP$_i$, pH 7.6 and at 2°C, were reacted with 50 μM substrate (both final concentrations) in the stopped-flow instrument (2-cm path cell). The data points shown are the numerical average from three single shots. The lines are the best fits obtained using a two-exponential algorithm.

Fig. 5. Temperature dependence of the activity of human wild-type and [Glu304]MCADH. Purified enzymes were incubated in 100 mM KP$_i$, pH 7.6, at the temperatures shown for 15 min and the activity was then measured using the ferricenium assay. The data points shown are the average of at least three individual determinations, and the bars indicate the actual scatter of the data.
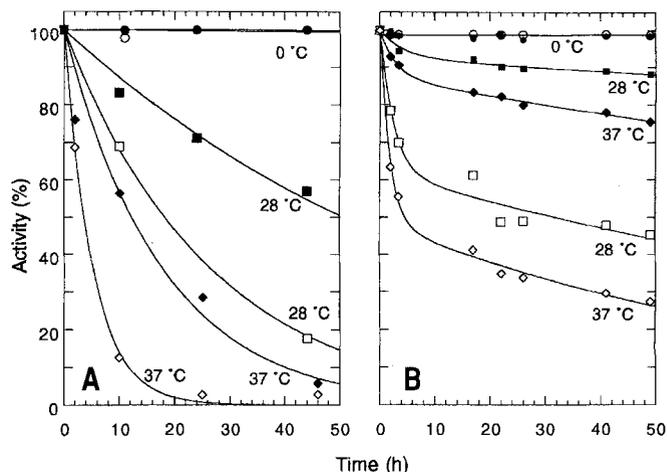
which show slightly lower and significantly higher activities respectively (see Table 3). Comparison of the data listed in Table 3 indicates that the ferricenium assay [36] is adequate for assessing the catalytic properties of various MCADHs and their chain-length dependence of the activity. This contrasts with the Cl$_2$Ind-based assays which appear to underestimate the maximally attainable rates in a way not linearly dependent on the substrate chain length (*cf.* e.g. values for C$_4$–C$_8$-CoA, Table 3). For this reason the catalytic properties of [Glu304]MCADH were studied using primarily the ferricenium assay, the large quantities of purified ETF required for the direct fluorescence assay precluding its routine use. Qualitatively, our results for MCADH are in line with those by Finocchiaro et al. [5].

The reductive half-reaction. The rate of turnover of most acyl-CoA dehydrogenases is limited by the oxidative half-reaction, which, in turn, is governed by factors influencing product dissociation [7, 9, 49], oxygen reactivity [50], and interaction with the acceptor [51]. Evaluation of the catalytic efficiency in the reductive half-reaction thus requires its direct measurement which is best done by stopped-flow spectrophotometry. Comparison of the course of reduction of MCADH and of [Glu304]-MCADH is difficult using the best substrate (C$_8$-CoA), even at 2°C because the rates are very fast (>300 s^{-1}, see also [7]), although it is apparent that the two proteins behave in a similar fashion. We have thus selected C$_{12}$-CoA, which, from the data of Fig. 2, is a good substrate for MCADH and the mutant. From the traces of the course of flavin reduction depicted in Fig. 4, it can be concluded that the rates for MCADH and [Glu304]M-CADH are very similar with the substrate C$_{12}$-CoA. The reaction is biphasic in both cases with a fast (55 s^{-1}) and a second, slower phase (14 s^{-1}), which are followed by a much slower phase involving equilibration [9].

Thermal effects, time dependence of inactivation and Arrhenius energy of activation. The effect of temperature on the activity of purified human wild-type and the [Glu304]MCADH was determined upon incubation of samples for 15 min at the temperatures shown in Fig. 5. [Glu304]MCADH has about a 4°C lower temperature of 50% inactivation. This is in good agreement with the results obtained previously in extracts from *E. coli* cells expressing these proteins [30]. MCADH displays

Fig. 6. Time and temperature dependence of the activity of MCADH and [Glu304]MCADH. (A) Samples of MCADH (●, ■, ◆) and of [Glu304]MCADH (○, □, ◇) from crude *E. coli* cells extracts were incubated for the indicated time in KP$_i$ pH 7.6 at 0°C (●, ○), 28°C (■, □) or 37°C (◆, ◇) and aliquots were assayed with C$_8$-CoA at 25°C. The half-times for inactivation were ≈50 h and ≈12 h for MCADH at 28°C and 37°C, respectively, and ≈18 h and ≈3.5 h for [Glu304]MCADH at the same temperatures. (B) The same experiment was performed using purified MCADH (●, ■, ◆) and [Glu304]MCADH (○, □, ◇). Note the biphasic course of the inactivation process at >0°C. The half-times of inactivation of MCADH were ≈3 (fast phase) and >700 h (slow phase) at 28°C and ≈2 (fast phase) and ≈230 h (slow phase) at 37°C. The corresponding values for [Glu304]MCADH were ≈1.7, ≈100, ≈1.25, and ≈55 h. The curves in A are the best fits for a monophasic, and in B for a biphasic exponential process.

an unusual region of increased stability at 42–45°C where the activity returns to the level observed at lower temperatures. This is most probably not due to an artefact because we reproduced the effect in multiple measurements of different preparations. Purified [Glu304]MCADH and MCADH do not differ significantly in proteolytic susceptibility when digested in the absence or presence of 0.05% SDS and as judged from SDS/PAGE analysis (not shown). The Arrhenius activation energies were estimated as 46 kJ/mol for MCADH and 59 kJ/mol for the

[Glu304]MCADH mutant. The Arrhenius plots of both proteins (not shown) are linear over the temperature range of $5-50\,^\circ C$; at $>50\,^\circ C$ the activity rapidly decreases, probably coinciding with the onset of thermal dissociation and irreversible inactivation. From the coincidence of these parameters, it is likely that the same basic mechanism(s) of thermal inactivation are operative for MCADH and [Glu304]MCADH.

The stability of MCADH and [Glu304]MCADH was investigated by measuring the decrease of activity at increasing time, and at different temperatures in *E. coli* crude extracts (Fig. 6A) and in purified enzyme samples (Fig. 6B). Both enzymes are stable on ice for more than 50 h. At $28\,^\circ C$ and at $37\,^\circ C$ the inactivation of the mutant enzyme in *E. coli* extracts (Fig. 6A) is ≈ 3-fold faster than observed with MCADH (for the half-times of inactivation see legend to Fig. 6). With purified enzyme samples the course of inactivation is biphasic (Fig. 6B). The differences in the fast rate of inactivation between MCADH and [Glu304]-MCADH is ≈ 2-fold; it is much more pronounced (4-7-fold) in the slow phase.

DISCUSSION

The new expression system is a crucial improvement over the previous ones [6, 7] because it allows the expression in crude extracts of [Glu304]MCADH to about 20% the total activity content found with MCADH under comparable conditions. It also permits the isolation of apparently homogeneous [Glu304]-MCADH in two chromatographic steps in about 20% yield (Table 1). Our results contrast with previous reports that this mutant is unstable and cannot be purified [28, 52]. In our hands, the greatest loss of mutant MCADH occurs in the first ammonium sulfate cut (Table 1) and may be due to the presence of incorrectly folded or partially degraded active [Glu304]MCADH in the crude extract.

The physicochemical properties of highly purified MCADH, [Glu304]MCADH and of pig kidney MCADH are, overall, quite similar (Table 2). There are, however, some significant differences among them. First, the redox potential of the human enzyme is some 30 mV higher than that of pig kidney MCADH. This is reflected in the larger extent of bleaching of pig kidney MCADH with substrate (Table 2, Fig. 1). The differences in bleaching between MCADH and [Glu304]MCADH are probably due to differences in affinity for substrate/product. Second, and more importantly, some interesting aspects emerge upon inspection of the catalytic parameters of [Glu304]MCADH as compared to those of MCADH. It is likely that the differences in activity observed with substrates C_6 to C_{14} are due not to lower rates in the reductive half-reaction, which are essentially identical (Fig. 4), but to the mode of interaction with the electron acceptor. Third, the most astonishing difference is the shift in substrate specificity (Fig. 2) with the [Glu304]MCADH, which might more appropriately be named a long-chain acyl-CoA dehydrogenase because it exhibits highest efficiency with C_{12}/C_{14}-CoA substrates rather than with C_8-CoA, as with normal human MCADH. Recent insights [32], suggest that the volume of the substrate binding pocket is important in determining substrate chain-length specificity. We therefore infer that the active site of [Glu304]MCADH may be more flexible and tolerant with respect to substrate chain length, i.e. size. In fact, the mutant MCADH is more active than MCADH with C_4-CoA, and is active, albeit at low levels, with C_{18}-CoA, whereas MCADH is not (Table 3). Fourth, the data shown in Figs 5 and 6 document an important decrease in stability both with time and temperature for [Glu304]MCADH. This confirms previous results obtained with crude cell extracts [30]. The biphasic inactivation

behavior of the purified enzymes (Fig. 6B) is compatible with a transition into a less active form, which might be much more susceptible to proteolytic degradation in crude extracts (Fig. 6A) and, more relevantly so, *in vivo*. This instability possibly originates from the modified interactions of the H and I helices around positions 304 in the center of the MCADH tetramer and may be secondary to the higher flexibility of the substrate binding pocket.

The significantly decreased activity of [Glu304]MCADH compared to MCADH observed with the natural electron acceptor, ETF, and the higher K_m values for medium-chain substrates may well contribute to the disease mechanism in patients homozygous for this mutation. It would be of interest to know whether these effects also occur in hetero-tetrameric MCADH, consisting of two different types of monomers, which would be expected to occur in individuals heterozygous for the K304E mutation. Our groups [26] and others [28, 29] have shown earlier that the K304E mutation has a major impact on folding and assembly of the polypeptide. Higher levels of chaperonins increase the yield of active [Glu304]MCADH in bacteria in agreement with impaired formation of tertiary and quarternary structures [30]. The difference in [Glu304]MACDH activity produced at $28\,^\circ C$ and $37\,^\circ C$ is in line with the notion that folding and assembly are a major factor in the generation of disease. In addition to that, the modified chain-length spectrum and K_m/V_{max} of [Glu304]MCADH could adversely affect the substrate specificity pattern of the β-oxidation process. Decreased levels of MCADH due to impaired folding and tetramer assembly may thus be further potentiated by a hole in the substrate affinity for C_8-acyl-CoAs. The persistent occurrence of elevated levels of glycine conjugates with medium-length acyl chains observed also in asymptomatic periods [53] may thus be due to a combination of this hole in the substrate affinity and the decreased level of [Glu304]MCADH resulting from impaired folding, assembly and stability of the mutant variant. The contribution of each of these multiple effects of the K304E mutation observed experimentally to the phenotypes expressed in the patient organism remains to be established.

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