Biochemical characterization of a variant human medium-chain acyl-CoA dehydrogenase with a disease-associated mutation localized in the active site

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Medium-chain acyl-CoA dehydrogenase (MCADH) deficiency, an autosomal recessive inherited disorder, is the most common genetic disorder in mitochondrial β-oxidation in humans. In addition to one prevalent disease-causing mutation (K304E), a series of rarer mutations has been reported, but none of these has yet been characterized in detail. We report here on the biochemical characterization of the purified recombinant mutant protein in which threonine is replaced by alanine at position 168 of the mature protein (T168A-MCADH). It is the first mutation to be found in patients that is located in the active site of the enzyme. Thr-168 is hydrogen-bonded to the flavin N(5) of the cofactor FAD. The thermostability of T168A-MCADH is markedly decreased compared with human wild-type MCADH (hwt-MCADH). Catalytic activity with ferricenium as acceptor is lowered by 80 % and with the natural acceptor electron-transferring flavoprotein by over 90 % compared with hwt-MCADH. In the mutant the extent of flavin semiquinone formed on reduction is approx. 50 % that of hwt-MCADH. The pK reflected by the pH-dependence of V_{max} is shifted from approx. 8.2 (hwt-MCADH) to approx. 7 (T168A-MCADH) and the rates of enzyme flavin reduction (stopped-flow measurements) are only approx. 1/10 those of the parent enzyme. These properties are discussed in the light of the possible mechanisms leading to disease in humans.

INTRODUCTION

Acyl-CoA dehydrogenases catalyse the initial and rate-limiting step of mitochondrial β-oxidation by performing the α,β-dehydrogenation of fatty acyl-CoA *S*-esters. Several members of this flavoprotein family are known; they can be subdivided by their specificity for the chain length and branching of acyl-CoA substrates (reviewed in [1,2]). The best studied member of this protein family is the homotetrameric medium-chain acyl-CoA dehydrogenase (MCADH). Deficiency in this enzyme is the most common known genetic disorder in mitochondrial β-oxidation in humans [3,4]. It is an autosomal recessive inherited disorder that usually occurs in the first years of life. The variety of the phenotypic occurrence is wide, but usually it includes fasting-induced non-ketotic hypoglycaemia and lethargy, sometimes resulting in coma [5–7]. Between 20 % and 25 % of the patients die suddenly at the first presentation of the disease [6,8,9]. In contrast, there are patients who are affected but remain without symptoms for years [9–12]. Approx. 90 % of the alleles in patients carry the prevalent G985A point mutation, which results in a replacement of lysine with glutamic acid at position 304 of the mature enzyme (K304E-MCADH). Lys-304 is positioned at the interface between the homotetramer-forming subunits of the enzyme (helix H) [13]. The mutant protein shows a specific activity comparable with that of human wild-type MCADH (hwt-MCADH) [14] but is present only at much decreased levels in patient cells [15–18]. The reason for this is impaired folding and tetramer assembly, which presumably results in premature degradation of the enzyme [14,19]. The investigation of four further mis-sense mutations detected in patients indicated that in all cases the folding and/or the assembly of the protein are affected [3,19–24]. Recently Andresen et al. [7] studied 52 families with MCADH deficiency not caused by homozygosity for the K304E mutation and found seven new mutations. One of these is a point mutation (A577G) resulting in the replacement of a threonine residue with alanine at position 168 in the amino acid sequence of the mature protein. The patient and his father had previously been reported to be heterozygous for a 13 bp insertion mutation (999-12) in exon 11. The mother was found to be heterozygous for the A577G mutation too. The study indicated that the steady-state amounts of MCADH mRNA from both mutant alleles were decreased [7,10].

What sets the T168A mutation apart from all other previously known mutations is that it constitutes the first case of a modification within the active site of the protein. Thr-168 is located in contact with the FAD cofactor and forms a hydrogen-bond with the flavin N(5) position. This is the point of entry of the substrate-derived hydride during catalysis [25–30], so it is conceivable that the modification affects the chemistry of catalysis (Figure 1). To investigate these aspects we have expressed, purified and partly characterized the recombinant mutant protein and report on some of its properties in comparison with those of hwt-MCADH and of K304E-MCADH.

EXPERIMENTAL

Materials and enzymes

CoA was from Sigma; ferricenium hexafluorophosphate and free fatty acids of different chain lengths were from Aldrich; and glucose oxidase and catalase were from Boehringer Mannheim. Acyl-CoAs were prepared by the mixed-anhydride procedure [31]; C_{16}-CoA and C_{18}-CoA were prepared by the method of Kawaguchi et al. [32] and purified by preparative HPLC. Sepharose Q (fast flow) and hydroxyapatite (fast flow) were from Pharmacia and Fluka respectively. Yeast extract,

Abbreviations used: ETF, electron-transferring flavoprotein; hwt-MCADH, human wild-type medium chain acyl-CoA dehydrogenase.

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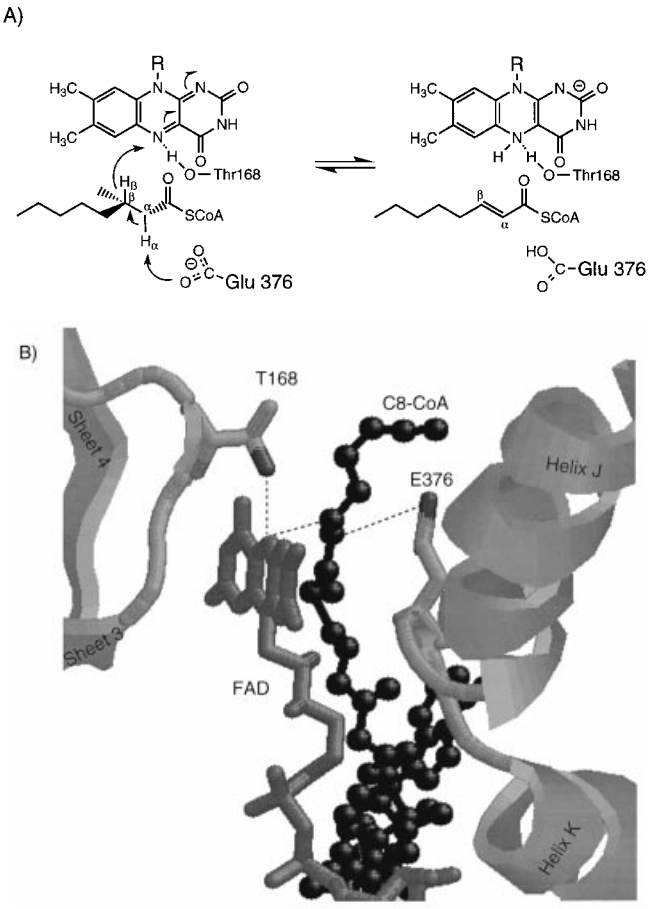

Figure 1 Stereochemical course of the α,β-dehydrogenation and arrangement of relevant functional groups, flavin and substrate at the active centre of MCADH

(**A**) Stereochemistry of the α,β-dehydrogenation. The substrate α-hydrogen is abstracted as H^+ by Glu-376 in a concerted process and the β-hydrogen is transferred as hydride to the flavin N(5) position. The latter builds a hydrogen bond to Thr-168-OH (broken line). (**B**) Arrangement of functional groups at the active centre. Thr-168 lies on the loop between sheets 3 and 4 on the Si-side of the flavin. The substrate C_8-CoA is sandwiched between the isoalloxazine plane and Glu-376, which is linked to loop JK. (Illustration based on coordinates of MCADH [13].)

peptone and isopropyl β-D-thiogalactoside were from Roth, and molecular-mass standards for SDS/PAGE and gel filtration were from Bio-Rad. All restriction enzymes used were from Boehringer Mannheim; T4 ligase was from MBI Biotech; DNA extraction used Geneclean from Bio 101. DNA sequencing was performed with the *Taq* dye terminator kit (Applied Biosystems/Perkin Elmer) and products were analysed on an ABI 373 automated sequencer.

Instrumentation and conditions

Visible and UV spectra and activity measurements were recorded with a Kontron Uvikon 930 spectrophotometer. Absorbance spectra were corrected for dilution if necessary. Solutions of acyl-CoA substrates in 10 mM potassium phosphate, pH 6.0, were standardized spectrophotometrically with ϵ_{260} 15.4 $mM^{-1}\cdot cm^{-1}$ for fatty acyl-CoAs [33]. Single-turnover experiments were performed by using a stopped-flow spectrophotometer with a 2.0 cm path-length cell [34,35], a 'dead time' of 3–4 ms and equipped with a diode array detector (Spectroscopy Instruments, Gilching, Germany) interfaced with a Macintosh IIcx computer for data acquisition (POSMA 2.3 k software from Spectroscopy Instruments). Rapid reaction kinetics were measured by recording spectra in the range 300–650 nm by using the normal scan mode with a scan time of 10 ms per spectrum and with a resolution of 2 pixels/nm. From at least five reproducible kinetic traces obtained under the same conditions the arithmetical mean was computed and then fitted with program A (Dr. D. P. Ballou, University of Michigan, Ann Arbor, MI, U.S.A.). The deviation of the values from the mean of three independent measurements was at most 10 %. Other fitting routines were performed with KALEIDAGRAPH (Synergy Software) and appropriate algorithms.

Enzyme assay

All enzyme assays were conducted with ferricenium hexafluorophosphate as electron acceptor, as detailed by Lehman et al. [36]. Alternatively the assay, which uses electron-transferring flavoprotein (ETF) as acceptor, was employed (fluorescence assay [37]; Kontron SFM 25 fluorimeter). All ferricenium assays were performed at 25 °C in 100 mM potassium phosphate, pH 7.6, unless stated otherwise. For the ETF assay, recombinant human ETF or its Y16L mutant [38] was used. Cloning, expression and purification of the latter will be described elsewhere (A. Ghany, B. Küchler, P. Bross and S. Ghisla, unpublished work).

Miscellanous methods

For the estimation of ϵ_{max} of the FAD in the visible range, SDS was added to a final concentration of 1 % (w/v) to a solution of approx. 15 mM T168A-MCADH in 20 mM sodium phosphate, pH 7.8. The resulting spectrum of the flavin was recorded and the ϵ_{max} was calculated on the basis of an ϵ_{450} of 11.3 $mM^{-1}\cdot cm^{-1}$ for free FAD [39]. The resulting coefficient (ϵ_{439} 14.25 $mM^{-1}\cdot cm^{-1}$) was used in all determinations of enzyme concentrations of the mutant.

Construction of pTrc hMCADH T168A

The plasmid pT168A was obtained as described elsewhere [7]. To generate the expression plasmid pTrc hMACDH T168A, the plasmid pTrc hMCADH [2] and pT168A were restricted by double digestion at the unique *Eco*RI/*Pst*I sites within the MCADH coding region. The resulting 5422 bp vector fragment of pTrc hMCADH and the 498 bp fragment of pT168A, including the mutation site, were separated by agarose-gel electrophoresis and purified. Ligation of the two fragments led to the complete plasmid pTrc hMCADH T168A that was used for transformation in accordance with the conditions provided by MBI Biotech.

Expression and purification of the mutant protein

The plasmid pTrc hMCADH T168A was transfected into *Escherichia coli* cells (strain TG 1) in accordance with the protocol described by Hanahan et al. [40]. The expression in *E. coli* was performed as described by Nandy et al. [2]. The purification procedure was mainly adapted from that reported by Kieweg et al. [14] for human recombinant MCADH, except that all buffers contained 10 % (v/v) glycerol.

RESULTS

Purification and selected properties

A purification in the absence of glycerol based on the procedure described by Kieweg et al. [14] resulted in a yield of approx.

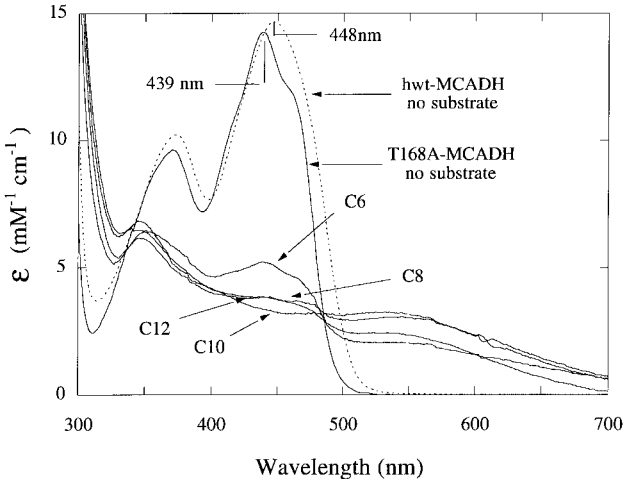

Figure 2 Reaction of T168A-MCADH with substrates of different chain lengths and effect on the extent of reduction

Acyl-CoA substrates with the indicated chain lengths were each added to the oxidized enzyme (approx. 12 μM in standard buffer) and the spectra were recorded after approx. 2 min, at which time spectral changes had ceased. The extent of bleaching of the oxidized flavin band (λ_{max} $\approx$ 440 nm) for the individual substrates is reported in Table 1 and is related to the bleaching obtained on anaerobic reduction of oxidized MCADH with dithionite at λ_{max} $\approx$ 450 nm, which was set to 100% (see the legend to Table 1 for further details).

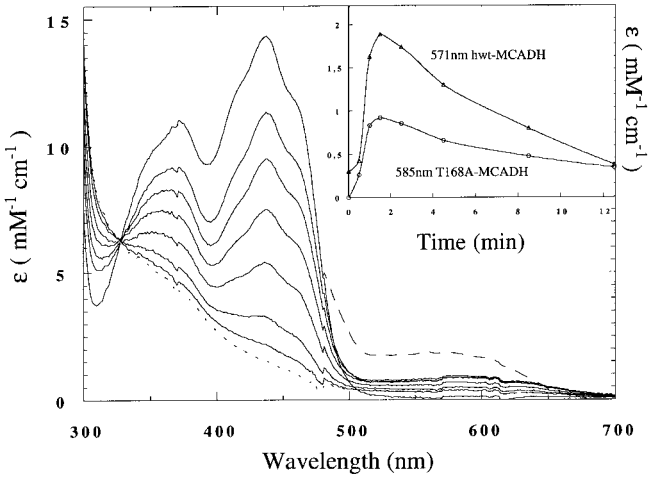

Figure 3 Course of photoreduction of T168A-MCADH

T168A-MCADH (approx. 10 μM), 2 μM 5-deaza-riboflavin and 5 mM EDTA were illuminated with a 250 W tungsten lamp (distance 11 cm) in 100 mM potassium phosphate, pH 8, at 20 °C. The spectra shown were recorded after 0, 1, 1.5, 2.5, 4.5, 8.5 and 12.5 min of illumination. The broken line shows the spectrum obtained in a parallel experiment with hwt-MCADH and at the maximal extent of neutral radical formation; the dotted line shows the spectrum calculated for full reduction of T168A-MCADH. The inset compares the time course for the formation of the long-wavelength bands with T168A-MCADH at 585 nm and with hwt-MCADH at 571 nm.

40 mg per 100 g of wet cell paste and a $A_{270/450}$ of approx. 8.5. This is 15–20% of the yield obtained for hwt-MCADH [14]. Under these conditions a large fraction of free FAD was also eluted from the Q-Sepharose column, suggesting a loss of FAD during the isolation. The addition of 10% (v/v) glycerol to all purification buffers resulted in a 50% yield increase and a better $A_{270/450}$ of approx. 6. Apparently homogeneous T168A-MCADH was therefore isolated in a yield of approx. 60 mg (57 and 60 mg per 100 g of wet cell paste in two preparations). It exhibited a single protein band of approx. 43 kDa on a silver-stained SDS/PAGE gel and was indistinguishable from hwt-MCADH used as reference (results not shown). The obtained ϵ_{439} value of 14.25 $mM^{-1} \cdot cm^{-1}$ was lower than that of hwt-MCADH (ϵ_{448} 14.8 $mM^{-1} \cdot cm^{-1}$) [14]. The absorption spectrum of the oxidized form of the mutant was significantly different from that of hwt-MCADH in that it showed pronounced shoulders at approx. 460, 415 and 360 nm and a 9 nm blue shift in λ_{max} in the visible region (Figure 2) ($A_{270/450}$ $\approx$ 6). This effect is indicative of a less hydrophilic environment for the flavin chromophore [41] and is consistent with the Thr $\rightarrow$ Ala exchange in the vicinity of the N(5) locus. The spectrum of the fully reduced form (Figure 3), in contrast, is essentially identical with that of hwt-MCADH, suggesting that Thr-168 has no relevant interactions with the reduced chromophore.

Reaction with substrates of various chain lengths and photoreduction

The reaction of acyl-CoA dehydrogenases with fatty-acyl-CoAs of various chain lengths leads to a complex set of reactions and equilibria, which include reduction of the oxidized flavin, formation of charge-transfer complexes of the reduced enzyme with enoyl-CoA products, dissociation of the latter, and binding of (excess) substrate to the reduced form [28]. The extent of oxidized flavin reduction and charge-transfer complex formation reflects the ratio of the redox potentials of the partners involved [42] and the affinity of the oxidized enzyme for a given chain length of the substrate [2,28]. Thus with hwt-MCADH the maximal extent of reduction was obtained with C_8-CoA and decreased with elongation of the substrate chain (Table 1) [14]. With the T168A mutant the maximal extent of reduction was shifted to C_{10}-CoA (Table 1 and Figure 2).

The photoreduction of flavin enzymes is a convenient tool for obtaining and characterizing half-reduced and fully reduced species in the absence of chemical reagents or substrates [42]. Figure 3 depicts the course of this reaction. On comparison of the behaviour of the mutant protein with that of hwt-MCADH, two differences become apparent: the maximal extent of blue (neutral) radical formation is approx. 50% of that observed with hwt-MCADH and its long-wavelength maximum is shifted from approx. 571 to 585 nm. The first effect is taken as reflecting a smaller extent of stabilization of the semiquinone in the mutant, which results from an increase in the redox potential of the first half-reduction step. This can clearly be attributed to a difference in the interaction of the flavin N(5) position with the protein environment.

Catalytic properties and substrate specificity

Some of the properties of T168A-MCADH are listed in Table 1 and are shown in Figures 4–9, where they are compared with those of hwt-MCADH and also of the K304E mutant. With the ferricenium assay the T168A mutant has approx. 20% of the specific activity of hwt-MCADH, each with its best substrate. The maximal activity of the mutant is shifted to C_8–C_{10} and is lower with C_6 (Figure 4). Similarly to the case of the K304E-MCADH, the T168A mutant shows little activity with C_{18}-CoA; however, it is higher than that of hwt-MCADH. The K_m values of T168A-MCADH with the best substrates are comparable and marginally lower than those of hwt-MCADH and show a similar dependence on chain length (Figure 5). Figure 6 shows and compares for the mutant and hwt-MCADH the dependence of

Table 1 Substrate chain-length dependence of selected parameters for hwt-MCADH, and comparison with K304E-MCADH and T168A-MCADH

Reduction (%) is the percentage bleaching of the flavin absorbance at 439 nm observed 2 min after the addition of a 10-fold molar excess of substrate. The deviation in determinations was ±5% and the value was related to the absorbance of hwt-MCADH reduced with dithionite (ϵ_{450} approx. 1500 $M^{-1} \cdot cm^{-1}$). k_{red} is the rate of the absorption decrease at 450 nm measured with the stopped-flow instrument on aerobic mixing of enzyme and C_8-CoA (see the Experimental section for details). $V_{reox\text{-}02}$ is the rate of increase of the flavin absorption at 450 nm, which corresponds to its reoxidation after aerobic reduction with approx. 0.7 equiv. of substrate (measured as described previously [2]; the deviation of the values from the mean of three independent measurements was at most 5%). Values for hwt-MCADH were adapted from Nandy et al. [2]; those for K304E-MCADH from Kieweg et al. [14].

		Chain length of substrate					
Enzyme	Parameter, units and conditions	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}
hwt-MCADH	Reduction (%)		91	90	88		53
	k_{red} (min^{-1})		> 20000	> 20000	5400	420	
	$V_{reox\text{-}02}$ (min^{-1})	0.028	0.004	0.004	0.01	0.021	0.018
K304E-MCADH	Reduction (%)		80				70
	k_{red} (min^{-1})		> 20000				
T168A-MCADH	Reduction (%)	71	81	88	82		
	k_{red} (min^{-1})		1800				
	$V_{reox\text{-}02}$ (min^{-1})		0.008				

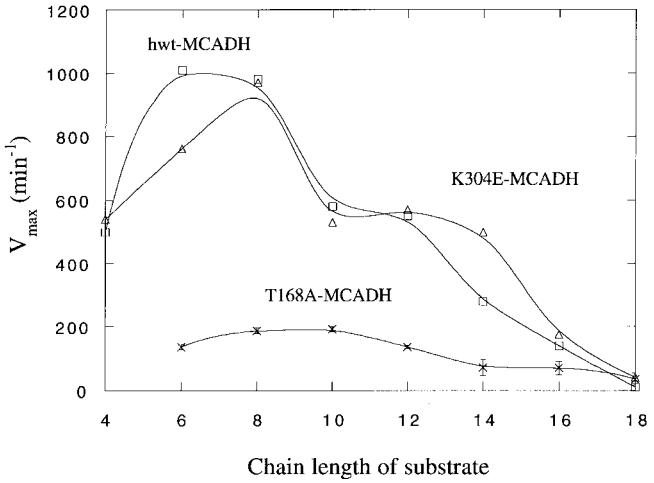

Figure 4 Chain-length dependence of V_{max} for T168A-MCADH, and comparison with hwt-MCADH and K304E-MCADH

All activities were measured with the ferricenium assay in 0.1 M potassium phosphate, pH 7.6, at 25 °C [36]. Error bars are S.E.M.

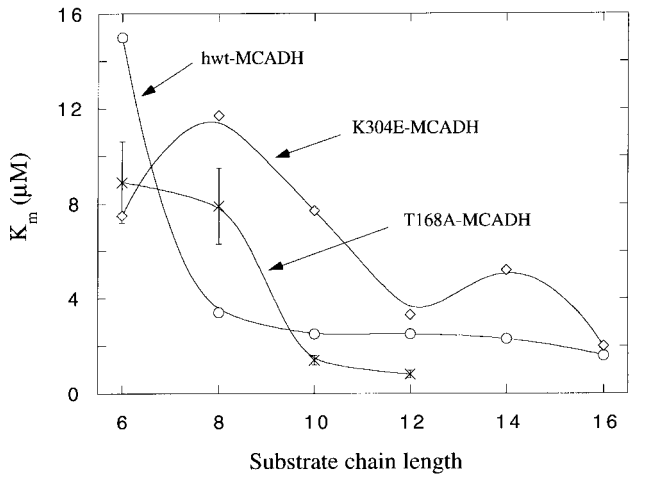

Figure 5 Substrate chain-length dependence of K_m values for T168A-MCADH, and comparison with hwt-MCADH and K304E-MCADH

All activities were measured with the ferricenium assay [35]. Error bars are S.E.M.

the catalytic efficiency, V_{max}/K_m, on the substrate chain length. With this parameter as a criterion it is apparent that the mutant has more the character of a 'long-chain'-dehydrogenase, as opposed to a 'medium-chain' one, an effect that is incidentally also observed with the K304E mutant and is correlated with the chain-length dependence of the extent of bleaching described above (Figure 2).

The activity assay that probably best reflects the situation *in vivo* is that with ETF, the physiological electron acceptor [37]. We therefore used purified human recombinant ETF for this assay. The activity of the T168A mutant was less than 10% that of hwt-MCADH (V_{max} approx. 60 and approx. 880 min^{-1} with human ETF, and approx. 100 and approx. 1100 min^{-1} with human ETF-β-Y16L as electron acceptor, a variant with a higher fluorescence yield [38]) (Figure 7). This means that the mutant is more than twice as active with the artificial assay, whereas with hwt-MCADH the opposite is true. These results suggest that the mutation affects the interaction with ETF, that is the oxidative half-reaction.

pH-dependence of the activity

We have recently shown that the activity of acyl-CoA dehydrogenases is markedly dependent on the pH, having apparent pK values in the range 7–8 and that this pK is dependent on the substrate chain length [43]. Therefore it is most important that activity comparisons take these factors into account. Figure 8 shows the pH-dependence of the activity of T168A-MCADH in comparison with that of hwt-MCADH, and documents a shift in the apparent pK of the mutant to approx. 7.0. The activities of the mutant and of hwt-MCADH were approx. 20 and 430 min^{-1} at pH < pK, and 180 and 5800 min^{-1} at pH > pK. This demonstrates that the comparison of activities restricted to the 'standard pH = 7.6' can lead to gross underestimates or overestimates. The molecular reasons for the observed shift cannot be deduced readily, because the activity of acyl-CoA dehydrogenases arises from the interplay of several steps [2,28] and the pK values in question cannot be attributed to single (microscopic) ionizations. In contrast, it is tempting to speculate that the hydrogen bridge of Thr-168-OH towards the N(5) position of FAD isoalloxazine affects the microscopic pK values of the neutral (blue) flavin semiquinone.

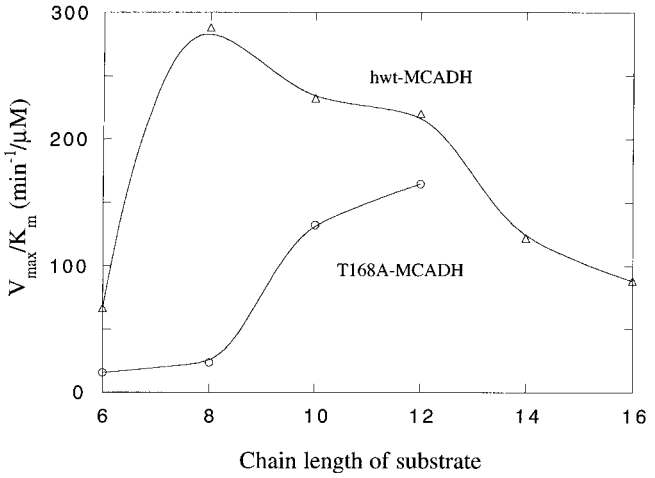

Figure 6 Chain-length dependence of V_{max}/K_m for T168A-MCADH, and comparison with hwt-MCADH

Conditions were as described in the legend to Figure 4. The values for hwt-MCADH were taken from [2].

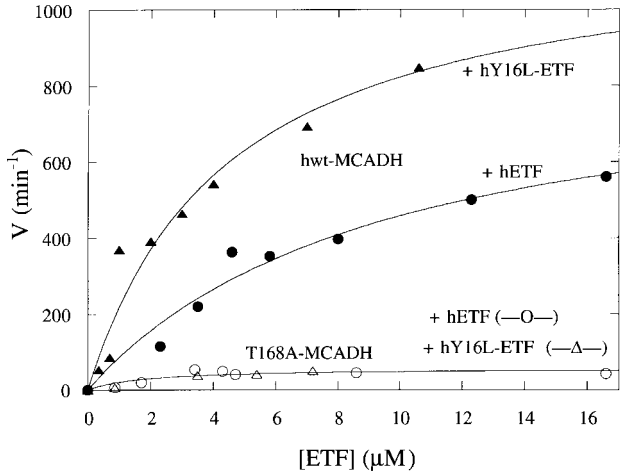

Figure 7 Activity of T168A-MCADH measured with the fluorimetric assay by using human ETF or human Y16L-ETF, and comparison with hwt-MCADH

Samples containing 20 mM Tris, pH 8, 80 μM C_8-CoA and 1–16 μM human ETF at 25 °C were made anaerobic (see the Experimental secion) and the reaction was started by the addition of enzyme (final concentrations 1–10 nM). The decrease in fluorescence emission at 500 nm was monitored (excitation was at 435 nm). The V_{max} obtained with human ETF as electron acceptor was estimated as approx. 880 ± 143.8 min^{-1} (mean $\pm$ S.E.M.) and approx. 60 ± 11.6 min^{-1} for hwt-MCADH and T168A-MCADH; with hY16L-ETF these values were 1100 ± 74.4 and approx. 100 ± 9.8 min^{-1}. The velocity is defined as electrons transferred/ETF–MCADH active centre per min.

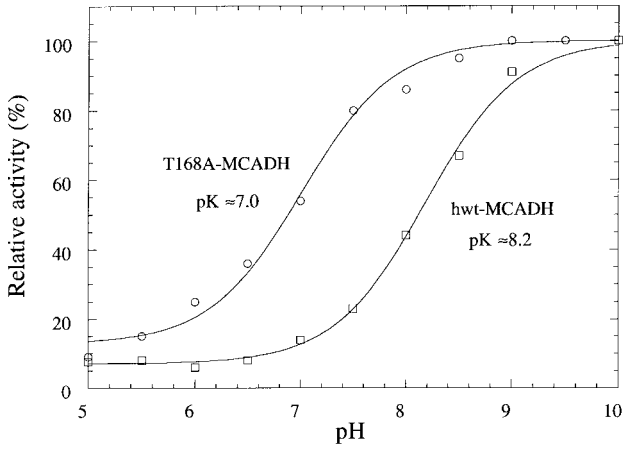

Figure 8 pH dependence of the activity of T168A-MCADH, and comparison with hwt-MCADH

The velocities are standardized to 100% for comparison. Activity (ferricenium assay) was measured by incubation with 100 μM C_8-CoA in 250 mM KCl/50 mM acetate/HCl, Mes/KOH, Hepes/KOH, Tricine/KOH, Tris/HCl or glycine/HCl buffers adjusted to the pH values shown [the activity was found not to depend on the ionic strength (KCl) of the medium in the range 0.01–0.1 M]. The maximum/minimum velocities extrapolated to pH $>$ pK and pH $<$ pK were 180/20 min^{-1} (S.E.M. 3.4/4.2) for T168A-MCADH and 5800/430 min^{-1} for hwt-MCADH; they were obtained from the pH–activity fits shown with the standard velocity–pH-dependence equation. The curve for MCADH is shown for comparison and was taken from Ghisla et al. [30].

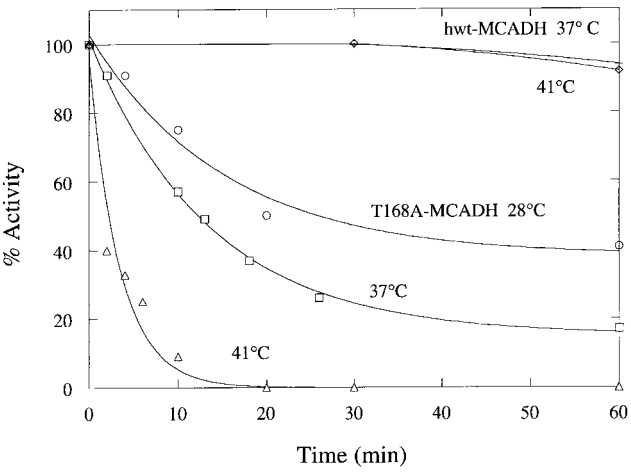

Figure 9 Dependence of incubation time at different temperatures of the activity of human wild-type MCADH and T168A-MCADH

Enzyme (5 μM) was incubated in 0.1 M potassium phosphate, pH 7.6. All activities were measured with the ferricenium assay [35].

Stability and thermal effects

From its behaviour after expression in crude *E. coli* extracts [7] and during purification, T168A-MCADH was assumed to be much less stable than hwt-MCADH. This was confirmed by the time-dependent decrease in activity at various temperatures shown in Figure 9. It should be noted that at 41 °C T168A-MCADH was inactivated completely within approx. 20 min, whereas hwt-MCADH was essentially unaffected under the same conditions.

The reductive half-reaction

An important point in dissecting the effect of specific mutations is the study of the rates of isolated steps involved in catalysis. The rate of enzyme flavin reduction is directly linked to substrate dehydrogenation, the chemically most demanding step. The rates of flavin reduction of T168A-MCADH, hwt-MCADH and K304E-MCADH by C_8-CoA were determined by stopped-flow spectrophotometry at pH 7.6 and are listed in Table 1. A comparison of these results indicates that the rate of reduction of the mutant is at most 10% that of hwt-MCADH.

DISCUSSION

T168A-MCADH is the first acyl-CoA dehydrogenase mutant occurring in humans in which the mutation has been shown to affect properties of the enzyme that are important for the chemistry of catalysis. All other mutants studied so far are thought to be defective in the folding and/or assembly of a functional tetramer. The two effects seem to be cumulatively operative in the present case too, because the quantity of tetrameric active enzyme is severely decreased in the presence or absence of co-overexpressed GroESL [7]. The molecular reasons for the decreased thermal stability, which is apparent from the experiments shown in Figure 9, and the probably related low expression of active tetrameric enzyme can only be inferred at present: it is likely that the hydrogen bridge between the Thr-168-OH and the flavin N(5) serves both in anchoring the cofactor and in modulating its activity. Inactivation would thus result from a loss of the FAD and the consequent lability of the apoenzyme. The rapid loss of activity at temperatures near 40 °C compared with the relative stability of hwt-MCADH could be an important factor determining the consequences of this mutation in stressed individuals, as has been inferred also for K304E-MCADH. The adverse effects of the mutation could also be augmented by the pK shift, and the shift in specificity towards 'long-chain' substrates as documented in Figures 8 and 6.

Of particular mechanistic interest also are the effects of the absence of the Thr-168-OH–N(5) hydrogen bridge on the stability of the (intermediate, blue) neutral semiquinone. Because the transfer of electrons in the oxidative half-reaction obligatorily involves the semiquinone, its destabilization could also be a factor influencing activity. This could be related to the lowered activities found with ETF as acceptor because the latter must interact with the semiquinone form of MCADH [43]. The three-dimensional structure [13] of MCADH shows that Glu-376-COO^-, the substrate α-H and β-H bonds and the flavin N(5) (empty) π-orbital are aligned precisely in the orientation expected for hydride transfer. A function of the Thr-168-OH–N(5) interaction would thus set up the correct alignment of the reaction partners mentioned, and its absence could explain the marked decrease in the rate of substrate dehydrogenation.

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