

## Characterization of human and pig kidney long-chain-acyl-CoA dehydrogenases and their role in $\beta$ -oxidation

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Long-chain-acyl-CoA dehydrogenase (LCADH) has been produced by recombinant techniques from the human cDNA and purified after expression in *Escherichia coli*. Pig kidney LCADH was purified using an optimized method which also produces apparently pure short-chain-acyl-CoA dehydrogenase (SCADH) and medium-chain-acyl-CoA dehydrogenase (MCADH) in good yields. LCADH from both sources has a maximal turnover rate ( $V_{\max}$  of 650–700  $\text{min}^{-1}$  at pH 7.6) with the best substrates, which is approximately fivefold higher than reported previously. The human enzyme has an approximately fivefold higher  $K_m$  compared with the pig kidney enzyme with substrates of chain length from  $C_{10}$  to  $C_{18}$  and a significantly different dependence of  $V_{\max}$  on the chain length. Pig kidney LCADH has a similar  $V_{\max}/K_m$  with  $C_{10}$  to  $C_{14}$  substrates as MCADH does with  $C_6$  to  $C_{10}$  substrates. Recombinant human LCADH, however, is significantly less efficient (approximately fourfold with  $C_{12}$ ) than purified pig kidney enzyme. We conclude that human LCADH is either quantitatively less important in  $\beta$ -oxidation than in the pig, or that post-translational modifications, not present in the recombinant human enzyme, are required to optimize human LCADH activity. Our results demonstrate that LCADH is as important as the other acyl-CoA dehydrogenases in fatty acid oxidation at physiological, mitochondrial pH with optimal substrates of chain length  $C_{10}$ – $C_{14}$ . The extent of the LCADH-flavin cofactor reduction observed with most substrates and the rate of the subsequent reoxidation with oxygen are markedly different from those found with human medium chain acyl-CoA dehydrogenase. Both LCADH are inactivated by the substrate analogue 2-octynoyl-CoA, possibly via covalent modification of Glu261, the active-site residue involved in deprotonation of the substrate ( $\alpha$ )C-H.

**Keywords:**  $\beta$ -oxidation; long chain; acyl-CoA; dehydrogenase; electron-transferring flavoprotein.

Long-chain-acyl-CoA dehydrogenase (LCADH) is a member of a family of enzymes (Beinert, 1963; Nandy et al., 1996a; Tanaka and Indo, 1992), which catalyze in sequence the desaturation of fatty acyl-CoA conjugates. It differs from the other dehydrogenases of the family in its specificity ranging from medium( $C_8$ )-to long-chain( $C_{18}$ ) acyl-CoA substrates as well as in important parts of its sequence (Nandy et al., 1996a). As in isovaleryl-CoA dehydrogenase, the specific glutamate (conjugate) base which functions in the abstraction of the substrate ( $\alpha$ )C-H as a proton, i.e. in the initiation of catalysis, was determined to reside at position 261 (position 255; medium-chain-acyl-CoA dehydrogenase MCADH numbering; Djordjevic et al., 1994; Mohsen and Vockley, 1995). This position is located on helix G (Kim et al., 1993) and is different from that encountered within

all other acyl-CoA dehydrogenases, i.e. position 376 in MCADH (Nandy et al., 1996b).

LCADH from various sources has a low activity when compared with other members of the family, e.g. MCADH. The differences in  $V_{\max}$  between these two enzymes vary with substrate chain length, but, for substrates with highest activity with each enzyme, the  $V_{\max}$  values for rat liver MCADH are approximately fivefold higher than for rat liver LCADH (Ikeda et al., 1985). Taken at face value this would suggest that the activity of MCADH with  $C_{10}$ -CoA and  $C_{12}$ -CoA (the best substrates of LCADH) would be higher than that of LCADH with the same substrates. The information about chain-length dependence of activity parameters also is uncertain due largely to the use of different assay systems by different groups (Beinert, 1963; Davidson and Schulz, 1982; Dommès and Kunau, 1984; Ikeda et al., 1985) and different degrees of purity of enzymes.

The seeming occurrence of genetic defects of LCADH was reported (Hale et al., 1985, 1990); although these defects are now known to originate from mutations of the related enzyme very-long-chain-acyl-CoA dehydrogenase (VLCADH; Strauss et al., 1995). This was a further stimulus to carry out a basic characterisation of human LCADH, which, now, can be obtained by means of recombinant techniques (Djordjevic et al., 1994), and thus lay the basis for a better assessment of its role *in vivo*. We observed that the activity of LCADH isolated either from recombinant sources or from pig kidney under optimal condi-

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**Abbreviations.**  $C_x$ -CoA, straight chain acyl-CoA thioesters, where X denotes the length of the carbon chain; hwt- and pkMCADH, human wild-type and pig kidney medium-chain acyl-CoA dehydrogenase; hwt- and pkLCADH, human wild-type and pig kidney long-chain acyl-CoA dehydrogenase; pkSCADH, pig kidney short-chain acyl-CoA dehydrogenase; ETF, pig kidney electron-transfer flavoprotein.

**Enzymes.** Medium-chain-acyl-CoA dehydrogenase (EC 1.3.99.3); short-chain-acyl-CoA dehydrogenase (EC 1.3.99.2); long-chain-acyl-CoA dehydrogenase (EC 1.3.99.13).

tions is significantly higher than previously reported and is comparable to that of MCADH (Kieweg et al., 1997). Furthermore the activity versus chain-length profile of human wild-type (hwt)LCADH is different from that of pig kidney (pk)LCADH and from that of LCADH from other sources reported earlier (Davidson and Schulz, 1982; Dommès and Kunau, 1984).

## MATERIALS AND METHODS

**Materials and reagents.** These were from the companies listed: coenzyme A (Waldhof), ferricenium hexafluorophosphate, free fatty acids and 2-octynoic acid (Aldrich), glucose oxidase and catalase (Boehringer Mannheim), fast flow Sepharose Q (Pharmacia), fast flow hydroxyapatite (Fluka), yeast extract, peptone and isopropyl-thio- $\beta$ -D-galactoside (Roth), molecular mass standards for SDS/PAGE and gel filtration (Bio-Rad). Acyl-CoAs, 2-octynoyl-CoA and 3-thiooctanoyl-CoA were prepared using the mixed anhydride procedure (Bernert and Sprecher, 1977) and purified by preparative HPLC. pNO<sub>2</sub>- and pCN-phenyl acetyl-CoA were synthesized as will be described later (Vock, P. and Ghisla, S., unpublished results). Analytical and preparative HPLC was performed using C<sub>18</sub> reverse-phase columns LiChroCART 250-4 (Merck) equipped with a diode array detector (Kontron 440).

**Miscellaneous.** Solutions of acyl-CoA substrates and analogues in 10 mM potassium phosphate, pH 6.0, were standardized spectrophotometrically with absorption coefficients of 15.4 mM<sup>-1</sup> cm<sup>-1</sup> for fatty acyl-CoAs at  $\lambda = 260$  nm, 20.9 mM<sup>-1</sup> cm<sup>-1</sup> at  $\lambda = 260$  nm for 2-octynoyl-CoA (Freund et al., 1985) and 16 mM<sup>-1</sup> cm<sup>-1</sup> for 3-thiooctanoyl-CoA at  $\lambda = 260$  nm (Lau et al., 1988). Unless otherwise stated, all spectrophotometric measurements were performed at 25°C in 50 mM potassium phosphate, pH 7.6, containing 10% glycerol, using a Kontron Uvikon 930 spectrophotometer. All absorbance spectra were corrected for dilution as appropriate. Enzymes were assayed either with ferricenium hexafluorophosphate as the terminal electron acceptor (Lehman et al., 1990), (Kontron Uvikon 820 spectrophotometer), or using the electron-transfer flavoprotein (ETF) fluorescence assay (Frerman and Goodman, 1985; Kontron SFM 25 fluorimeter). Absorption coefficients for pk-LCADH and hwtLCADH ( $\lambda_{max}$  in the 450-nm region) were determined upon extraction with trichloroacetic acid based on an  $\epsilon$  of 11.3 mM<sup>-1</sup> cm<sup>-1</sup> for free FAD at 448 nm. The averaged values from three determinations each are 15.4  $\pm$  0.2 mM<sup>-1</sup> cm<sup>-1</sup> for pkLCADH and 14.4  $\pm$  0.3 mM<sup>-1</sup> cm<sup>-1</sup> for hwtLCADH. SDS/PAGE was with 4%/12% polyacrylamide gels, staining with Serva blue R. For N-terminal sequencing, 15- $\mu$ g portions of each purified enzyme were subjected to 7–10 cycles on a Applied Biosystems 477A instrument. Native molecular mass was estimated on a Superdex pg 200 (XK26/60) column (100 mM sodium phosphate, pH 7.6 at 4°C, 1 ml/min; standards: dextran, ferritin, aldolase, ovalbumin and myoglobin).

**Expression and growth of host cells of recombinant human LCADH.** The expression plasmid pET11a-LCAD (Dong, J. J. and Strauss, A. W., unpublished results) was transformed into *Escherichia coli* strain BL21(DE3). The cells were grown in 40-l cultures in 2YT medium with 100  $\mu$ g ampicillin/ml at 28°C to an A<sub>600</sub> of 0.9–1.0. Expression of hwtLCADH was induced by addition of isopropyl-thio- $\beta$ -D-galactoside to a final concentration of 0.1 mM and the cells were harvested 17 h after induction.

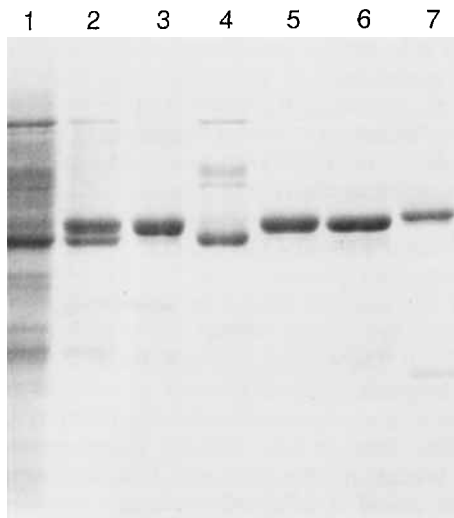
**Isolation and purification of recombinant human LCADH.** The purification procedure of human LCADH (135 g of wet cell paste from 20 l *E. coli* cell culture) was adapted from the method described by Kieweg et al. (1997) for recombinant

human MCADH. Unless otherwise stated, all operations were performed at 0–4°C and all buffers contained 10% glycerol. Content of protein in fractions was estimated spectrophotometrically at 280 nm, LCADH activity (ferricenium assay) was tested using C<sub>12</sub>-CoA. Harvested cells were centrifuged at 8400 g for 15 min, and the pellet was resuspended in 350 ml buffer containing 20 mM Tris/HCl, pH 7.8, 50 mM sucrose, 10 mM EDTA and 1  $\mu$ M FAD. The cells were sonicated 3 times for 5 min and centrifuged 20 min at 16500 g. The supernatant was fractionated at 40–80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and the pellet was dialyzed against 50 mM Tris/HCl, pH 7.8, 10 mM EDTA, 4  $\mu$ M FAD. The dialyzed sample was loaded on a Sepharose Q column (3 cm $\times$ 40 cm, bed volume 280 ml, flow 4 ml/min), equilibrated with 50 mM Tris/HCl, pH 7.8, 10 mM EDTA and 1  $\mu$ M FAD. LCADH binds weakly to the Sepharose Q, and was eluted with a NaCl gradient (0 to 300 mM in 300 min, 300 mM to 600 mM in 60 min). LCADH-containing fractions elute at 50–100 mM NaCl. They were pooled, precipitated with 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, the pellet was resuspended in, and dialyzed against, 20 mM Tris/HCl, pH 7.8, containing 0.5 mM EDTA and 4  $\mu$ M FAD. The solution was loaded on a second Sepharose Q column (50 mM Tris/HCl, pH 7.8, 0.5 mM EDTA and 1  $\mu$ M FAD) and the same elution procedure as above was applied. LCADH elutes as a narrow, yellow band at 150–200 mM NaCl. Pooled fractions were precipitated with 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The pellet was dialyzed (10 mM potassium phosphate, pH 7.8, 0.5 mM EDTA, 4  $\mu$ M FAD) and the solution loaded on a hydroxyapatite column (2 cm $\times$ 30 cm, bed volume 95 ml, flow 1 ml/min, same buffer, but 1  $\mu$ M FAD). The enzyme was eluted with a linear 50 mM to 500 mM phosphate gradient (50 mM to 500 mM in 180 min, started after elution of unbound proteins). LCADH eluted as an intense yellow band at 330–400 mM phosphate. Active fractions were brought to 10 mg/ml with Amicon Centripreps (molecular-mass cut-off 30 kDa) and stored at –196°C.

**Purification of pig kidney LCADH.** The pkLCADH was co-isolated during purification of pkMCADH. Batch material (730 ml) obtained during the procedure described by Gorelick et al. (1982) was applied to a Sepharose Q column (5 cm $\times$ 56 cm, bed volume 1100 ml, flow 5 ml/min, 50 mM potassium phosphate, pH 7.2, containing 0.3 mM EDTA, 1  $\mu$ M FAD). LCADH-containing fractions were separated from MCADH using a linear gradient (50 mM to 400 mM phosphate in 480 min), started after elution of unbound protein. The first yellow fractions eluting at 310 mM to 350 mM phosphate contained a mixture of pkLCADH and pkSCADH, as judged by assays using C<sub>4</sub>-, C<sub>8</sub>-, and C<sub>14</sub>-CoA. MCADH elutes at 370–400 mM phosphate and was further purified according to Gorelick et al. (1982). For a complete separation of pkLCADH and pkSCADH two chromatography steps on hydroxyapatite are necessary. The first (hydroxyapatite I, column 3 cm $\times$ 30 cm, bed volume 210 ml, flow 1.5 ml/min, linear gradient during 240 min, 50 mM to 500 mM phosphate, 0.3 mM EDTA, 1  $\mu$ M FAD) was at pH 7.2. Three pools were obtained, the first contains mainly pkSCADH (Fig. 1); the second is a mixture of already fairly enriched pkSCADH and pkLCADH, and the third consists of pkLCADH at a purity of at least 70% (A<sub>280</sub>/A<sub>445</sub>  $\approx$  12). The second pool from this step was then applied to the second column (hydroxyapatite II, 2 cm $\times$ 30 cm, bed volume 95 ml, flow 1 ml/min, equilibrated with 100 mM potassium phosphate, pH 7.8, 0.3 mM EDTA, 1  $\mu$ M FAD, 10% glycerol). Protein was eluted with a linear phosphate gradient (100 mM to 500 mM in 240 min, started after elution of unbound proteins). pkSCADH elutes first at 180–280 mM phosphate, pkLCADH follows at 300–420 mM phosphate and was concentrated with Amicon Centripreps (molecular-mass cut-off 30 kDa) to 5 mg/ml and stored in liquid nitrogen without apparent loss of activity for weeks.

**Table 1. Summary of purification of recombinant human long-chain-acyl-CoA dehydrogenase.** The purification was started from the amount of bacterial paste obtained from 20 l of *E. coli* culture. Protein concentration was estimated from the absorbance at 280 nm or, for pure samples, that at 445 nm of the FAD cofactor. Activities were determined with the standard ferricenium assay using  $C_{10}$ -CoA (100  $\mu$ M) as substrate (see Materials and Methods). 1 U is 1  $\mu$ mol ferricenium reduced/min.

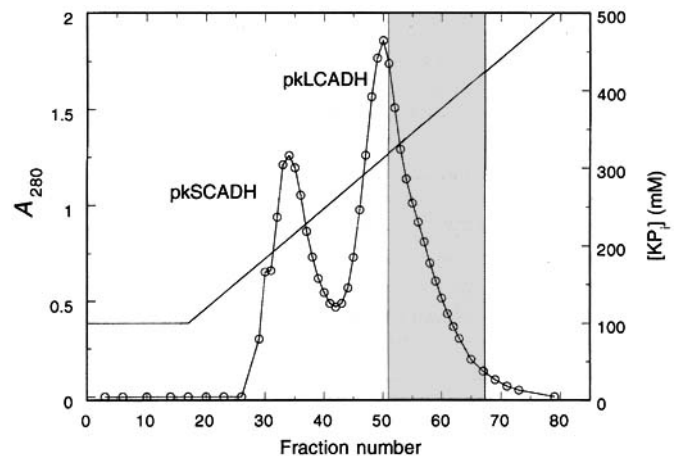
Purification step	Total protein	Total activity	Specific activity	Absorbance ratio $A_{275}/A_{444}$	Purification	Yield
	mg	U	U/mg		-fold	%
Crude extract	17 350	3080	0.18	—	1	100
40–80% ammonium sulfate precipitation	7960	2240	0.28	—	1.6	72.7
Q-Sepharose I	2130	2170	1.02	26	5.7	70.3
Q-Sepharose II	612	2080	3.40	13.8	19.1	67.3
Hydroxyapatite	69	1054	15.27	7.8	84.9	34.2



**Fig. 1. Comparison of various acyl-CoA dehydrogenases on SDS/PAGE.** Lanes 1, 2 and 3 are pooled fractions obtained from the first hydroxyapatite column (hydroxyapatite I). Lane 1 is from the fractions eluting first, and containing mainly pkSCADH; lane 2 is an intermediate fraction and corresponds to the material used for the second hydroxyapatite column (hydroxyapatite II) the elution profile of which is shown in Fig. 2. Lane 3 is from the pool of later fractions from hydroxyapatite I, and contains pkLCADH already free from other acyl-CoA dehydrogenases. Lane 4 represents pkSCADH (cf. lane 1) upon further purification on Mono-Q. Lane 5 is pkLCADH obtained from the pool corresponding to lane 2, and after final purification on hydroxyapatite II (shaded area in Fig. 2, see Materials and Methods for details). Lane 6 and 7 show, for comparison, purified hwtLCADH and hwtMCADH, respectively.

## RESULTS

**Purification.** The hwtLCADH was isolated with a yield of 50–55 mg (35%)/100 g wet cell paste obtained from 40 l *E. coli* cultures (Table 1). The presence of 10% glycerol in all purification steps increased the stability of the protein and prevented the loss of FAD when the concentration of LCADH was less than 5  $\mu$ M. Purified hwtLCADH is a single band of approximately 44 kDa on SDS/PAGE (Fig. 1), without detectable amounts of contaminating proteins. The approximate molecular mass of the native protein was estimated as approximately 180 000 Da by calibrated gel filtration. The N-terminal sequence was (M)SGGEER... matching that predicted from the cDNA (Indo et al., 1991). For the isolation of pkLCADH, the procedure for the purification of pkMCADH (Gorelick et al., 1982) was modified by substituting in the first step Whatman DE-52 with Sepharose Q and by eluting with a NaCl gradient (see Materials and Methods). By this procedure, a mixture of pkLCADH and pkSCADH



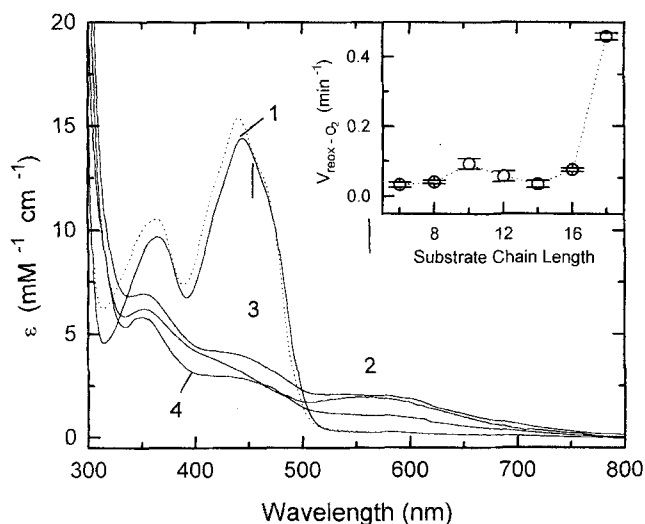
**Fig. 2. Separation of pig kidney LCADH from pig kidney SCADH by chromatography on hydroxyapatite.** Fractions corresponding to lane 2 in Fig. 1 were applied to the hydroxyapatite II column (see Materials and Methods section for details). Elution was followed by recording the protein absorbance  $A_{280}$  ( $\circ$ ) and was obtained using the linear KP gradient shown. The shaded area indicates the fractions of pkLCADH which are essentially free of pkSCADH (see lane 5, Fig. 1) and which were used in the present experiments. The pool of fractions 30–35 similarly yielded apparently homogeneous pkSCADH.

was obtained, which was essentially free of MCADH. At this stage of purification and from the specific activities determined with the purified enzymes, we estimate the quantities of pkSCADH as 30–40 mg, of pkLCADH as approximately 100 mg and of pkMCADH as 250–300 mg, which should be a fair representation of the ratios present in crude extracts from approximately 4 kg of pig kidney cortex. Further purification was carried out by chromatography first at pH 7.2 and then at pH 7.8 over hydroxyapatite (hydroxyapatite I and II, see Materials and Methods). A typical separation of pkLCADH from pkSCADH, is shown by the elution profile in Fig. 2. The purified enzyme shows a single band at approximately 44 kDa on SDS/PAGE indistinguishable from the human enzyme (Fig. 1). The N-terminal sequence for pig kidney LCADH was SGGEERLES..., revealing a conservative S→T change at position 9, compared with hwtLCADH (Indo et al., 1991).

**Spectral properties and reaction with substrates.** The ultraviolet–visible spectra of oxidized pkLCADH and hwtLCADH are shown in Fig. 3. Notably the flavin absorbance of pkLCADH in the visible range is more resolved with a distinct shoulder at 465 nm. Since this shoulder is not present in the spectra of pkSCADH and pkMCADH, it is helpful in differentiating these enzymes during purification. The ratio  $A_{275}/A_{444}$  in MCADH is

**Table 2. Summary of catalytic properties of pig kidney and human wild-type LCADH and comparison with human wild-type MCADH.** Enzyme activity was determined with the ferricenium assay at  $[\text{FePF}_6] = 200\text{--}300\ \mu\text{M}$ , in 50 mM potassium phosphate, pH 7.6 at 25°C (Lehmann et al., 1990).  $V_{\text{max}}$  is expressed as turnover numbers ( $\text{FePF}_6$  reduced  $\cdot \text{FAD}^{-1} \cdot \text{min}^{-1}$ ). Reoxidation rates ( $V_{\text{reox-O}_2}$ ) were determined in air-saturated 50 mM potassium phosphate, pH 7.6, containing 10% glycerol at 25°C. Upon reduction with 1 mol substrate, the time dependence of the reoxidation was followed spectrophotometrically at 444 nm and  $V_{\text{reox-O}_2}$  was estimated from the fits of the numerical average of 3–4 absorbance traces using an equation with a single exponential.  $V_{\text{max}}$  values at pH 8.0 were determined with the ferricenium assay (Lehmann et al., 1990) in 50 mM Tris/HCl, 250 mM KCl at 25°C.  $V_{\text{max}}$  values are expressed as turnover numbers ( $\text{FePF}_6$  reduced  $\cdot \text{FAD}^{-1} \cdot \text{min}^{-1}$ ).  $V_{\text{max}}$  data were obtained from 2–4 single determinations at  $[\text{substrate}] = 0.15\ \text{mM}\text{--}0.25\ \text{mM}$ , i.e.  $\gg K_m$ . The values in brackets indicate the maximal scatter of the data.  $K_m$  was estimated from secondary plots of  $V_{\text{max}}$  at 5–6 different substrate concentrations, and according to Michaelis-Menten; the values in brackets are the standard errors obtained from such plots.  $V_{\text{max}}$  values obtained by the two methods vary within 10%.

Substrate	Pig kidney LCADH			Human wild type LCADH					Human wild type MCADH (pH 8) $V_{\text{max}}$
	$V_{\text{max}}$	$K_m$	$V_{\text{max}}/K_m$	$V_{\text{max}}$	$K_m$	$V_{\text{max}}/K_m$	$V_{\text{reox-O}_2}$	$V_{\text{max}}$ (pH 8)	
C <sub>4</sub> -CoA	50 (±5)	n.d.	—	≈0	—	—	—	—	—
C <sub>6</sub> -CoA	215 (±15)	6.3 (±1)	34 (8)	380 (±5)	29 (2)	13 (±1)	0.03 (±0.01)	—	—
C <sub>8</sub> -CoA	320 (±5)	3.3 (±0.5)	100 (±15)	450 (±5)	8 (±1)	56 (±5)	0.04 (±0.01)	—	—
C <sub>10</sub> -CoA	650 (±5)	2.2 (±0.2)	300 (±30)	700 (±30)	10 (±2)	70 (±15)	0.09 (±0.01)	580 (±60)	700 (±50)
C <sub>12</sub> -CoA	620 (±5)	1.5 (±0.2)	410 (±70)	700 (±15)	7 (±1)	100 (±20)	0.06 (±0.01)	1380 (±80)	420 (±40)
C <sub>14</sub> -CoA	570 (±5)	2.3 (±0.1)	250 (±10)	400 (±20)	10 (±2)	40 (±10)	0.03 (±0.01)	940 (±70)	360 (±30)
C <sub>16</sub> -CoA	565 (±15)	3.6 (±0.5)	160 (±30)	360 (±10)	14 (±2)	26 (±5)	0.08 (±0.01)	—	—
C <sub>18</sub> -CoA	585 (±10)	3.7 (±0.4)	160 (±20)	320 (±10)	8 (±1)	40 (±10)	0.46 (±0.01)	—	—
C <sub>20</sub> -CoA	70 (±10)	n.d.	—	45 (±5)	n.d.	—	—	—	—



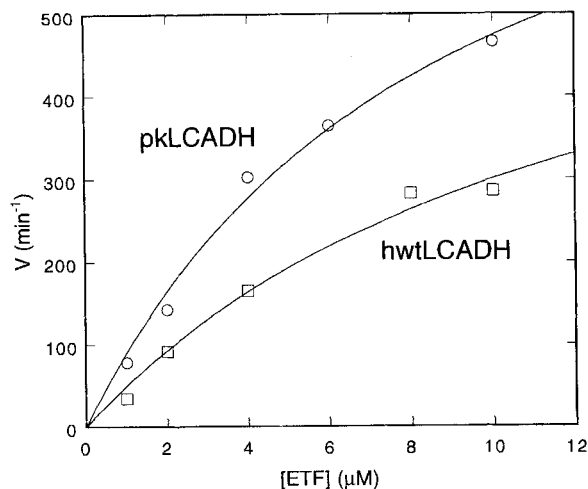
**Fig. 3. Absorbance spectra of pig kidney LCADH, human wild-type LCADH and reaction spectra with substrates of varying chain length.** The enzymes were approximately 10  $\mu\text{M}$  in 50 mM potassium phosphate, pH 7.6, containing 10% glycerol at 25°C. pkLCADH: (·····), (Ratios  $A_{275}/A_{365}/A_{441} = 8.4:0.69:1.0$ ;  $\epsilon_{444\ \text{nm}} = 15.4$ ); hwtLCADH: Curve 1. (Ratios  $A_{275}/A_{365}/A_{441} = 8.4:0.69:1.0$ ;  $\epsilon_{444\ \text{nm}} = 14.4$ ). Curves 2, 3 and 4 were recorded upon aerobic addition of approximately 7 mol of C<sub>8</sub>-, C<sub>12</sub>-, and C<sub>16</sub>-CoA/mol hwtLCADH. With pkLCADH similar results were obtained (data not shown). The inset shows the chain-length dependence of the rate of reoxidation by oxygen ( $V_{\text{reox-O}_2}$ ) obtained from the absorbance changes ( $\Delta A_{444}$ ) occurring subsequent to reduction with approximately 1 mol of C<sub>n</sub>-CoA/mol; the data points were obtained from fits of the  $\Delta A_{444}$ /time traces using a single exponential equation and the bars indicate the corresponding standard errors (see Table 2 for rates).

approximately 5 [calculated from the content of aromatic amino acids (Thorpe et al. (1979))] and that of LCADH is approximately 7–8 (legend to Fig. 3, calculated value approximately 7). Aerobic addition of acyl-CoA substrates of varying chain length to hwtLCADH leads to extensive bleaching of the oxidized flavin spectrum and is accompanied by appearance of a

long-wavelength, charge-transfer band, typical for acyl-CoA dehydrogenases (Beinert, 1963; Thorpe et al., 1979) (Fig. 3). A similar extent of reduction was found for pkLCADH. When the same type of experiment is carried out using approximately equimolar concentrations of substrates, the reduction phase is followed by a much slower phase of reoxidation, which leads essentially to complete reformation of the (original amount of) oxidized enzyme. The rate of this reoxidation is essentially independent of the length of the substrate aliphatic chain from C<sub>6</sub> to C<sub>16</sub> (Fig. 3). This contrasts with what has been found with hwtMCADH, where the rates of reoxidation are approximately 10-fold slower and exhibit a marked dependence on the substrate chain length with a break at C<sub>10</sub> (Nandy et al., 1996b).

#### Catalytic properties of human wild-type and of pig kidney LCADH.

Steady-state kinetic parameters for both enzymes (10–25 nM) and various substrates were determined using the ferricenium hexafluorophosphate assay (Lehman et al., 1990; Table 2). It should be noted that the ferricenium assay, while being the most convenient one, has not been compared in its efficiency with the fluorimetric ETF assay (Frerman and Goodman, 1985). The latter is assumed to best reflect the situation *in vivo*, because ETF is the natural electron acceptor (Crane et al., 1956). We have studied this system using the best substrate for LCADH, C<sub>12</sub>-CoA at a saturating concentration ( $[\text{C}_{12}\text{-CoA}] \approx 10 \cdot K_m$ , Table 2). The interaction between LCADH and pig kidney ETF (Fig. 4) follows saturation kinetics, implying formation of a complex prior to electron transfer. pkLCADH and hwtLCADH have the same apparent  $K_m$  for this interaction (legend to Fig. 4). The higher velocity for pkLCADH may reflect a more effective interaction with the ETF from the same species. Comparison of the  $V_{\text{max}}$  values obtained using ETF with those from the ferricenium assay (Table 2) shows that both acceptors are equivalent with hwtLCADH, and that the difference of approximately 30% for pkLCADH is at the limit of significance. This indicates that ferricenium is adequate for assaying LCADH. In Fig. 5, the substrate chain length dependencies of  $V_{\text{max}}$  and  $V_{\text{max}}/K_m$  of hwtLCADH and pkLCADH are compared and contrasted with that of hwtMCADH [data from Kieweg et al.



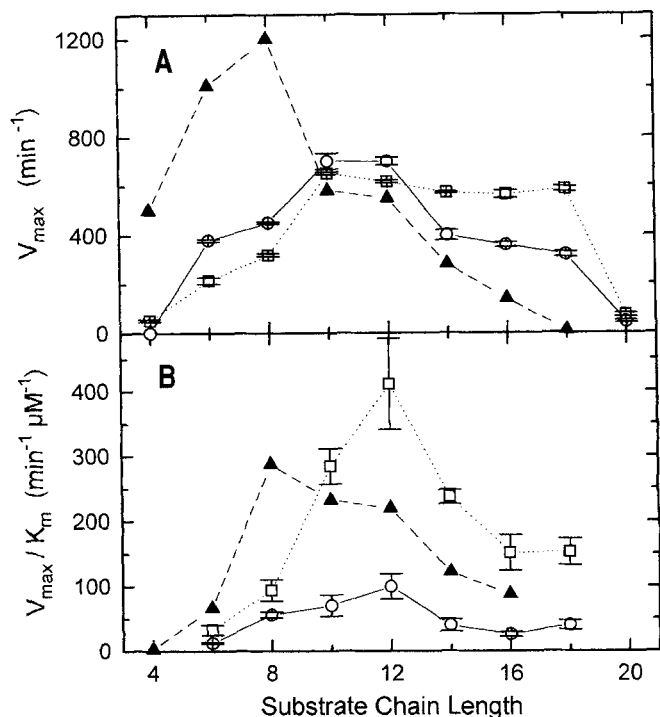
**Fig. 4.** Dependence of human wild type LCADH (—□—) and pig kidney LCADH (—○—) activity from the pig kidney ETF concentration. Conditions: [hwtLCADH] or [pkLCADH] were approximately  $1 \times 10^{-9}$  M and  $C_{12}$ -CoA was kept invariant at a saturating concentration of 80  $\mu$ M in 20 mM Tris, pH 8.0, 10% glycerol and at 25°C (see Materials and Methods for further details). The velocities were obtained from the initial slope of fluorescence decrease ( $\lambda_{exc.} = 435$  nm,  $\lambda_{emiss.} = 490$  nm). The values of  $K_m$  and of  $V$  extrapolated to  $[ETF] = \infty$  are approximately  $9 (\pm 2)$   $\mu$ M and  $900 \pm 140$   $\text{min}^{-1}$  ( $R^2 = 0.99$ ), respectively, approximately 12  $\mu$ M and  $675 \pm 170$   $\text{min}^{-1}$  ( $R^2 = 0.98$ ) for pkLCADH and hwtLCADH. The curves represent the best fits through the data points using the Michaelis-Menten equation.

(1997)]. The relatively broad substrate specificity of pkLCADH and hwtLCADH is noteworthy and complements that of MCADH. The pig kidney enzyme also is superior in its catalytic efficiency compared with hwtLCADH because of lower  $K_m$  values throughout (Table 2).

The  $V_{max}$  values for pkLCADH and hwtLCADH ( $\approx 700$   $\text{min}^{-1}$ ) determined in the present work approach those of MCADH ( $\approx 1100$   $\text{min}^{-1}$ ; Kieweg et al., 1997; Peterson et al., 1995). With  $C_{12}$ -CoA, one of the best substrates for LCADH (Fig. 5, Table 2),  $V_{max}$  values are approximately fivefold higher compared with those reported previously by Djordjevic et al. (1994). A comparison with catalytic values of LCADH published earlier is not straightforward, since most of the data were obtained using the (less efficient) phenazine methosulfate/dichlorophenolindophenol or other assays, instead of the ferricenium or ETF assay (Dommes and Kunau, 1984; Ikeda et al., 1985). Using the phenazine methosulfate/dichlorophenolindophenol assay, Ikeda et al. (1985) reported for rat liver LCADH ( $C_{14}$ -CoA,  $V_{max}$  6.0  $\text{s}^{-1}$ )  $V_{max}$  values approximately 20% of those of the corresponding MCADH ( $C_6$ -CoA,  $V_{max}$  31.2  $\text{s}^{-1}$ ). However, Dommes and Kunau (1984) found for LCADH from bovine liver using iodonitrotetrazolium chloride and meldola blue as electron acceptor, 36-fold more activity compared with MCADH.

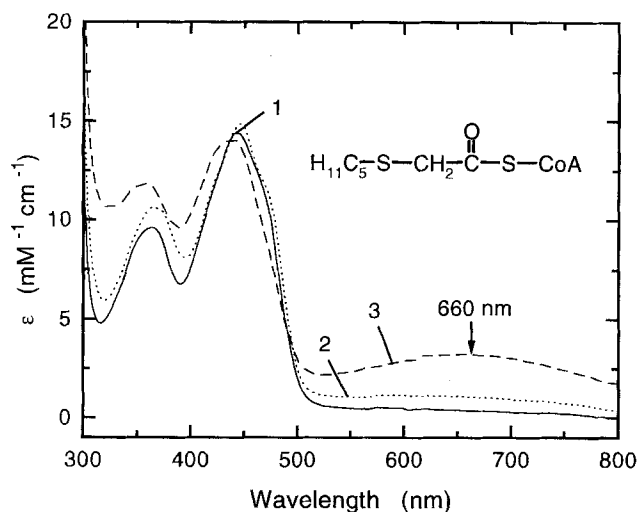
The relevant differences between the pig kidney and human wild-type enzymes are in  $V_{max}$  and  $K_m$ . With both enzymes,  $V_{max}$  is maximal with  $C_{10}$ - $C_{12}$ -CoA (Fig. 5A).  $K_m$  values are approximately fivefold lower in the case of pkLCADH in the range  $C_{10}$ - $C_{18}$  (Table 2). The chain-length profile of the catalytic efficiency  $V_{max}/K_m$  is similar for LCADH from both sources (Fig. 5B), however, it is approximately fivefold lower for hwtLCADH over the  $C_{10}$ - $C_{18}$  range.

**Interaction of human wild-type and pig kidney LCADH with acyl-CoA substrate analogues.** The Glu261 base abstracting the substrate ( $\alpha$ )C-H in LCADH is located on a different seg-



**Fig. 5.** Dependence of catalytic parameters of human wild-type LCADH (—●—), pig kidney LCADH (—■—) and comparison with human wild-type MCADH (—○—) using substrates of varying chain length. (A) shows an activity profile using  $V_{max}$  data obtained with the ferricenium assay (Lehman et al., 1990) under standard conditions and at fixed substrate concentrations (140  $\mu$ M, see Materials and Methods). (B) The term  $V_{max}/K_m$  is plotted versus substrate chain length.  $K_m$  values of LCADH with  $C_4$ -CoA and  $C_{20}$ -CoA could not be obtained due to too low activity. The error bars indicate the data scatter as outlined in the legend to Table 2. The data for hwtMCADH are taken from Kieweg et al. (1997).

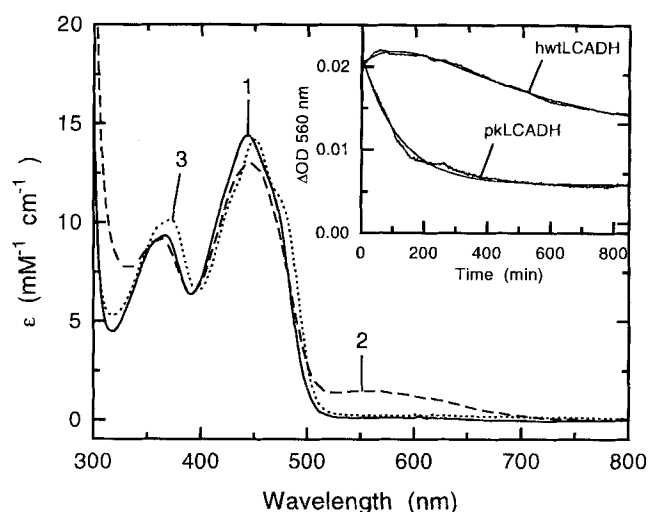
ment of the protein compared with the other members of the family (Glu376 in MCADH; Lee et al., 1996), it was of interest to find out whether this transposition affects the general reactivity of the glutamic residue in question. This has been probed using some substrate analogues, which yield typical reactions with MCADH. 3-Thiooctanoyl-CoA is a redox-inactive substrate analogue (Lau et al., 1988) and a probe for the capacity of the acyl-CoA dehydrogenases to abstract the substrate ( $\alpha$ )C-H. It binds to MCADH and forms a deep green charge-transfer complex with the oxidized flavin ( $K_d \approx 0.5$   $\mu$ M,  $\lambda_{max} = 804$  nm;  $\epsilon_{804} = 8.7$   $\text{mM}^{-1} \text{cm}^{-1}$  at pH 7.6). The effects induced by addition of a 10-fold molar excess of 3-thiooctanoyl-CoA to hwtLCADH and pkLCADH are depicted in Fig. 6. With hwtLCADH, binding is indicated by the perturbation of the oxidized flavin spectrum and only a weak, long-wavelength band is formed, in contrast to the pkLCADH where the band has a  $\lambda_{max}$  of approximately 660 nm and a substantial  $\epsilon_{600}$  approximately 2.9  $\text{mM}^{-1} \text{cm}^{-1}$ . From the spectral changes  $K_d$  values of approximately 12.7  $\mu$ M and 9.4  $\mu$ M can be estimated for hwtLCADH and pkLCADH, respectively, which are approximately 20-fold larger than those observed with MCADH (Lau et al., 1988). 4-Nitrophenylacetyl-CoA is a related aromatic substrate analogue, which also binds to MCADH ( $K_d \approx 23$   $\mu$ M; Engst and Ghisla, 1991), becomes deprotonated at position ( $\alpha$ )C-H, and induces an intense charge-transfer band (Engst and Ghisla, 1991). Addition of this analogue to hwtLCADH at pH 7.6 ( $K_d$  approximately 170  $\mu$ M) induces primarily a perturbation of the oxidized flavin spectrum (data not shown) consistent



**Fig. 6.** Reaction of human wild-type LCADH with the 3-thiooctanoyl-CoA. The enzyme, 10  $\mu\text{M}$  in 50 mM potassium phosphate, pH 7.6, containing 10% glycerol at 25°C (curve 1, —), was incubated with 10 mol of the analogue/mol. Curves 2 (·····) and 3 (---) represent the spectra obtained after addition of 10 mol 3-thiooctanoyl-CoA to 1 mol hwtLCADH and pkLCADH, respectively.

with a simple binding interaction. The extent of long-wavelength absorption formed and of absorbance at approximately 490 nm, which reflects directly formation of the anion of 4-nitrophenylacetyl-CoA, corresponds to deprotonation of at the most 10% of the ligand compared with MCADH. With 4-CN-phenylacetyl-CoA, no deprotonation of the ligand was observed, although binding occurs.

2-Octynoyl-CoA inactivates medium-chain-acyl-CoA dehydrogenase rapidly, stoichiometrically and irreversibly, secondary to covalent reaction with the active-site base, E376-COO<sup>-</sup> (Freund et al., 1985; Powell and Thorpe, 1988). In contrast to this, it was reported that 2-octynoyl-CoA inactivates beef liver long-chain-acyl-CoA dehydrogenase reversibly without covalent modification of the protein moiety (Ankele et al., 1991). In the cases of pkLCADH and hwtLCADH, addition of 1 mol 2-octynoyl-CoA/mol to the oxidized enzymes leads to a rapid ( $k_{\text{obs}} \approx 1.2 \text{ min}^{-1}$ ) formation of a long-wavelength band, centered at 562 nm (Fig. 7). The increase is biphasic, the main first phase being followed by a much slower, second one of minor extent ( $k_{\text{obs}} \leq 0.01 \text{ min}^{-1}$ ). These absorbancies subsequently decrease slowly ( $k_{\text{obs}} \approx 0.004 \text{ min}^{-1}$  and  $\approx 0.009 \text{ min}^{-1}$  for hwt and pkLCADH) and completely. These processes are approximately 10-fold slower than in the case of pkMCADH ( $k_{\text{obs}} \approx 0.06 \text{ min}^{-1}$ , Freund et al., 1985). In contrast to this, with hwtLCADH (but not with pkLCADH) when a fivefold excess of 2-octynoyl-CoA was used, no long-wavelength band was observed; and the flavin absorbance peak of the resulting spectrum was approximately 7 nm red shifted and more resolved (Fig. 7), reflecting the presence of bound ligand. The enzyme activity was followed using C<sub>12</sub>-CoA. When hwtLCADH was incubated with 5 mol 2-octynoyl-CoA/mol, approximately 90% of the original activity was lost immediately, whereas with pkLCADH, the loss was less ( $\approx 65\%$ ). Activity could not be recovered upon ultrafiltration, in contrast to what was reported by Ankele et al. (1991) for the beef liver enzyme and approximately 40% of the theoretically possible amount of free CoA-SH was found in the ultrafiltrate by HPLC analysis. In analogy to the results by Thorpe's group with pkMCADH (Freund et al., 1985), this is compatible with turnover of the inactivator and with covalent modification of the protein.



**Fig. 7.** Reaction of human wild-type LCADH with 2-octynoyl-CoA. The enzyme, 10  $\mu\text{M}$  in 50 mM potassium phosphate, pH 7.6, containing 10% glycerol at 25°C (curve 1, —), was treated with 1 mol 2-octynoyl-CoA/mol. Curve 2 (---) represents the spectrum recorded 5 min after mixing. With pkLCADH the same results were obtained (data not shown). The inset shows time-course of the  $\lambda_{560}$  absorbance observed upon addition of 1 mol of inhibitor to 1 mol hwtLCADH and pkLCADH. Note that a biphasic increase (not observable on the time-scale shown) is followed by a monophasic decrease. The lines are the best fits for triphasic processes with the following rate constants: 1.2  $\text{min}^{-1}$  and 0.01  $\text{min}^{-1}$  for the increasing processes and  $\approx 0.004 \text{ min}^{-1}$  and  $\approx 0.009 \text{ min}^{-1}$  for hwtLCADH and pkLCADH, respectively. Main figure: curve 3 (·····) is the spectrum obtained in a companion experiment immediately upon addition of 5 mol 2-octynoyl-CoA in one portion to 1 mol hwtLCADH.

## DISCUSSION

A puzzling aspect of LCADH from various sources has been their low activity compared with that of MCADH, and the considerable overlap of the substrate chain-length activity spectra of both enzymes. Here we show that both recombinant hwtLCADH and pkLCADH have activities quite similar to those of MCADH (Kieweg et al., 1997; Peterson et al., 1995) and that pkMCADH and pkLCADH are present in comparable quantities in kidneys. The similarity of activities is probably more comparable to the relationship of MCADH and LCADH enzyme activities *in vivo* because we used ETF as acceptor (Fig. 4). Our data suggest that LCADH probably has a significant role in fatty acid  $\beta$ -oxidation *in vivo*, especially with C<sub>10</sub>–C<sub>14</sub> substrates. With C<sub>12</sub>-CoA and C<sub>14</sub>-CoA, the results of Fig. 5 suggest that, in quantitative terms, pkLCADH is more important than MCADH. Our published results (Hainline et al., 1993; Strauss et al., 1996) show remarkably similar patterns of rodent MCADH, LCADH and VLCADH mRNA expression among tissues, with highest levels in heart, brown fat, and vascular smooth muscle and substantial mRNA levels in liver and skeletal muscle. This suggest that all four enzymes are required for the sequential oxidation of long-chain fatty acids.

We conclude, in contrast to previously reported results with LCADH isolated from other sources (Ikeda et al., 1985) that pkLCADH has efficiency with its optimal substrates (C<sub>10</sub>–C<sub>14</sub>-CoA) comparable to that of MCADH with C<sub>6</sub>–C<sub>10</sub>-CoA. This is confirmed by preliminary results (Ghisla et al., 1997) which show that the activities of short-chain, medium-chain, long-chain and very-long-chain-acyl-CoA dehydrogenases are strongly dependent upon pH. The apparent pK values of these effects are, in turn, dependent upon the chain length of the substrate and upon the enzyme. While the pK values in question

vary from 7 to above 9, they are around 8 for the best substrates of a given enzyme. This should be considered in view of a recent report by Lemasters et al. (1995) that the pH in the mitochondrial matrix is also around 8. The possibility that pH is a factor in the regulation of the activities of acyl-CoA dehydrogenases should be seriously considered.

We have compared the activities of hwtMCADH and hwtLCADH with C<sub>10</sub>-, C<sub>12</sub>- and C<sub>14</sub>-CoA at pH 8.0, as opposed to pH 7.6 as has been done routinely in the past. The activities of both enzymes are comparable for C<sub>10</sub>-CoA. However, with C<sub>12</sub>-CoA and C<sub>14</sub>-CoA, the activity of LCADH is superior by factors of approximately 3.2 and 2.6, respectively, compared with that of MCADH. These results support the concept that LCADH is an essential member of the acyl-CoA dehydrogenase family required for oxidation of long-chain fatty acids in the  $\beta$ -oxidation spiral, especially in those tissues with high energy requirements, such as heart and brown fat.

To consider this, we have pursued our goal of finding a practical method for differentiating between LCADH and MCADH enzymes. The results obtained with 2-octynoyl-CoA and hwtLCADH or pKLCADH agree with the irreversible inactivation reported for MCADH (Freund et al., 1985). They contrast with those reported earlier by our group for beef liver LCADH (Ankele et al., 1991) which were interpreted as reflecting the absence of covalent protein modification. It should be noted that hwtLCADH (this work), hwtMCADH (Nandy et al., 1996b; Peterson et al., 1995) and a chimeric human MCADH which has the active-site Glu at position 261 instead of 376 (MCADH numbering; Nandy et al., 1996b) all differ substantially in their mode of reaction with 2-octynoyl-CoA. For this reason we conclude that the differences between MCADH and LCADH and those shown by LCADH from diverse sources are real. Probably the same basic mechanism of abstraction of the inhibitor C( $\gamma$ )-H by Glu261 (or equivalent number) occurs in all cases, and the formation of the product is the result of competition among covalent modification, rearrangements and acyl-CoA dissociation. In view of most recent data on the three-dimensional structural positioning of the catalytic Glu in MCADH located at either position 376 or 255 (MCADH numbering; Lee et al., 1996), it is likely that accessibility of this Glu-COO<sup>-</sup> to the inhibitor/product  $\gamma$ -function (to yield a covalent adduct) is the key factor in determining the final product distribution.

Human MCADH and LCADH can easily be distinguished spectrophotometrically using 1 mol 2-octynoyl-CoA/mol. With MCADH the formed complex has consistently a  $\lambda_{\max}$  of 800 nm with  $\epsilon_{800}$  of 4.5 mM<sup>-1</sup> cm<sup>-1</sup> (Freund et al., 1985) while the absorption maximum of the LCADH complex is blue shifted ( $\lambda_{\max}$  = 560 nm) and has only about 20% its intensity ( $\epsilon_{560}$   $\approx$  1 mM<sup>-1</sup> cm<sup>-1</sup>; see Fig. 7). Differentiation between (purified) MCADH and LCADH could be achieved also based on the spectra obtained upon reaction with 3-thiooctanoyl-CoA. With MCADH a product with a  $\lambda_{\max}$  of approximately 810 nm can be observed, while with LCADH  $\lambda_{\max}$  is approximately 660 nm with lower intensity. This probably reflects a major difference in the extent of ligand anion formation coupled with a substantially different orientation of the orbitals of the ligand donor and those of the flavin (acceptor) in the ground-state complex. Using 4-nitrophenylacetyl-CoA, the amount of ligand anion formed complex with pKLCADH or hwtLCADH is negligible, in contrast to MCADH, where large absorbance changes can be observed upon binding of 4-NO<sub>2</sub>-phenylacetyl-CoA ( $\Delta\epsilon_{494}$  of 12.6 mM<sup>-1</sup> cm<sup>-1</sup> and  $\Delta\epsilon_{740}$  of 4.3 mM<sup>-1</sup> cm<sup>-1</sup>). This clearly reflects a substantial ( $\geq$  pK 1) difference in the microscopic pK values of the species involved at the respective active centers, and thus the extent of activation of the substrate  $\alpha$ -CH. This, in turn, correlates with the results of Hazekawa et al. (1995) who have

found a substantial difference in the Raman frequencies of the C(1)=O groups of 3-ketoacyl-CoA ligands bound to either SCADH and MCADH compared with LCADH, reflecting the extent of activation of the  $\alpha$ -CH-C(=O)-S moiety. The difference in pK (i.e. activation) might also reflect a different polarity due to the absence of Glu99 in the active-center cavity of LCADH (Lee et al., 1996). In a general sense the differences between MCADH and LCADH are of a gradual nature. Thus the position of the catalytically essential Glu residue (position 261 in LCADH compared with 376 in MCADH) is most unlikely to affect its basic chemical/catalytic properties. This is in accord with the observation that the essential Glu is not linked to further functional groups in a pair or triad, as is often the case in other biochemical systems carrying out acid/base catalysis.

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