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Redox Properties of Human Medium-Chain Acyl-CoA Dehydrogenase, Modulation by Charged Active-Site Amino Acid Residues†

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ABSTRACT: The modulation of the electron-transfer properties of human medium-chain acyl-CoA dehydrogenase (hwtMCADH) has been studied using wild-type and site-directed mutants by determining their midpoint potentials at various pH values and estimating the involved p*K*s. The mutants used were E376D, in which the negative charge is retained; E376Q, in which one negative charge (p$K_a \approx 6.0$) is removed from the active center; E99G, in which a different negative charge (p$K_a \approx 7.3$) also is affected; and E376H (p$K_a \approx 9.3$) in which a positive charge is present. E_m for hwtMCADH at pH 7.6 is −0.114 V. Results for the site-directed mutants indicate that loss of a negative charge in the active site causes a +0.033 V potential shift. This is consistent with the assumption that electrostatic interactions (as in the case of flavodoxins) and specific charges are important in the modulation of the electron-transfer properties of this class of dehydrogenases. Specifically, these charge interactions appear to correlate with the positive E_m shift observed upon binding of substrate/product couple to MCADH [Lenn, N. D., Stankovich, M. T., and Liu, H. (1990) *Biochemistry* 29, 3709−3715], which coincides with a p*K* increase of Glu376-COOH from ∼6 to 8−9 [Rudik, I., Ghisla, S., and Thorpe, C. (1998) *Biochemistry 37*, 8437−8445]. From the pH dependence of the midpoint potentials of hwtMCADH two mechanistically important ionizations are estimated. The pK_a value of ∼6.0 is assigned to the catalytic base, Glu376-COOH, in the oxidized enzyme based on comparison with the pH behavior of the E376H mutant, it thus coincides with the p*K* value recently estimated [Vock, P., Engst, S., Eder, M., and Ghisla, S. (1998) *Biochemistry 37*, 1848−1860]. The pK_a of ∼7.1 is assigned to Glu376-COOH in reduced hwtMCADH. Comparable values for these pK_as for Glu376-COOH in pig kidney MCADH are $pK_{ox} = 6.5$ and $pK_{red} = 7.9$. The E_m measured for K304E-MCADH (a major mutant resulting in a deficiency syndrome) is essentially identical to that of hwtMCADH, indicating that the disordered enzyme has an intact active site.

Mitochondrial fatty acid oxidation is a major energy source for mammals (*1*). The initial and key enzymes in β-oxidation are the acyl-CoA dehydrogenases, members of a broad family, which catalyze the dehydrogenation of fatty acids with chain lengths of 4 up to >18 carbon atoms. Medium-chain acyl-CoA dehydrogenase (MCADH), with an optimum activity for C_8-substrates, is the best studied member of this family. The reaction it catalyzes occurs formally in two steps as shown in eqs 1 and 2 (*2*, *3*):

$$\text{reductive half reaction: } E_{ox} + SH_2 \leftrightarrow E_{red}\text{-}P \quad (1)$$

$$\text{oxidative half-reaction: } E_{red}\text{-}P + 2ETF_{ox} \leftrightarrow E_{ox} + 2ETF^{\bullet} \quad (2)$$

where E = MCADH, SH_2 = reduced substrate, P = product, ETF = electron transferring flavoprotein, and its (•) semiquinone form.[1]

The reductive half-reaction proceeds via the abstraction of both a proton and a hydride ion from the α- and β-carbons of the thioester-CoA substrate, respectively, (*4*). Glu376-COO^- is the proton abstracting base (*5*, *6*). The carbonyl oxygen of the thioester is involved in two essential hydrogen bonds, one to the ribityl 2-hydroxyl group of the FAD (*7*, *8*), and the second to the main-chain amide nitrogen of Glu376 (*7*). These interactions serve to lower the pK_a of the α-H of the acyl-CoA ligand (*9*) and are also likely to be responsible for the precise alignment of the substrate, with the flavin and Glu376 for catalysis.

The main focus of this laboratory has been the study of the reductive half-reaction where it was discovered that binding of the substrate/product couple increases the redox potential of MCADH by over 100 mV making the unfavorable electron-transfer possible (*10*). Further studies discovered that it was the product rather than the substrate which was responsible for this considerable shift (*11*).

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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; $E_1^{o\prime}$, formal potential of first electron transfer; $E_2^{o\prime}$, formal potential of second electron transfer; E_m, midpoint potential; E_{ox}, oxidized MCADH; E_{red}, two-electron reduced MCADH; ETF, electron transferring flavoprotein [and its (•) semiquinone]; hwtMCADH, recombinant human medium-chain acyl-CoA dehydrogenase; pK_a, ionization constant; SH_2, substrate; thioester-CoA; P, product.

From parallel work, it is known that binding of ligands increases the pK of Glu376-COOH from ~6 in uncomplexed MCADH by 1−3 pK units, depending on the nature of the ligand (*9*, *12*). This can potentially lead to a net change in the charge balance at the active center and thus strongly affect the flavin E_m. In view of this, MCADH active-site mutants which should lead to changes of 1 or 2 charges at the active center have been constructed, and the corresponding effect on E_m investigated. An important point in the context of electron transfer of MCADH is the ionization state of the 1 and 2 electron-reduced flavin. For the semiquinone form, the blue neutral form is stabilized when observed. In two-electron reduced MCADH, the state of ionization is more difficult to determine, but evidence exists for the anionic form of reduced flavin (*13*, *14*).

The pH dependence of the redox potentials of hwtMCADH were determined to obtain information about the protons transferred with reduction and to estimate pK_a values for the protein. Studying the potential vs pH profile of E376H obtained evidence that the pK_a observed with wild-type enzyme was due to Glu376. In addition, the K304E mutant was constructed in hopes that knowledge of its properties would provide insight into an MCADH disorder known as MCADH deficiency syndrome.

MATERIALS AND METHODS

Materials and Enzymes. The purification of hwtMCADH and of active-site mutants was done as described elsewhere (*15*); the yield of mutants was essentially the same as that of hwtMCADH. For storage, purified proteins were incubated on ice for 30 min with a 5-fold molar excess of FAD for 30 min in buffer (50 mM Tris, pH 7.8, containing 200 mM NaCl) and then brought to 85% ammonium sulfate and the precipitate stored at 4 °C or at −20 °C. Before use, the enzymes were resuspended in the desired buffer and desalted over a Pharmacia Sephadex G-25 M column (bed volume approximately equal to 9 mL) or dialyzed with the desired buffer. For "degreening", removal of tightly bound CoA-persulfide (*16*), 200 μL of a saturated solution of dithionite in the desired buffer was added to 800 μL of a cold, concentrated MCADH suspension in the same buffer. The enzyme becomes colorless while it dissolves, and after 1−2 min it is passed through a Sephadex G-25 M column during which process it reoxidizes to the yellow form.

The following dyes were used for the spectroelectrochemical experiments: methyl viologen (Sigma); indigo disulfonate (Aldrich); and 8-chlororiboflavin (a generous gift of Dr. J. P. Lambooy, University of Maryland). The buffer systems used were potassium phosphate (KPi) and CHES (Sigma). All experiments utilized glass-distilled water.

Methods. The molar absorptivities of fully oxidized proteins were determined by initially dialyzing the proteins with 20 mM NaPi (pH 7.8) and recording the FAD spectrum. Thereafter, 10% SDS in H_2O was added to a final concentration of 0.1% and the spectrum of the released FAD was recorded when changes had ceased. An ϵ of 11.3 mM^{-1} cm^{-1} was used (*17*) for determination of FAD concentration. The following ϵ_{max} values reported earlier (*15*) were used: hwtMCADH = 14.8 mM^{-1} cm^{-1}; E376D-MCADH = 14.8 mM^{-1} cm^{-1}; E376Q-MCADH = 14.5 mM^{-1} cm^{-1}; E376H-MCADH = 14.8 mM^{-1} cm^{-1}. MCADH activity was measured at 25 °C with the ferricenium assay (*18*) in 100 mM KPi, pH 7.6 (standard buffer) using 200 μM ferricenium, a concentration which was found to be saturating for all proteins tested.

Potentiometric and coulometric titrations were performed as previously described (*19*, *20*). Visible spectra were obtained either with a Kontron Uvikon 930 or a Perkin-Elmer l2 UV−vis spectrophotometer equipped with thermostated cells. Coulometric and dithionite titrations were carried out at pH 7.6 in 50 mM potassium phosphate and at 25 °C in the presence of 0.1 mM methyl viologen as mediator dye. Protein concentrations were in the range 10−20 μM. The indicator dyes used for potentiometric titrations were indigo disulfonate (E_m = −0.098 V, pH = 7.6) and 8-chlororiboflavin (E_m = −0.145 V, pH = 7.6) at a 2 μM concentration. Equilibrium of the system was considered to be obtained when the ΔE was less than 0.001 V/10 min; this required typically around 1−2 h. All potential values are reported versus the standard hydrogen electrode.

For the pH-dependent study of hwtMCADH two sets of conditions were used. Initial experiments were performed from pH 6.5 to 8.0 in 50 mM KPi at 25 °C. To achieve a better stability at high pH values, 10% glycerol was subsequently added and the experiments done at 4 °C. Under these conditions, experiments performed at pH 6.5−8.0 were in 50 mM KPi buffer and experiments at pH 8.5−9.5 were in 50 mM KPi and 20 mM CHES buffers.

Calculations. Before quantitation of the redox species and if necessary, spectra were corrected for turbidity that occurred during the titration (*21*). The data were analyzed using the Nernst equation:

$$E = E_m + 2.303\frac{RT}{nF}\log\frac{[\text{oxidized}]}{[\text{reduced}]} \qquad (3)$$

where E is the measured equilibrium potential at each point in the titration; R is the gas constant (8.314 41 Vc/K mol); T is the experimental temperature; n is the number of electrons in the half-reaction; F is Faradays constant (9.648 538 1 × 10^4 C/mol). A Nernst plot (potential vs log [ox]/[red]) is obtained, E_m, the midpoint potential, is determined from the y-intercept, and n, the number of electrons transferred in the half-reaction, is calculated from the slope of the line.

The pK values were determined by using the following nonlinear equation (*22*, *23*).

$$E_m = E_0 - 0.05916\text{pH} + 2.303\frac{RT}{nf}\log\left[\frac{[H^+]^3 + [H^+]^2K_{red,1} + [H^+]K_{red,1}K_{red,2} + K_{red,1}K_{red,2}K_{red,3}}{[H^+]^3 + [H^+]^2K_{ox,1} + [H^+]K_{ox,1}K_{ox,2} + K_{ox,1}K_{ox,2}K_{ox,3}}\right] \qquad (4)$$

For this equation, E_m is the experimentally determined midpoint potential at a given pH, and E_0 is a calculated constant. The constants R, T, F, and n are defined above. The various Ks are as assigned in the text.

RESULTS

Characterization of Recombinant hwtMCADH. To compare the midpoint potentials of mutants, accurate information

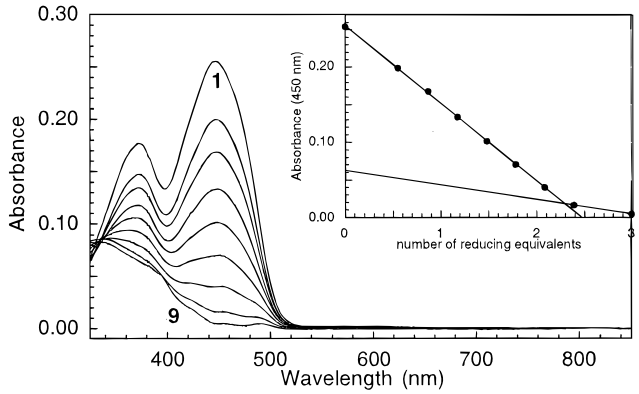

FIGURE 1: UV−vis absorption spectrum of hwtMCADH in a coulometric titration. 12 μM hwtMCADH and 100 μM methyl viologen in 50 mM potassium phosphate buffer, pH 7.6, at 25 °C. The inset depicts the absorbance vs reducing equivalents for a coulometric titration of hwtMCADH.

Table 1: Comparison of E_m for hwtMCADH Under Different Experimental Conditions[a]

pH	E_m (V), 25 °C, 50 mM KPi	E_m (V), 4 °C, 10% glycerol, 50 mM KPi	ΔE_m (V)
6.5	−0.054	−0.040	+0.014
7.0	−0.087	−0.080	+0.007
7.6	−0.114	−0.103	+0.011
8.0	−0.142	−0.128	+0.014
8.2	−0.155		
8.5	unstable	−0.145	
9.0	unstable	−0.162	
9.5	unstable	−0.184	

[a] The Average ΔE_m is 0.012 V.

about the hwtMCADH redox behavior is mandatory. A dithionite titration of hwtMCADH at pH 7.6, 50 mM KPi at 25 °C was initially performed to characterize the spectra of the half and fully reduced species of hwtMCADH. Approximately 35% blue neutral semiquinone is kinetically stabilized during this process. In contrast, Figure 1 shows the results of a companion, coulometric experiment, where less than 5% of the blue neutral semiquinone was observed. A similar trend, where more blue neutral semiquinone is stabilized during a dithionite titration, is observed with pig MCADH (*24*). This is attributed to the speed of reduction of a dithionite titration, wherein thermodynamic equilibrium is not obtained over the course of an experiment and more blue semiquinone results from kinetic stabilization. A potentiometric titration of recombinant human MCADH yielded a midpoint potential (E_m) of −0.114 V (Table 1), which is some 30 mV more positive than the value determined for pig MCADH (−0.145 V, 24) at pH 7.6. The formal potential values for the one-electron couples could not be estimated for hwtMCADH due to the very small amount of semiquinone stabilized thermodynamically.

Studies with Glu376 Mutants. The experiments for all the mutants were performed at pH 7.6, and the results of the potentiometric measurements are reported in Table 2. Essentially, the thermodynamic properties of E376D were identical to those of hwtMCADH. The same holds for the coulometric titration, however, more blue neutral semiquinone was stabilized, 7%, and the spectra are slightly more resolved compared to recombinant hwtMCADH. E376Q stabilized substantially more blue neutral semiquinone, 15%.

Table 2: Comparison of E_m Values of HwtMCADH with Those of Specific Mutants Which Induce a Modification of Charge[a]

hwtMCADH	E_m, V	ΔE_m, V	thermodynamic stabilization of blue, neutral semiquinone
hwtMCADH	−0.114		<5%
E376D	−0.114	0.000	7%
E376Q	−0.081	+0.033	15%
E376H	−0.069	+0.045	15%
E99G	−0.070	+0.044	<5%
K304E	−0.115	−0.001	<5%

[a] Conditions: pH 7.6 in 50 mM potassium phosphate, 25 °C.

The midpoint potential was shifted positive to −0.081 V, a 0.033 V shift compared to recombinant hwtMCADH. The same trend, yet smaller (0.011 V), was found for the corresponding mutant in short-chain acyl-CoA dehydrogenase (*11*). With regard to the E376H mutant, the p*K* of the imidazole is raised from ∼6 in the free state to ∼9.3 at the active center of uncomplexed hwtMCADH (*9*). From this, the residue will be positively charged in the neutral range and appears to lead to a 15% stabilization of the blue radical and a positive potential shift of 0.045 V (Table 2).

E99G-MCADH. hwtMCADH possesses a second glutamate residue, E99, at the bottom of the active site center cavity (*7*, *25*), the p*K* of which is ∼7.3 in uncomplexed enzyme (*9*). The midpoint potential of this mutant is also positively shifted by 0.044 to −0.070 V, compared to hwtMCADH (Table 2). Less than 5% neutral semiquinone is thermodynamically stabilized, approximately the same amount as found with hwtMCADH.

Effect of pH on the Spectral Properties of Oxidized and Reduced hwtMCADH. As a basis for and complement to the interpretation of the pH dependence of the redox potential, the UV−vis spectra of oxidized and reduced hwtMCADH were recorded as a function of pH in the range 4.5−10. It should be stated that the changes observable with both the oxidized and the reduced chromophore are very small and cannot be attributed to ionizations of the chromophore itself. They consist mainly in a perturbation of the visible, oxidized flavin band (Figure 2A, inset), which results in a shift of the corresponding maximum. Thus, at pH ∼10 the λ_{max} is at ∼444 nm and shifts to 450 nm at pH ≤5, and this pH dependence can be fitted based on two ionizations, one at ∼6 and the other at ∼7.3 (Figure 2B), where the major changes occur coincidentally with the second p*K*.

It was expected that both pK_as would be unequivocally identified by performing the same experiment on the E376H mutant, which should only exhibit the pK_a for Glu99. However, with the E376H mutant, the spectral changes in the pH range 6.5−10.5 are minimal and not interpretable in terms of specific ionizations (Figure 2B) (open diamonds). The similarity of the λ_{max} of hwtMCADH, in which both Glu376 and Glu99 are neutral (pH <6) with that of the E376H mutant suggests that in the latter the charges of the imidazole and of Glu99 neutralize each other (His376 positively charged-Glu99 negatively charged), resulting in absorbance looking like hwtMCADH when both Glu are protonated (neutral). This spectral similarity is indicated by the dashed line in Figure 2B. Because of the lack of spectral change in the pH study of the E376H mutant, the spectral

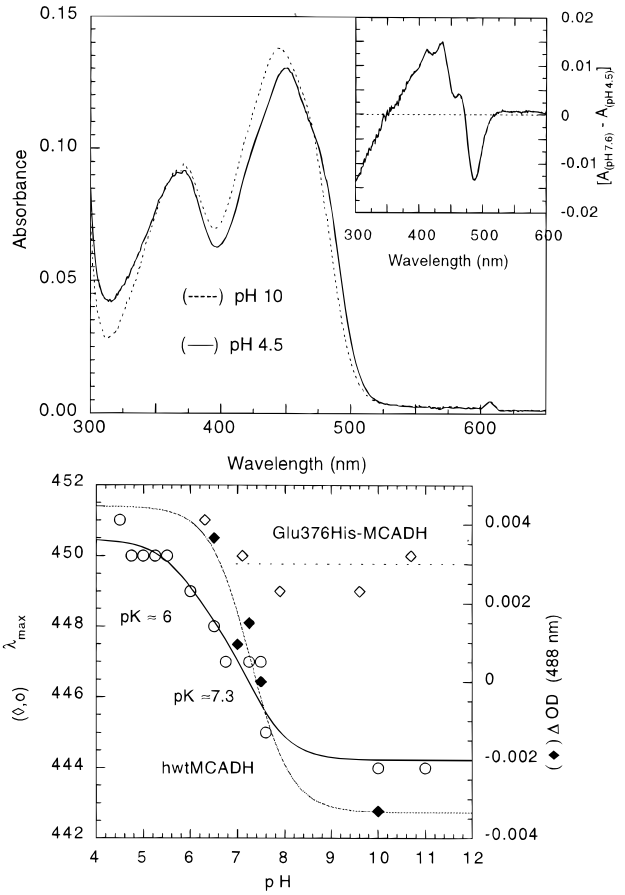

FIGURE 2: Effect of pH on the absorption spectra of hwtMCADH. (A) The spectra of the enzyme at ~10 μM were recorded in the presence of 250 mM KCl and using 10 mM concentrations of acetate, phosphate, tris, and carbonate as buffers. The inset depicts the difference between the spectra at pH 7.6 and 4.5. (B) pH dependence of (○) the λ_{max} of the visible band of the flavin chromophore in hwtMCADH; (◇) λ_{max} of the visible band for E376H-MCADH and (◆) the difference spectra at 488 nm for hwtMCADH of the same chromophore.

changes as a function of pH for the hwtMCADH were carefully reexamined to determine if the combined effects of Glu376 and Glu99 on the flavin spectra as a function of pH could be deconvoluted. The most prominent spectral effect detected by difference spectroscopy (Figure 2A, inset) is a difference peak maximal at ~487 nm, the pH dependence of which reflects essentially only changes coincident with a p$K \approx 7.3$ and none with the second p$K \approx 6$. Perturbations associated with the latter pK are observed mainly in the 440−460 nm area. On the basis of this and on the pKs estimated by Vock et al. (*9*) for the same groups and resulting from the behavior of spectrally active ligands, the p$K \approx 6$ was attributed to Glu376 and that at ~7 to Glu99.

The much stronger effect of Glu99 on the spectra of the flavin is unexpected. It is known that the carboxylate of the Glu376 is pointing to the FAD ring in the uncomplexed MCADH structure (and is parallel to the plane of the FAD ring in the complexed structure.) Furthermore, the Glu99 carboxylate is located further away from the isoalloxazine ring N(5) than the 376 carboxylate (7.5 vs 6.0 Å). Thus, Glu99 appears to have a stronger effect on both flavin spectra and redox potential than the distance to the flavin would warrant. However, the X-ray structure shows that Glu99 and Glu376 are linked by a chain of four ordered water

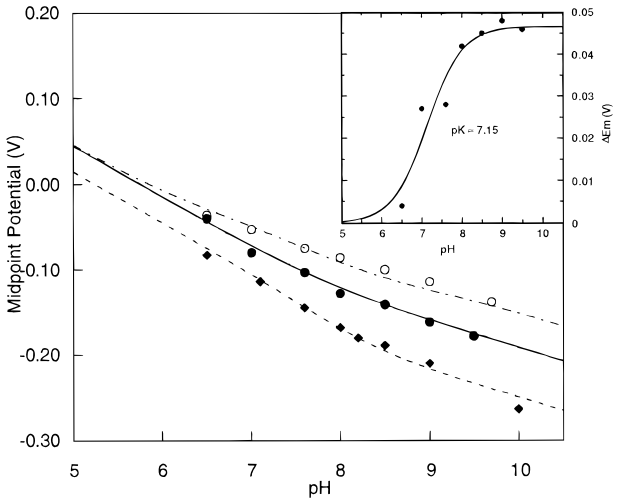

FIGURE 3: pH vs midpoint potential of hwtMCADH (●) at 4 °C, E376H-MCADH (○) at 4 °C and pig MCADH (◆) at 25 °C. (Inset) pH dependence of the difference in E_m between hwt- and E376H-MCADH.

molecules which are hydrogen bonded to each other and the two carboxylates (*7*). Thus, both are linked to N(5) and affect N(5). That hydrogen-bonding network of ordered waters probably remains intact when Glu376 is changed to glutamine. However, that series of hydrogen bonds may be disrupted when Glu99 is changed to glycine, for the carboxyl group that participated in the hydrogen bonding is lost. This would affect the hydrogen bonding at the flavin and thus change its potential and its spectral properties even more than when the Glu376 is mutated to glutamine. Other mutations at Glu99 will be investigated to test the influence of this carboxylate residue.

In reduced hwtMCADH, there is a minor effect on the spectra which occurs between pH 8.5−9 and 10. This perturbation is so small that it cannot be attributed to a change in ionization state of the flavin itself, noting that changes in ionization of reduced flavin are, in general, accompanied by definite spectral effects (*26*).

pH Dependence of the Redox Potential of MCADH. Initially, the potential vs pH dependence was determined from pH 6.5−8.2 at 25 °C and 50 mM KPi buffer. Unfortunately, hwtMCADH was too unstable at values greater than 8.5, thus impeding the further exploration of pH dependence. In an effort to better stabilize hwtMCADH over a broader pH range, glycerol (10%) was added and the temperature lowered to 4 °C. Under these new conditions, a more complete pH dependence was determined and is shown in Figure 3.

The overall slope of 38 mV/pH corresponds roughly to a $2e^-/1H^+$ transfer, the slight change in slope below pH 7 suggests a change in protonation state.

$$E{-}FAD_{ox} + 2e^- + H^+ \Leftrightarrow E{-}FADH^-_{red} \qquad (5)$$

When the data was fit to a nonlinear equation, the following pK values emerged: $pK_{ox,1} \approx 6.0$; $pK_{ox,2} \approx 7.3$; $pK_{ox,3} \approx 12$; $pK_{red,1} \approx 6.0$; $pK_{red,2} \approx 7.1$; and $pK_{red,3} \approx 8.0$. The value for $pK_{ox,3}$ corresponds to the ionization constant for N(3)−H of the oxidized isoalloxazine. This value was assigned based on a study of 6-OH FAD in which the oxidized pK shifted from approximately 7 in free 6-OH FAD

Table 3: pK Values Used for the Fitting of E_m Data Shown in Figure 3, and Assigned to the Species Involved in the Interconversion of Oxidized and Reduced Flavin at the Active Center of MCADH

pK	assignment	hwtMCADH	pig MCADH	E376H-MCADH
$pK_{ox,1}$	amino acid Glu376	6.0	6.5	7.3 (Glu99)
$pK_{ox,2}$	amino acid Glu99	7.3	7.5	9.3 (His376)
$pK_{ox,3}$	N(3) Flavin	12.0	11.5	12.0
$pK_{red,1}$	N(1) Flavin	6.0	6.5	6.0
$pK_{red,2}$	amino acid Glu376	7.1	7.9	8.0 (Glu99)
$pK_{red,3}$	amino acid Glu99	8.0	8.5	8.9 (His376)

to 9 when bound to the protein (*27*). Using the argument above, the p*K* of oxidized flavin would shift from 10 in the free form (*28*) to approximately 12 when bound to MCADH. Since no semiquinone is stabilized, the p*K* of N(5) need not be considered in the analysis. $pK_{ox,1}$ and $pK_{ox,2}$ were assigned to amino acids near the flavin, i.e., the $pK_a \approx 6.0$ recently derived for Glu376-COOH and $pK_a \approx 7.3$ for Glu99-COOH (*9*) were used as $pK_{ox,1}$ and $pK_{ox,2}$ in the nonlinear equation, respectively. The remaining pK_as of reduced MCADH were varied to fit the data. $pK_{red,1} \approx 6.0$ was assigned to N(1)−H of the flavin, which is in agreement to the lack of significant spectral change in the pH range 6−10. The remaining p*K*s of the reduced species, $pK_{red,2} \approx 7.1$ and $pK_{red,3} \approx 8.0$, were tentatively assigned to Glu376 and Glu99, respectively.

On comparing the midpoint potential values at 25 °C with those at 4 °C and in the presence of 10% glycerol, there is an average overall increase for the latter conditions of 0.012 V (Table 1). This in agreement with a control study performed with free 8-chlororiboflavin where E_m shifted from −0.145 to −0.130 V at 4 °C and in the presence of glycerol. Further control studies reveal that the differences in temperature, as opposed to glycerol, are responsible for the positive potential shift.

For the purpose of comparison, the E_m vs pH dependence of pig MCADH obtained at 25 °C (*24*) has been reanalyzed using the same equation as with hwtMCADH. The results indicate some marked differences. Thus, the overall slope for pig MCADH is 58 mV/pH corresponding to a $2e^-/2H^+$ transfer. There are slight changes in slope around pH 7 and 8, indicating possible pK_a values. Using the nonlinear equation and the hwtMCADH p*K* values as a starting point, the following p*K* values emerged: $pK_{ox,1} \approx 6.5$; $pK_{ox,2} \approx 7.5$; $pK_{ox,3} \approx 11.5$; $pK_{red,1} \approx 6.5$; $pK_{red,2} \approx 7.9$; and $pK_{red,3} \approx 8.5$, indicating an overall shift in p*K*. The p*K* values were assigned as in hwtMCADH, $pK_{ox,3}$ and $pK_{red,1}$ were assigned to the flavin, $pK_{ox,1}$ and $pK_{red,2}$ were assigned to Glu376, and $pK_{ox,2}$ and $pK_{red,3}$ were assigned to Glu99. These p*K* values are summarized in Table 3.

pH Dependence of the Redox Potential of E376H. To assess whether the redox-linked pK_a found for hwtMCADH is that of Glu376, a pH study was performed with the E376H-MCADH mutant in the presence of 10% glycerol and at 4 °C. Under these conditions, the pH dependence of E376H-MCADH is linear with a slope of 0.031 V/pH unit, Figure 3. This slope correlates to one proton transferred with two electrons over a pH range 6.5−9.5.

$$E{-}FAD_{ox} + 2e^- + H^+ \Leftrightarrow E{-}FADH^-_{red} \qquad (6)$$

From the electrochemical data, apparent p*K*s in the range 6.5−9.5 cannot be detected. This would apparently contrast with the finding of a pK_a of 9.3 for the imidazole of this mutant in its uncomplexed form (*9*). While the pH vs E_m data appears to be linear, a reasonable fit was also found using the nonlinear equation (see above) and $pK_{ox,2} = 9.3$ and $pK_{red,3} \approx 8.9$ (Figure 3). On the basis of this apparent linearity, the difference in E_m between hwt- and E376H-MCADH vs the pH has been plotted as shown in the insert of Figure 3. This representation clearly uncovers a $pK \approx 7.1$ for hwtMCADH enzyme, which is attributed to Glu99, in agreement with the above interpretations. The other p*K* values found for E376H-MCADH are the same as those for hwtMCADH. The pK_a being at the high end of the pH profile may account for the fact that both a linear and nonlinear fit can be obtained. Table 4 shows a comparison of midpoint potential for recombinant hwtMCADH and E376H-MCADH at different pH values.

Table 4: Comparison of pH Dependence of E_m for hwtMCADH and E376H-MCADH in 50 mM Potassium Phosphate, 10% Glycerol, 4 °C

pH	E_m (V) for hwtMCADH	E_m (V) for E376H-MCADH	ΔE_m (V)
6.5	−0.040	−0.036	+0.004
7.0	−0.080	−0.053	+0.027
7.6	−0.103	−0.075	+0.028
8.0	−0.128	−0.086	+0.042
8.5	−0.145	−0.100	+0.045
9.0	−0.162	−0.114	+0.048
9.5	−0.184	−0.138[a]	+0.046

[a] Experimental pH was 9.7.

Studies with K304E-MCADH. The midpoint potential of the K304E mutant was determined to be −0.115 V (Table 2). Thus, there is no change in the electron-transfer properties compared to the parent hwtMCADH.

DISCUSSION

The reduction of the (enzyme-bound) isoalloxazine system proceeds formally via two one-electron steps and is affected by the various ionizations of the chromophore itself at the oxidized, half and fully reduced level. Noticeably, very little blue neutral semiquinone is stabilized during all experiments which were carried out under thermodynamic control. From this finding, it can be concluded that the redox potential, E_1, of the $E{-}Fl_{ox}/E{-}FlH^\bullet$ couple is more negative than E_2, the $E{-}FlH^\bullet/E{-}Fl_{red}H^-$ couple. The values for E_1 and E_2 cannot be estimated because of the small amount of semiquinone thermodynamically stabilized (<5%), which is substantially less than the 20% found for pig kidney MCADH under similar conditions (*24*). The redox potential determined for hwtMCADH (−0.114 V) is an average of E_1 and E_2, and is thus best described as a midpoint potential. It is significantly more positive than that determined for pig kidney MCADH (−0.145 V) (*24*). The differences in potentials found for human and pig MCADH is reflected in the percentage of MCADH reduction attained at equilibrium with octanoyl-CoA, which is 74% (*15*) compared to 61% for pig kidney MCADH (*3*). Effectively, the E_m of hwtMCADH is closer to that of *M. elsdenii* SCADH (−0.075 V), which also stabilizes very little blue neutral semiquinone (<5%), compared to pig MCADH (*11*). The molecular origins for the 30 mV difference are not easy to determine. In fact the crystal structures of pig and human MCADH are essentially

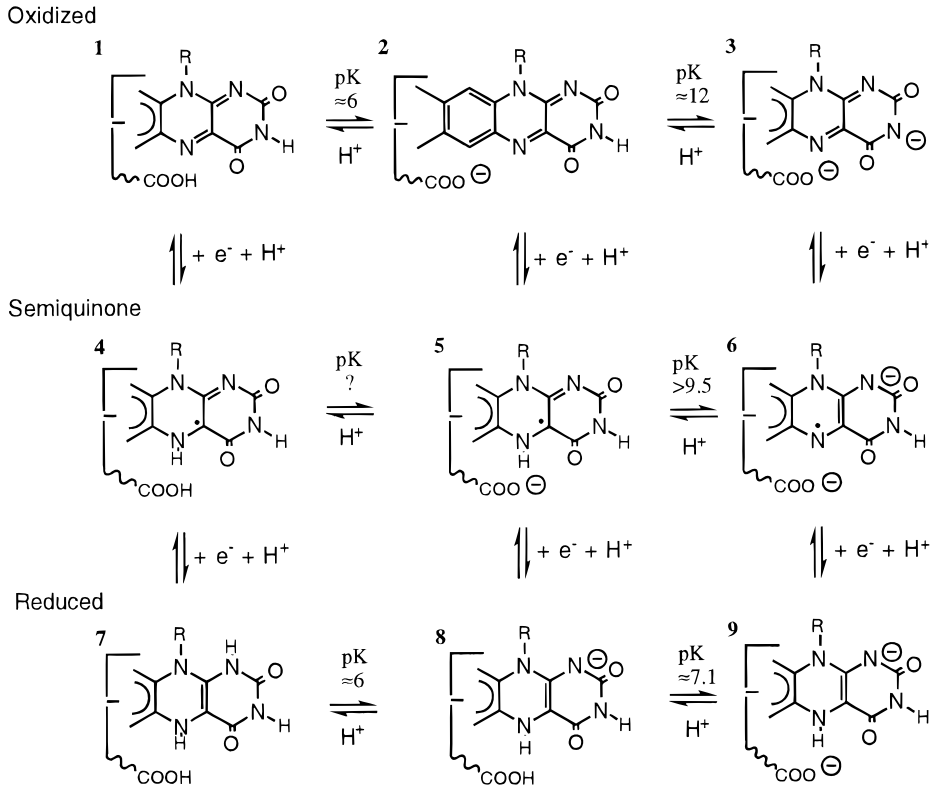

FIGURE 4: Redox and protonic equilibria proposed to be involved in the interconversion of oxidized and reduced flavin at the active center of hwtMCADH. Note that the ionizations of COOH are for Glu376 The ionization for Glu99-COOH, with a p$K \approx 7$ in the oxidized state of the flavin has been omitted for clarity.

identical (*7*), as are the charged amino acid residues flanking the active site.

There are two possible explanations for the difference in redox potential. The first is that there exists a more global difference in environment, namely that a 30% difference in sequence causes a variation in the arrangement of polypeptide chains and their interaction with each other and with the flavin. In support of this, it is known that the human MCADH is less stable than is the pig enzyme. The human MCADH appears to be more flexible, and more breathable, which in turn affects the active-site pK_as and redox potential. Second, the difference in potential might result from a variation in the balance of charges in the extended surroundings of the isoalloxazine between the two enzymes, as found by Swenson for flavodoxins (*29*, *30*).

This second explanation is in line with the results of the experiments in which the charge at amino acid position 376 is varied. This is found to have significant effect on the protein's redox potential. For E376Q-MCADH and E376H-MCADH, where the net change in charge is 1 and 2, there is a positive redox shift of 0.033 and 0.045 V, respectively. This again parallels the conclusion from Swenson's group obtained with various mutated flavodoxins and specifically with D66Q-flavodoxin for which a positive shift of 0.018 V for E_2, compared to wild-type was reported (*30*). Also, work from this laboratory using a flavodoxin from *Anabaena 7120* reveals that the similar mutation E61Q causes a positive shift of 0.052 V for E_2 and of 0.009 V for E_m (*31*). This confirms the expectation that the net charge affects the midpoint potential of the FAD in hwtMCADH. Additional support for this conclusion comes from the E99G-MCADH mutant, where a second negatively charged residue (p$K_a \approx 7.3$, *9*) at the active site is removed, resulting in a positive, 0.044 V shift compared to recombinant hwtMCADH.

The slope of the E_m vs pH shown in Figure 3 for hwtMCADH is approximately 30 mV/pH in the physiologically important pH range of 7−8 and is compatible with the transfer of $2e^-/1H^+$. The pK_a values estimated from the nonlinear fit, i.e., ∼6.0 and 7.3 coincide with those deduced from the use of transition state analogues (*9*). The E_m vs pH behavior is also compatible with the absence of changes in the ionization state of FAD above approximately pH 6.0, and the reduced flavin being in its anionic form. The one proton/two electron transfer is confirmed by the fact that blue neutral semiquinone is stabilized during the titration and the anionic two-electron reduced form is seen (*13*, *14*) (Figure 4, panels 2, 5, and 9) The pK_a for Glu99 does not shift as dramatically upon reduction of the flavin (7.3 vs 8.0).

A pK_a of 6.0 for Glu376 in oxidized hwtMCADH is in keeping with the mechanism of the reductive half-reaction, since it is logical that the catalytic base is deprotonated on its encounter with substrate in order to abstract the α-proton. The difference in the pK_as in the oxidized forms for pig (∼6.5) as compared to hwtMCADH (∼6.0) is significantly smaller compared to that between the pK_as for the same residue in the reduced form of the enzymes (∼7.9 and 7.1). This is in keeping with the differences in slope found for the E_m vs pH of the two enzymes. At pH 7.6, hwtMCADH transfers one proton to the FAD upon two electron reduction, while pig MCADH transfers one proton to FAD and one to Glu376 upon two electron reduction:

$$\mathrm{E{-}FAD_{ox} + B^- + 2e^- + H^+ \Leftrightarrow E{-}FAD_{red}H^- + BH} \quad (7)$$

The differences in p*K* values are also in agreement with the higher redox potential of hwt compared to pig MCADH.

Although the p*K* values were initially assigned based upon parallel spectral work (*9*), further insight is gained by considering the E376H-MCADH mutant. A comparison of the potential vs pH data of the wild-type enzyme and that of the E376H mutant shows clear differences. While both have a two electron/one proton dependence, that for hwtMCADH reflects the pK_as for Glu376 at 6.0 and 7.1, whereas the E376H mutant is linear and has no observable p*K* in the range studied. The absence of the pK_as at 6.0 and 7.1 with E376H-MCADH fully supports the p*K* attribution to Glu376. In support of this argument, the difference between the redox behavior of the hwtMCADH and the E376H-MCADH mutant shows maximal difference at 7.15, as shown in the insert in Figure 3. This is consistent with the loss of Glu 376, which had p$K_{a,ox}$ and p$K_{a,red}$ in the region of 6.1−7.1. Since the midpoint potential vs pH dependence for E376H-MCADH is essentially linear from pH 6.5−9.5, an ionization constant for the histidine residue cannot be adequately determined using the nonlinear fit algorithm. Any nonlinearity could be caused by the pK_as of Glu99. The pK_a of the E376H imidazole was estimated as 9.3 (*9*) in uncomplexed oxidized enzyme, it thus lies just at the upper borderline of the pH range studied. The histidine is protonated up to pH ∼9, accounting for the observed 0.031 V/pH unit dependence, in which the positive charge on the histidine stabilizes the anionic reduced form of the flavin by lowering its pK_a.

$$E{-}BH^{+}{-}Fl_{ox} + H^{+} + 2e^{-} \rightleftharpoons E{-}BH^{+}{-}Fl_{red}H^{-} \quad (8)$$

This is consistent with the positive shift in redox potential of this mutant. Such a shift of the redox potential of the flavin bound to MCADH compared to that of the free molecule is remarkable; its molecular basis is not readily apparent, as opposed to other cases. In general, an increase of the midpoint potential can be correlated with a stabilization of the negative charge on the isoalloxazine pyrimidine moiety, i.e., with a decrease of the pK_a of the reduced species [the N(1)−H pK_a of free flavin is ∼6.5 (*28*)]. This can be implemented by the presence of positively charged residue in the vicinity of the charge itself, i.e., the pyrimidine moiety of the isoalloxazine, as in lactate oxidase (*32*−*34*), glycollate oxidase (*35*), and cytochrome b5 (*36*, *37*). In other cases, such as glutathione reductase and D-amino acid oxidase, a dipole induced by a β-coil leads to the same effect (*38*, *39*).

Inspection of the three-dimensional structure of MCADH (*7*) does not reveal comparable functionalities. The pyrimidine moiety is, however, hydrogen bonded at several residues in the active site of the protein (*7*). The active center of MCADH is also predominantly hydrophobic, as reflected by the p*K* increases of Glu376 and Glu99 (both from 3.4 to ∼6 and ∼7.3 in uncomplexed MCADH). Recently, the Herschlag group has argued convincingly, that the strength of H bonds can be strongly increased in a hydrophobic environment (*40*). Application of the concept to the active center of MCADH has provided a rationale for the decrease of the p*K* of acyl-CoA ligand αC−H by approximately 10 pH units induced by two H bonds to the thioester carbonyl (*8*, *9*, *41*, *42*). It is thus likely that, in the present case, the driving force for the potential increase results from the stabilization of the negative charge by the named H bonds in the hydrophobic environments (*40*). There is, however, an apparent contradiction to this concept, which is worth discussing: the pK_a of (oxidized) 6OH−FAD bound to MCADH is increased from 7 to 9, i.e., it shows the opposite effect as that observed here for the reduced flavin N(1)−H. A hypothetical rationale for this discrepancy could be the difference in charge location between the two molecules, i.e., a stronger delocalization throughout the molecule and relevant negative charge on the C(6)-oxygen in the oxidized case, and localization around N(1)−C(2)═O in the reduced. Recently Rudik et al. (*43*) showed that binding the 6-OH flavin to apoprotein caused the pK_a of the 6-OH flavin to shift from 7 to 9, stabilizing the neutral form of 6-OH flavin. They also showed that binding a substrate analogue that could be polarized causes the pK_a to shift back to 6. These pK_a shifts occur because the analogue is polarized in the active site, causing the pK_a of the Glu376 at the active site to change so that now it is protonated. This protonation causes the pK_a of the flavin to be lowered so that the proton can be more easily lost and the flavin can exist in the anionic form.

The argument presented in the paper is analogous to the one presented by Rudik (*43*), namely if the Glu376 is protonated, the flavin is more likely to be deprotonated (anionic). In analogy to this work, upon reduction of the flavin, the pK_a of Glu376 shifts higher (from 6 to 7) so it is more likely to be protonated. This protonation causes the pK_a of the flavin to shift lower to 6 so it is less likely to be protonated. So, the negative charge density moves from glutamic acid to flavin upon reduction, as stated above. Thus, the Glu376 interaction with flavin changes with oxidation state.

This would also lead to a different interaction (repulsion) between the charge on the flavins, and that on Glu376-COO− for the two cases. In any event, the importance of changes in the net charge in modulating the redox potential of the flavin is clearly documented by the effects of mutations at position 376 reported in this work. In view of this, an assessment of effects of the net charge of the shell surrounding the active center of MCADH on the redox potential of the flavin is worth further consideration, based on similar effects observed in the flavodoxin family (*30*).

The p*K* values found for oxidized Glu376 should be compared to the apparent pK_a values of 8.2 and 8.3 from the pH/activity profiles of hwtMCADH and pig MCADH (*44*, *45*). This suggests that the binding of the substrate causes the pK_a to shift to higher values, perhaps by displacing water from the active site, thus making the site more hydrophobic. There is some evidence for this in earlier work (*12*), but it was not clear if the pK_a shift observed was for the Glu376 or for the substrate analogue, since both had pK_as in the range. Clearly, more work on the effect of substrate or product analogues, that do not themselves have a pK_a is necessary to define a change in pK_a of the enzyme.

The prevalent mutation found in patients with MCADH deficiency syndrome is a lysine at position 304 replaced by a glutamic acid (*46*). The mutation lies approximately 20 Å away from the FAD active site and is not involved in binding of the substrate or FAD (*7*). The identity of the redox potential of the flavin in K304E-MCADH with that of hwtMCADH fully confirms earlier suggestions (*15*) that the deficiency results from an instability of the mutant protein

as opposed to a functional difference. The lower rate of electron transfer of the mutant to the natural electron acceptor ETF (*15*) is thus likely to result from minor differences in quaternary structure affecting the ETF interaction, as opposed to electronic effects influencing the flavin potentials and the stabilization of the intermediate semiquinones.

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