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Probing the Electron Transfer Properties of human Medium-Chain Acyl-CoA Dehydrogenase and Site-Directed Mutants

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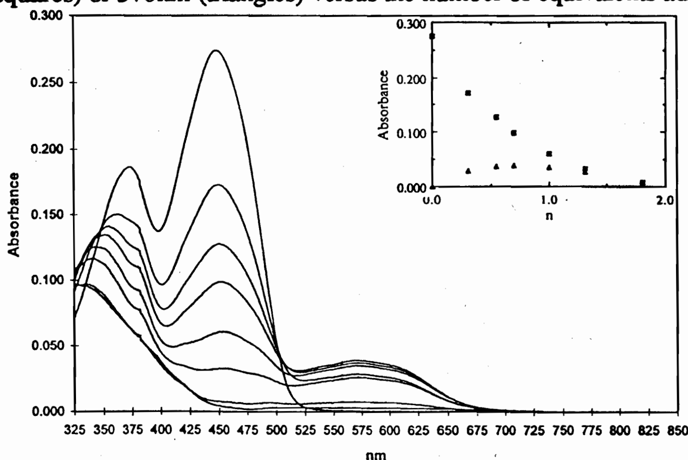
INTRODUCTION

Mitochondrial fatty acid oxidation is an important energy source for mammals. Approximately 40% of the total human energy requirement is provided by the β-oxidation cycle (1). The initial and key enzyme in β-oxidation, medium-chain acyl-CoA dehydrogenase (MCADH), catalyzes the two-electron oxidation of a broad range of fatty acids. Specifically, MCADH catalyzes the conversion of straight-chain fatty acyl-CoA thioesters to their corresponding α,β-enoyl-CoA products. MCADH consists of four identical monomers each containing one flavin adenine dinucleotide (FAD).

The main focus of our laboratory has been on the reductive half-reaction where it was discovered that substrate/product binding shifts the midpoint potential of MCADH positive by over 100 mV making the unfavorable electron-transfer possible (2). Using a newly cloned and over-expressed human medium-chain acyl-CoA dehydrogenase (hMCADH) along with site-directed hMCADH mutants, the interactions that modulate the electron transfer properties of the enzyme are under investigation. (3).

The objectives for this study were, first, to characterize the visible absorbance spectra as well as determine the midpoint potential of the enzyme. Second, establish the midpoint potential versus pH dependence for hMCADH. Third, investigate a series of site-directed mutants designed to test possible π-stacking between a highly conserved tyrosine, tyrosine 375, and the isoalloxazine rings of FAD.

Fig. 1. Reduction spectra of hMCADH using dithionite as the reductant at pH = 7.6 in 50 mM potassium phosphate buffer at 25°C. Inset is a plot of the absorbance at 450nm (squares) or 570nm (triangles) versus the number of equivalents added.



RESULTS

Characterization of hMCADH.

Initially, the enzyme was reduced with dithionite to monitor the reduction spectra, Figure 1. About 35% blue neutral semiquinone kinetically stabilizes. Interestingly, a coulometric reduction using methyl viologen as a mediator stabilized less than 5% blue neutral semiquinone. The smaller amount of blue neutral semiquinone stabilized is also observed under thermodynamic equilibrium in the potentiometric titrations.

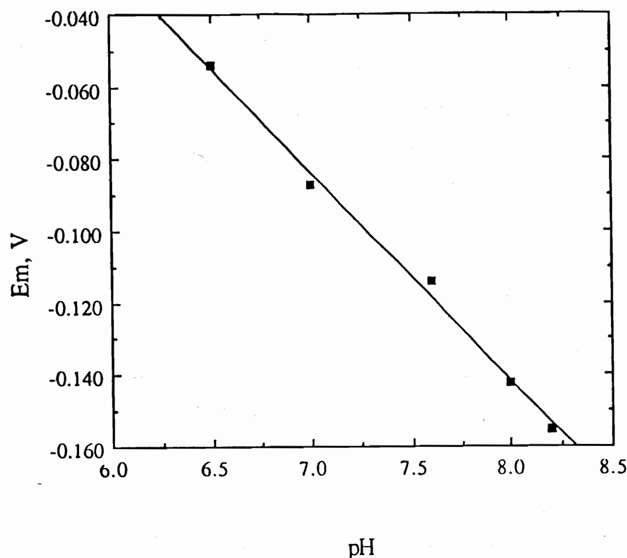
Using our spectroelectrochemical methodology, the midpoint potential of hMCADH was determined to be -0.114 V at pH = 7.6 in 50 mM potassium phosphate at 25°C versus a standard hydrogen electrode (4,5). This is a slightly more positive value than that determined for pig MCADH under the same conditions, -0.145 V (6).

pH dependence of hMCADH.

The potential versus pH dependence was determined from pH 6.5 - 8.2. These experiments were done at 25°C in 50 mM potassium phosphate buffer. The results are shown in Figure 2. The relationship between Em and pH is linear with a slope of 0.058 V/pH unit. The slope is compatible with the transfer of one proton with each electron transferred.

$$E\text{-}FAD_{(ox)} + 2e^- + 2H^+ \text{ -----> } EFADH_{2(red)} \quad [1]$$

Fig. 2. Midpoint potential versus pH dependence of hMCADH. All experiments were in 50 mM potassium phosphate buffer at 25°C.



At more acidic pH levels, 6.5 - 7.6, no semiquinone stabilized. However, at pH 8.0 and 8.2, 10-15% blue neutral semiquinone is thermodynamically stabilized, suggesting the electron transfer and proton transfer properties are changing in this region. These findings are still under investigation.

Tyrosine 375 hMCADH mutants.

The interactions between tyrosine 375 and the isoalloxazine rings of the FAD were studied by constructing two site-directed mutants. The hypothesis is that the tyrosine may have some π-stacking interaction with the rings of the FAD. The first mutation changes the tyrosine to a phenylalanine which eliminates the hydroxyl portion of the tyrosine residue, Y375F. The second mutation changes the tyrosine to a glycine, removing the hydroxyl portion of the residue as well as the phenyl ring of the tyrosine, Y375G. This allows the effects of the individual interactions on the redox properties to be examined.

TABLE I: **Midpoint potential comparison of tyrosine 375 site-directed hMCADH mutants to recombinant hMCADH. All at pH = 7.6 in 50 mM potassium phosphate buffer at 25°C. All potentials are versus a standard hydrogen electrode.**

hMCADH	Em, V	ΔEm, V
recombinant	-0.114	-
Y375F	-0.132	-0.018
Y375G	-0.158	-0.044

The midpoint potential results, shown in Table I, were somewhat surprising. Both mutants have more negative midpoint potentials compared to recombinant hMCADH which indicates that tyrosine 375 is important in modulating the midpoint potential of hMCADH. However, the fact that Y375F hMCADH has a slightly more negative midpoint potential, 0.018 V, compared to recombinant hMCADH reveals the importance of the hydroxyl portion of the tyrosine residue, possibly indicating some important hydrogen bonding behavior. In addition, an even larger negative shift in midpoint potential was observed for Y375G, 0.044 V. This supports the hypothesis that the phenyl ring is involved in π-stacking with the isoalloxazine rings of the FAD.

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