First publ. in: Flavins and flavoproteins 2002 : proceedings of the fourteenth international symposium, St. John's College, University of Cambridge, UK, July 14 - 18, 2002, pp. 193-198

On the Role of the 376-Functional Group in Catalysis by Medium Chain Acyl-CoA-dehydrogenase

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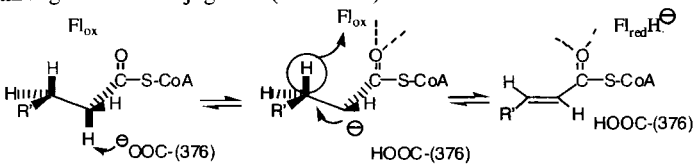
Introduction

Acyl-CoA dehydrogenases catalyze the α,β-desaturation of fatty acids activated through S/CoA conjugation (Scheme 1):



Scheme 1. Chemical mechanism and stereochemistry of acyl-CoA α,β-dehydrogenation by acyl-CoA dehydrogenases.

In a two-step process substrate docks into the active site and then two strong H–bonds form between the CoA carbonyl and active center functional groups [1,2] (see also Scheme 2, below). This induces a strong acidification (polarisation /-activation) of the substrate C_α-H (pK shift >10 pK units from >20 to ≈8) [3,4]. Catalysis then proceeds *via* abstraction of the acyl-CoA C_α-H as H^+ by the carboxylate of Glu-376 (numbering of human medium chain acyl-CoA dehydrogenase, wtMCADH). Next, in a concerted process [5,6], a hydride is expulsed from the substrate pos. β and is transferred to the flavin pos. N(5) [7]. The role of Glu-376 was demonstrated by mutagenesis to 376Gln, this having $\approx 1/10^5$ of the wtMCADH activity [8]. While this clearly identifies Glu376 as the H^+ abstracting base, the observed residual activity was surprising since from the 3D/structure [1] there are no functional groups at the active site that might substitute for Glu376. To clarify this aspect we have reinvestigated some properties of Glu376Gln-MCADH and constructed 376-mutants carrying glycine, histidine, and cysteine. Selected properties of these proteins are reported along with corresponding ones of Glu99Gly-MCADH [9]. The rationale underlying this study was as follows: The Gln group is approx. isosteric with Glu. This should not alter steric interactions at the active center while eliminating the catalytic Glu-group. With Gly376 the space occupied by Glu376 might be replaced by water. This, in turn, could affect the polarity and accessibility of the active site. His is of interest in that it would lead to a much different, possibly to an opposite charge distribution during catalysis. Cys would be a candidate for a role as a base, it would, however, possess a substantially higher microscopic pK_a compared to Glu. At the "bottom" of the active site of MCADH a

second glutamate, Glu99, is present [1,9] that affects the behavior of ligands [9] and might affect ionizations inside the active center cavity. The Glu99Gly replacement would also create a larger cavity at the bottom of the active center, this possibly leading to a modification of polar interactions and of the chain length specificity of MCADH [9].

Materials and methods

E376G, E376H, E376Q, E99G, and E376C-MCADH were obtained as described earlier [9]. After transformation in *E. coli* TG1 the proteins were overexpressed and purified as detailed in [10]. Activities were assessed using the ferricenium assay [11] (10-30 nM wt-MCADH, respectively 0.01-2 μM mutant and 150-200 μM acyl-CoA in 50 mM buffer containing 250 mM KCl at 25 °C).

Results

E376Q-MCADH exhibits an unexpected, and unexplained "residual" activity that we have reported earlier [8]. Also the other mutants studied, E376G, E376H, and E376C appear to be competent in catalysis although at different degrees. For these mutants, the specific activity, or the rate of enzyme flavin reduction (k_{red}) are compared in Table 1. With E376C-MCADH addition of C8-CoA induces a very rapid reduction of enzyme flavin. The enzyme is, however, subsequently converted into an inactive state via a still unclear mechanism.

Table 1. Selected properties of MCADH mutants and comparison with wtMCADH.

Method	Ferricenium assay (V)			Anaerobic reduction (k_{red})		
MCADH	wt	E376H	E99G	wt	E376Q	E376G
V (s^{-1}), k_{red}, pH 8	39	0.01	10	>330[a)]	0.0026	0.0053
V (s^{-1}), k_{red}, extrapol[b)]	97	0.22	14.7	-	0.05	0.01
apparent pK_{app}[c)]	8.2	9.5	7.6	-	8.4	8.5

a) pH = 7.6. b) Extrapolated values at either high or low pH from plot such as in Figs 1 and 2. c) derived from the pH dependence of either k, or V_{max} as shown in Figs 1 and 2. Data for wtMCADH respectively E99G are from [4] and [9].

Remarkably, while with E376H, and E376Q-MCADH the activity is low at low pH and increases with pH reflecting the indicated apparent pK's, with E376G-MCADH the activity/pH profile has the opposite shape. This suggests that the pH dependence (Fig. 1) is, at least in part, due (also) to the ionisation of E99G, this being the only other candidate group at the active center possibly having such a pK.

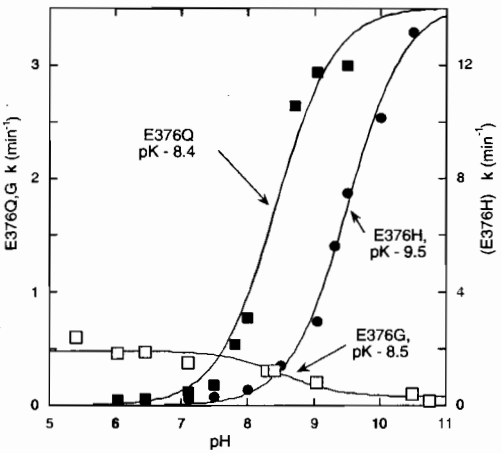


Figure 1: pH dependence of activity parameters for E376H-, E376G- and E376Q-MCADH. With E376G- and E376Q-MCADH the enzyme solution, 6.5 μM was made anaerobic in Thunberg cuvets in the presence of 0.2 μM glucose oxidase and 10 mM glucose. An aliquot of a solution of C_8CoA to yield 50 μM final concentrations was then added from a side arm, and the course of the reaction was monitored by following the A_{450nm} decrease with time. For E376H-MCADH the activity was measured with the ferricenium assay [11]. The fits to the data points were obtained using the pH equation and one ionization. In the case of E376Q and E376H-MCADH the fits extrapolate to zero values for k at low and to 3.5, respectively 14 (min^{-1}) at high pH. For E376G at the low and high pH values are 0.5 and 0.01 (min^{-1}).

The observed activity is strongly dependent on the substrate chain length as has been reported earlier also for wtMCADH [12]. Since E376Q- and E376G-MCADH show a pH dependence of their activity it was suspected that this might reflect also properties of E99-COOH, the group located at the "bottom" of the active site [1]. The behavior of this mutant was thus studied. As demonstrated in Fig. 2. this mutant exhibits maximal activites approaching those of wtMCADH [9]. The profiles of V_{max} versus substrate chain length show that E99G-MCADH has very high activity with "long" substrates rendering it similar to long-chain and to very long-chain acyl-CoA dehydrogenase [9]. Importantly E99G-MCADH functional group did not lead to the disappearance of the pH dependence. Fig. 2 depicts the pH dependence of the term $\log V_{max}$ according to Dixon's conventions [13].

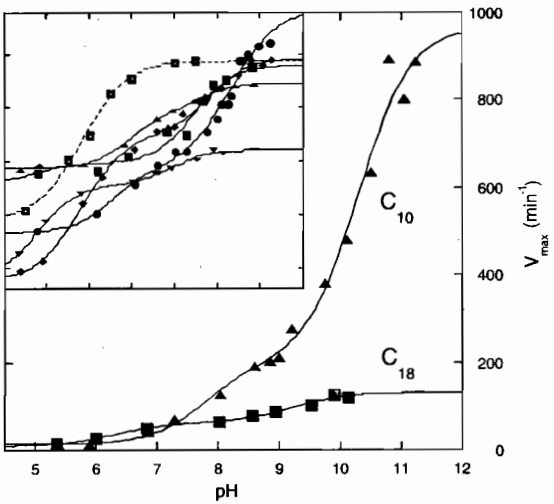
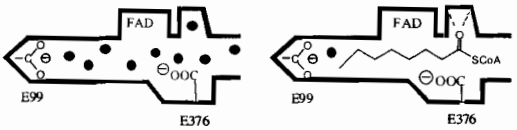


Figure 2. pH dependence of the activity (V_{max}) of E99G-MCADH with the substrates of varying chain length indicated on the graph. *Logarithmic and linear representation.* The fits to the data points (as detailed in Materials and methods) were obtained using a pH equation for two ionizations (respectively single ionization for C8).

Discussion and conclusions

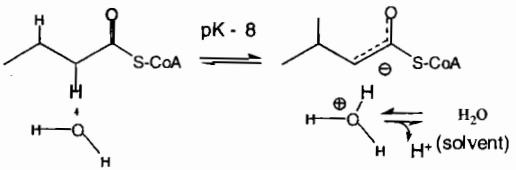
The following points emerge from the present study: a) In most cases activity parameters approach finite values at low pH. b) The inflection points of the logV/pH profiles reflect two apparent pK's for C_8CoA and four for all other substrates. c) The values of the apparent pK's depends strongly from the substrate chain length, however, not in a linear manner (compare e.g. C8, C12, and C18 profiles in Fig. 2). From earlier experiments the pK of the active center base E376-COOH in wtMCADH was estimated as 7-9 [3,4]. The dependence of k_{red} for E376Q- and E376G-MCADH reflects a pK ≈8.5 (Fig. 1). This implies that the (apparent) pK ≈8 of wtMCADH cannot be attributed simply and solely to E376-COOH, although its microscopic ionisation might participate in the observed effect. The second second ionising group at the active center, E99-COOH (See Scheme 2), also cannot be solely responsible for the dependence simply because the E99G mutant shows apparent pK's in the same range as wtMCADH and has similar activity. The E99-COOH group also is too distant from the locus of proton abstraction in order to act directly as a base [1]. A further point that needs clarification is the finite activity at low pH observed in most cases and particularly with the E376G mutant, its k_{red}/ pH profile also being opposite to that of e.g. E376Q-MCADH. In the case of direct involvement of a specific base in a pH dependent activity, the latter should converge towards zero with decreasing pH.

The present results cannot provide a precise answer to the intriguing question about the nature of the base involved in abstraction of the αC-H as H^+ in the case of the E376Q or E376G mutants. Possibly E99G-COO^- could line up with water molecule(s) and mediate the process. Planned studies with a E376X, E99X double mutant could help elucidate this point if they have sufficient residual activity. An involvement of E99-COOH, however, would not explain the activity observed at low pH, where E99-COOH should be protonated (pK ≈ 8, [4]).



Scheme 2. Diagram of the active site of wtMCADH. Left: in the absence of ligand containing H_2O (•) [8]. Right: with bound octanoyl-CoA.

An intriguing possibility is that the observed, low pH reactivity is related to the intrinsic, (pH independent) rate of αC-H dissociation. This could be mediated by water molecules transferring the abstracted H^+ to solvent or to a yet unidentified acceptor as shown in Scheme 3.



Scheme 3. Self-dissociation of C-H in enzyme bound Acyl-CoA, and transfer of the H^+ to solvent. Note that the acidification of the αC-H is induced by the interaction with the protein [3,4].

The present results also demonstrate that the size of the substrate and the presence/absence of specific functional groups that can be modified by mutagenesis have a profound effect on some properties of the active site. This will affect the number of water molecules present and this will modulate the dielectric. The latter, in turn, will affect the ionisations reflected by the pH dependence of activity parameters.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Gh 2/6-4). We thank Drs. C. Thorpe (Univ. Delaware) and J. J. Kim (Univ. Wisconsin) for many fruitful discussions.

References

1. Kim, J. J., Wang, M., and Paschke, R. (1993): Crystal structures of medium-chain acyl-CoA dehydrogenase from pig liver mitochondria with and without substrate. Proc Natl Acad Sci U S A **90**, 7523-7527.

2. 2. Engst, S., Vock, P., Wang, M., Kim, J. J., and Ghisla, S. (1999): Mechanism of Activation of Acyl-CoA Substrates by Medium Chain Acyl-CoA Dehydrogenase:

Interaction of the Thioester Carbonyl with the Flavin Adenine Dinucleotide Ribityl Side Chain. Biochemistry **38**, 257-267.

3. Rudik, I., Ghisla, S., and Thorpe, C. (1998): Protonic equilibria in the reductive half-reaction of the medium-chain acyl-CoA dehydrogenase. Biochemistry **37**, 8437-8445.

4. Vock, P., Engst, S., Eder, M., and Ghisla, S. (1998): Substrate Activation by Acyl-CoA Dehydrogenases: Transition-State Stabilization and pKs of Involved Functional Groups. Biochemistry **37**, 1848-1860.

5. Pohl, B., Raichle, T., and Ghisla, S. (1986): Studies on the reaction mechanism of general acyl-CoA dehydrogenase. Determination of selective isotope effects in the dehydrogenation of butyryl-CoA. Eur J Biochem. **160**, 109-115.

6. Schopfer, L. M., Massey, V., Ghisla, S., and Thorpe, C. (1988): Oxidation-Reduction of General Acyl-CoA Dehydrogenase by the Butyryl-CoA/Crotonyl-CoA Couple. A New Investigation of the Rapid Reaction Kinetics. Biochemistry **27**, 6599-6611.

7. Ghisla, S., Thorpe, C., and Massey, V. (1984): Mechanistic studies with general acyl-CoA dehydrogenase and butyryl-CoA dehydrogenase: evidence for the transfer of the beta-hydrogen to the flavin N (5)-position as a hydride. Biochemistry **23**, 3154-3161.

8. Bross, P., Engst, S., Strauss, A. W., Kelly, D. P., Rasched, I., and Ghisla, S. (1990): Characterization of wild-type and an active site mutant of human medium chain acyl-CoA dehydrogenase after expression in Escherichia coli. *J* Biol Chem **265**, 7116-7119.

9. Küchler, B., Nandy, A., Ghany, A., and Ghisla, S.: Substrate chain length specificity of acyl-CoA dehydrogenases: Studies on different mutants. In: Ghisla, S., Kroneck, P., Macheroux, P., and Sund H. (Eds.): Flavins and Flavoproteins 1999, Weber, R., Berlin (1999), pp. 523-526.

10. Kieweg, V., Kräutle, F.-G., Nandy, A, Engst, S., Vock, P., Abdel-Ghany, A-G, Bross, P., Gregersen, N., Rasched, I., Strauss, A. and Ghisla, S. (1997): Biochemical characterization of purified, human recombinant Lys304Glu medium-chain acyl dehydrogenase containing the common disease-causing mutation and comparison with the normal enzyme. Eur. J. Biochem **246**, 548-556.

11. Lehman, T. C., and Thorpe, C. (1990): Alternate Electro Acceptors for Medium-Chain Acyl-CoA Dehydrogenase : Use of Ferricenium Salts. Biochemistry **27**, 10594-10602.

12. Nandy, A, Kieweg, V., Kräutle, F.-G., Vock, P., Küchler, B., Bross, P., Kim, J.-J., Rasched, I., and Ghisla, S. (1996): Medium/Long Chain Chimeric Human Acyl-CoA Dehydrogenase: Medium Chain Enzyme with the Active Center Base Arrangement of Long Chain Acyl-CoA Dehydrogenase. Biochemistry **35**, 12402-12411.

13. Dixon, M., Webb., E. C., Thorne, C., I., R., and Tipton K. F., Enzymes, Longman, New York (1979).