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MECHANISM OF α,β-DEHYDROGENATION BY ACYL-COA DEHYDROGENASES

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Introduction

Flavin dependent acyl-CoA dehydrogenases constitute a class of some seven mitochondrial enzymes, which are thought to share the same chemical mechanism, but which differ in their specificity for the acyl residue linked to CoA. This class belongs to the acyl-CoA dehydrogenase/oxidase superfamily [1]. For reviews and specific information on the different enzymes we refer to several articles which have appeared recently [2,3]. Acyl-CoA dehydrogenases catalyze the two chemical steps shown in Scheme 1, which have also been termed "reductive half-reaction" (dehydrogenation, 1) and "oxidative half-reaction" (transfer of electrons to electron transferring flavoprotein or ETF, 2):

$$\text{ADH}\sim\text{Fl}_{ox} + \text{R-CH}_2\text{-CH}_2\text{-COS-CoA} \xrightarrow{(1)} \text{ADH}\sim\text{Fl}_{red}\text{H}_2 + \text{R-CH=CH-COS-CoA}$$

$$\text{ADH}\sim\text{Fl}_{red}\text{H}_2 + \text{ETF}_{ox} \xrightarrow{(2)} \text{ADH}\sim\text{Fl}_{ox} + \text{ETF}_{red}\text{H}_2$$

Scheme 1. Reactions catalyzed by acyl-CoA dehydrogenases (ADH). (1) and (2) are the reductive and the oxidative half-reactions. ETF: electron transferring flavoprotein.

These dehydrogenases are essentially "transhydrogenases" and transfer redox equivalents from activated fatty acids to ETF from which they proceed towards the respiratory chain. One of these dehydrogenases, medium chain acyl-CoA dehydrogenase (MCADH), has elicited scientific interest since it was found that a (point) mutation in the gene of MCADH causes one of the most common genetic defects in caucasian populations [2]. Moreover it is possible that such defects play a role in the "sudden infant death syndrome" (SIDS) [2]. MCADH also appears to be the acyl-CoA dehydrogenase with a broad chain length specificity reflected by its earlier name "general acyl-CoA dehydrogenase" [4]. It is also the one having received most

attention from the enzymological, biochemical and mechanistic points of view. Its basic mechanism was outlined in one of the earliest works by Beinert's group, which dealt with its discovery, purification and partial characterization [5]. In the present paper we update our progress achieved in the study of the mechanism of α,β- dehydrogenation reaction and focus on the work from our groups.

The chemistry of the α,β-dehydrogenation of acyl-CoA substrates by flavin enzymes.

This reaction (1, Scheme 1) is rather unusual because it involves the rupture of two kinetically stable C-H bonds as well as the transfer of the resulting redox equivalents to the oxidized flavin cofactor. The following mechanistic points have been elucidated in the recent past and shall only be recalled briefly:

a) The stereochemistry of rupture of the α- and β-C-H bonds is proR, proR trans [6,7], i.e. the two hydrogens "leave" the respective carbons in opposite directions during double bond formation. This demands that the functions involved must be placed on opposite sites of the substrate (see also Scheme 4, below).

b) The α-hydrogen is abstracted as a proton. This results from experiments demonstrating that elimination can occur from position β [8], from rearrangement reactions of substrate analogs [9], and from the finding of exchange of hydrogen exchange at this position [10,11].

c) The ß-hydrogen is transferred to the flavin as a hydride and specifically to the flavin position N(5) [10]. Because this occurs *via* the empty π-orbital (LUMO) of the N(5), substrate and the ß-CH bond are positioned on the flavin plane (see also Scheme 4, below).

d) Cleavage of the α- and the β-bonds occurs in a concerted fashion [12]. This is opposed to the two other possible ways, occurrence of intermediates such as an α-carbanion and a synchronous mode.

e) From a thermodynamic point of view, the reductive half-reaction does not proceed to completion upon saturation of the binding step with substrate. It reaches an equilibrium

$$E_{ox} + S \rightleftharpoons E_{ox}{\sim}S \rightleftharpoons E_{red}{\sim}P \rightleftharpoons E_{red} + P$$

which depends on the chain length of the substrate [13,14]. The kinetic of substrate dehydrogenation is consequently an approach to equilibrium [15].

It should be pointed out that the statements under a-d) do not preclude the fast, sequential transfer of electrons, in other words: hydride transfer could be mimicked by rapid transfer of two electrons and one proton. However, with other flavin dependent dehydrogenases, radical intermediates have never been observed during flavin reduction by C-H substrates.

Activation of acyl-CoA substrates and modulation of ADH reactivity.

To bring about the chemical events outlined above (Scheme 1) the following is required:

1) A basic group which initiates abstraction of the α-hydrogen as H^+.
2) Interactions which lower the pK_a of the substrate α-H and increase that of the base in order to give comparable values as required for efficient catalysis.
3) Interactions which modulate the redox potential of the flavin, and affect the relative strength of binding of substrate and product.
4) An effective shielding preventing "loss" of redox equivalents e.g. by reaction of the reduced enzyme with oxygen .

1) Glutamine 376 is the base involved in α-H^+ abstraction in MCADH.

Fendrich and Abeles work [10] with suicide inhibitors of the acetylenic type was crucial in suggesting that a glutamic acid residue was responsible for this reaction. This was supported later by Powell et al. [16], who identified a glutamic acid containing peptide from pig kidney MCADH linked to a rearranged suicide inhibitor. The three dimensional structure of MCADH shows that indeed Glu376 is placed adjacent to the substrate α-H [17,18]. We have shown [3,19] that exchange of Glu376 with Gln in MCADH yields a mutant with drastically lowered catalytic activity confirming the role of this glutamate. Other mutations at the same position affect similar catalytic activity.

While this evidence appears to be conclusive in demonstrating that Glu376 is indeed the base referred to, several collateral questions remain open: A survey of 15 acyl-CoA dehydrogenase sequences (Figure 1) shows that Glu is conserved at positions corresponding to 376 in MCADH in most acyl-CoA dehydrogenases. Moreover, isovaleryl- (IVDH) and long-chain acyl-CoA dehydrogenase (LCADH) have a Gly- or Ala-residue at the position corresponding to Glu 376. The question thus arises about the functional group involved in the α-H^+-abstracting role in these two latter enzymes. The three-dimensional structure of MCADH shows the presence of a Thr at position 255, this residue is located on the G-domain α-helix parallel to the K-domain α-helix on which one finds Glu376. At the corresponding positions in LCADH and IVDH there is a Glu which could thus play the role of Glu376 in MCADH. As reported in this volume [19] the "double mutant" Glu376Gly + Thr255Glu, called "MLCADH", has catalytic properties similar to those of LCADH. This proves that indeed Glu261 and Glu254 are the proton abstracting bases in LCADH and IVDH.

A further point of interest is the observation of a "residual activity" of the Glu376Gln MCADH mutant. Its rate of flavin reduction by octanoyl-CoA is $\approx 3 \cdot 10^6$-fold smaller than that of

wtMCADH; this activity is, however, still substantially higher than that of the uncatalyzed reaction. We do not have a plausible answer for this, although some speculative possibilities might be mentioned: The observed rate might be due to the "intrinsic" (uncatalyzed or solvent mediated) rate of α-deprotonation of substrate at the active center due to the activation of the latter by its interaction with the protein (cf. also 2)). The specific activity of the purified Glu376Asp mutant is ≈10 % that of wtMCADH, thus in line with expectations, that of the Glu376Gly MCADH <1%. We are currently constructing mutants carrying other residues at position 376 and are planning to study this point systematically. Ongoing crystallographic work in the laboratories of J.J. Kim with the Glu376Gln mutant should clarify this issue.

Enzyme	Source	Sequence
		350 370 380
AcOx	candida t.	RQAC**GGHG**HSSYNGFGKAYNDWVVQCTW **E** **G**DNNVLGMSVGK
AcOx	yeast	RQTC**GGHG**YSQYNGFGKGYDDWVVQCTW **E** **G**DNNVLSLTSAK
AcOx	rat	RMAC**GGHG**YSHSSGIPNIYVTFTPACTF **E** **G**ENTVMMLQTAR
Consensus	*Oxidases*	*R CGGHG S G Y CT* ***E G*** *V*
GCDH	human	RDML**GGNG**ISDEYHVIRHAMNLEAVNTY **E** **G**THDIHALILGR
GCDH	pig	RDML**GGNG**ISDEYHVIRHAMNLEAVNTY **E** **G**THDIHALILGR
SCADH	rat liver	IQIL**GGMG**YVTEMPAERYYRDARITEIY **E** **G**TSEIQRLVIAG
SCADH	human	IQIL**GGMG**YVTEMPAERHYRDARITEIY **E** **G**TSEIQRLVIAG
SCADH	rat	IQIL**GGMG**YVTEMPAERHYRDARITEIY **E** **G**TSEIQRLVIAG
MCADH	rat	VQIF**GGYG**FNTEYPVEKLMRDAKIYQIY **E** **G**TAQIQRLIIAR
MCADH	pig liver	VQVF**GGNG**FNTEYPVEKLMRDAKIYQIY **E** **G**TAQIQRIIIAR
MCADH	human	VQIL**GGNG**FNTEYPVEKLMRDAKIYQIY **E** **G**TSQIQRLIVAR
Cons. G+S+M CADH		*GG G E Y* ***E GT*** *I*
IVDH	rat liver	IQCL**GGNG**YINDFPMGRFLRDAKLYEIG G **G**TSEVRRLVIGR
IVDH	human	IQCF**GGNG**YINDFPMGRFLRDAKLYEIG A **G**TSEVRRLVIGR
LCADH	rat liver	VQLH**GGWG**YMWEYPIAKAYVDARVQPIY G **G**TNEIMKELIAR
LCADH	human liver	VQLH**GGWG**YMWEYPIAKAYVDARVQPIY G **G**TNEIMKELIAR
Consensus	*IV+L CADH*	*Q* ***GG GY*** *P DA I* ***GT E*** *I R*
Consensus	*all CADH*	*Q* ***GG G*** *DA I* ***GT***
		↑ ↑
		352 376
Consensus, all		***GG G*** ***G***

Figure 1: Comparison of the C-terminal sequences of enzymes capable to carry out the α,β-dehydrogenation reaction. The numbering refers to that of mature MCADH. Abbreviations: AcOx: Acyl-CoA oxidase; GCDH: Glutaryl-CoA-dehydrogenase: SCADH: short-chain acyl-CoA dehydrogenase; MCADH: medium-chain acyl-CoA dehydrogenase; IVDH: iso-valeryl-CoA dehydrogenase; LCADH: long-chain acyl-CoA dehydrogenase. The sequence of GCDH is from [21], that of pig liver MCADH from [22], all others are from the PIR protein data bank of MIPS, Martinsried, Germany.

On the pK of Glu376

Turnover by MCADH was first reported to be pH dependent by Murfin [23]. Apparent pK's between 7 and 8 - depending on the substrate - were observed also by others [24, 25]. The hypothesis was then put forward [3] that this pK might reflect the ionisation of Glu376. We have investigated this issue in some detail and have found that with both human wt- and pig kidney MCADH, octanoyl-CoA as substrate, and using the ferricenium assay the activity increases approx. 13-fold on going from pH 5 to pH 9.5, and reflects an apparent pK ≈ 8.2, respectively ≈ 8.3 as shown in Figure 2.

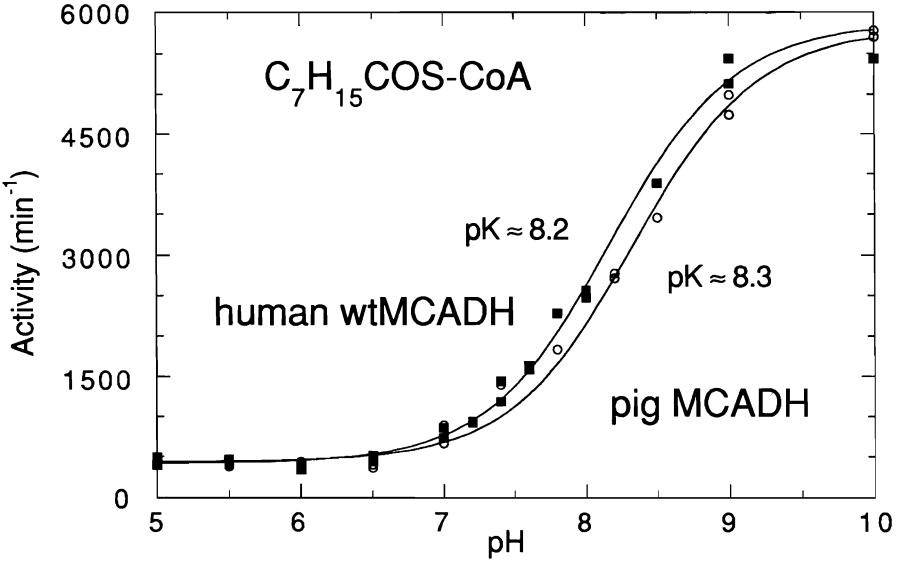


Figure 2. pH dependence of the activity (ferricenium assay [26]) of human wt- and of pig kidney MCADH using a fixed concentration of 100 μM octanoyl-CoA. K_m determinations at low and high pH have shown that this concentration is saturating. The pH values were adjusted with KOH for MES-, HEPES- and TRICINE- or HCl for TRIS-buffer (20mM) and in the presence of 100 mM KCl. The curves represent the best fit obtained using the pH/velocity relationship equation.

While the specific activities at the lower and upper ends of the dependence appear to be the same within the limit of the experimental error for both enzymes (Figure 2), we think that the difference in pK is real since parallel measurements of activities around the pK region consistently show that human wtMCADH has a slightly higher activity as compared to the pig one. This might be due to minor differences between the two active centers. At position 379 of human wtMCADH there is a Ser while in the pig enzyme one finds an Ala (Figure 1). Along this line the Tyr375Phe mutant shows a pK ≈ 7.3 (octanoyl-CoA, not shown), which is thus

lowered by a full unit compared to wtMCADH. This illustrates the pitfalls of such activity comparisons, in particular when dealing with mutants: In a previous report [3] we have stated that this mutant is "more active" than wtMCADH. While this is correct for pH 7.6, where the measurements were done, at high pH values the mutant has approx. 55% of the wildtype-activity.

The pK of the activity profile is also dependent from the chain length of the substrate, and with protio-butyryl-CoA we find a pK ≈ 7.3 (Figure 3). The activity with perdeuterobutyryl-CoA is approx. the same in the lower pH region (i.e. there is no deuterium isotope effect), while at high pH values the deuterium isotope effect is ≈ 2.3. Surprisingly there is a large pK shift for the deuterated analog, the reasons for which are still unclear. For the step of flavin reduction (Scheme 1,1) a very large isotope effect of ≈ 30 has been found at pH 7.6 [12]. It is thus clear that at low pH values the rate of flavin reduction (Scheme 1, 1) is not rate limiting, and that at high values it is probably only partially so. The shift in the apparent pK might also reflect a change in mechanism on going from low to high pH.

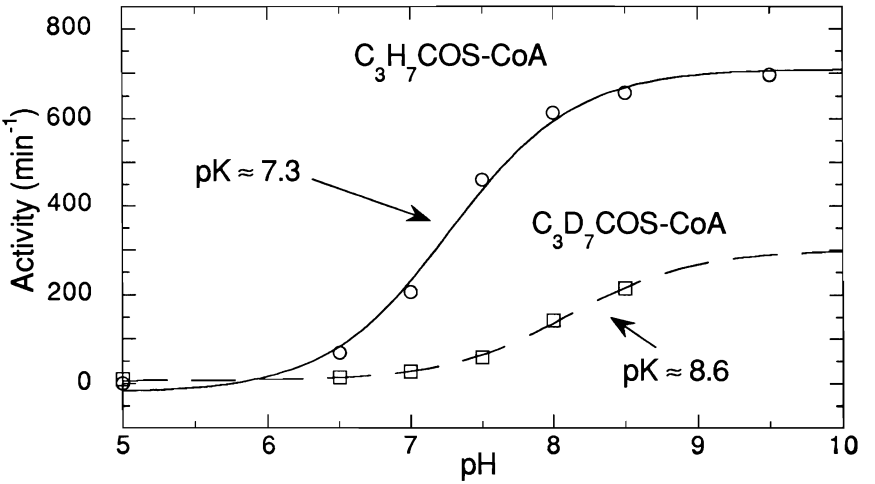


Figure 3. pH dependence of the activity of wtMCADH (ferricenium assay [26]) determined using butyryl-CoA and its fully deuterated analogue. The values shown were determined by extrapolation from Michaelis-Menten plots. Conditions as detailed in the legend of Figure 2.

As mentioned above, the Glu376Gln MCADH mutant shows a low, but authentic flavin reduction activity by octanoyl-CoA [3]. Puzzling is the pH dependence of this reaction, which increases ≈ 50-fold on going from pH 6 to 9, and which reflects a pK ≈ 8.4 (Figure 4). Glu376 cannot have the same pK as Gln376 and this result thus also argues against the pK in question being that of Glu376. Results obtained with the p-nitrophenylacetyl-CoA analog, while still preliminary, also support this conclusion.

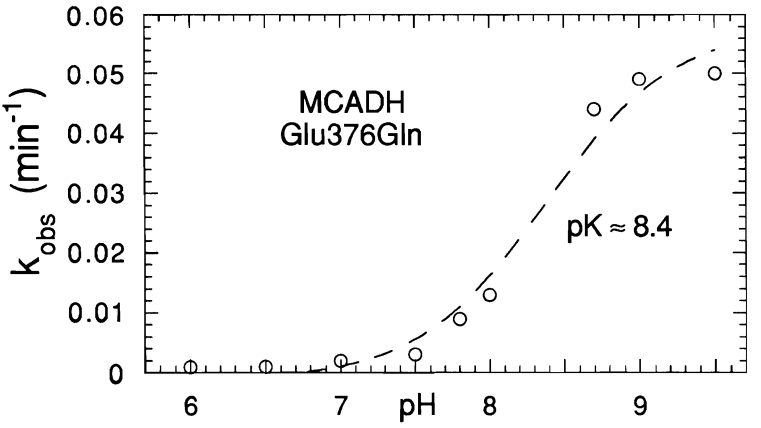
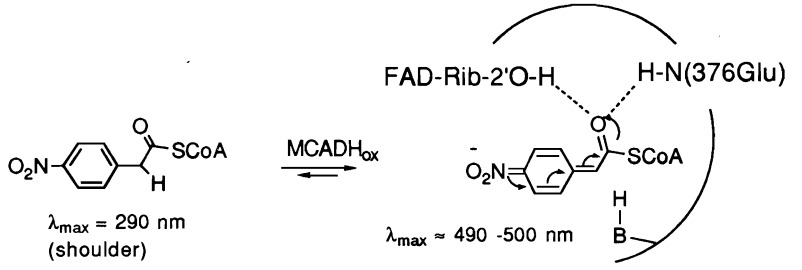


Figure 4. pH dependence of the rate of Glu376Gln-MCADH-flavin reduction measured in anaerobic experiments as detailed earlier [3]. Buffer conditions see Fig. 2.



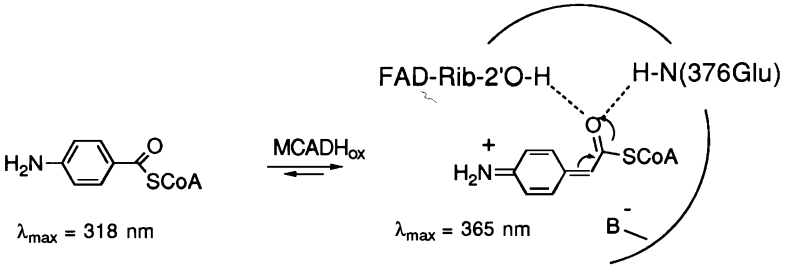
Scheme 2: p-Nitrophenylacetyl-CoA and postulated mode of interaction with two active center hydrogen bridges (R-H). The base -B deprotonates the analog to form the delocalized carbanion. See also legend to Scheme 3.

This analog can undergo deprotonation at position α, but cannot reduce the flavin, and the anionic species formed can be regarded as a transition state analog. That deprotonation occurs is manifested by the formation of a new chromophore belonging to the anionic form (Scheme 2). Glu376 is most probably involved in H^+ abstraction since the rate of deprotonation is drastically reduced with the Gln376 mutant. With wt, i.e. Glu376-MCADH, however, there is no pH dependence on the rate of formation of the anion. In this specific case some caution is due in the interpretation, since p-nitrophenylacetyl-CoA is a large molecule and at present it cannot be excluded that the rate limiting step in the observed process could be one preceding proton abstraction. Unfortunately, with p-nitrophenylacetyl-CoA verification of this assumption e.g, *via* measurement of a deuterium isotope effect on the rate of anion formation, is not feasible since hydrogen exchange at position α is too rapid. Attempts to resolve the issue using

similar analogs are underway. In conclusion to this paragraph, we feel that other functional groups or the fully reduced flavin (pK of free form ≈ 6.7) should be considered as having the pK in question.

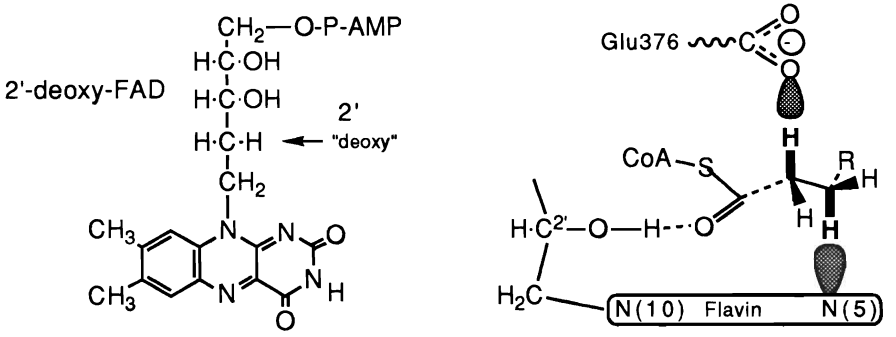
2) Modes of activation of the substrate α-H.

The mechanism of this activation is straight forward and has been suggested by various authors in the past. It consists in an interaction of a (partial) positive charge with the substrate thioester carbonyl as shown in scheme 2. The finding that the Glu376Gln mutant does catalyze the deprotonation of p-nitrophenylacetyl-CoA (scheme 2) and does stabilize its anionic form supports this concept. For p-nitrophenylacetone, the α-pK_a of which should be comparable to that of a thioester [27], a pK_a ≈13.6 was determined. Since only the anionic form of the MCADH bound species is found in the pH range 6 - 9 the α-pK_a of 4-nitrophenylacetylCoA must be lowered by at least 7 units. p-Aminobenzoyl-CoA is a further analog which we have found to reflect the electronic interactions and dipoles present at the active center. Upon binding to MCADH a new absorption band appears, which is attributed to delocalization of the free electron pairs of the amino group as shown in scheme 3.



Scheme 3. Mode of interactions of the substrate or analog thioester carbonyl group with the protein. In this specific case of p-aminobenzoyl-CoA the free electron pair of the p-amino group is delocalized towards the thioester carbonyl group as a consequence of the two hydrogen bridges formed by the ribityl-2'OH and by the Glu376 backbone N-H.

The interactions which bring about these effects have been identified and consist of two hydrogen bridges pointing towards the thioester carbonyl as shown in Schemes 2, 3 and 4. One involves the riboflavin 2'-OH function. This interaction has been suggested from the three-dimensional structure [17,18]. In order to prove it we have synthesized 2-deoxy-FAD (Scheme 4) and used it to reconstitute pig kidney MCADH from apo enzyme. The artificial 2-deoxy-FAD-MCADH binds octanoyl-CoA, albeit weaker as compared to wtMCADH. It is essentially devoid of activity, the flavin being reduced at a rate ≈ 10^6 times smaller than that of wtMCADH. The extent of reduction is also much smaller, ≈50% of the total flavin, compared to wtMCADH (> 95%) and indicating a modification of the redox potentials involved in the couples (Scheme 1, 1).



Scheme 4. Structure of 2'deoxy-FAD, left, and schematic representation of the interaction of the ribityl-2'-OH with the substrate thioester carbonyl (right). The flavin in the latter is shown from its side, with the empty N(5) orbital in a position to accept the substrate β-hydride. Substrate is placed on top of the flavin plane, and its α- and β-hydrogens are located in a proR, proR-trans orientation. Glu376 interacts with the α-hydrogen from the opposite side.

These results confirm the involvement of the ribityl-2'-OH function in the activation of substrate, and represent the first demonstration of a role of a flavin function different from the isoalloxazine ring in catalysis. The difference in activation energy between the 2'-deoxy-FAD and normal enzyme is ≈ 9 kcal and thus more than the amount which can be provided by a single hydrogen bond.The second hydrogen bridge is between the thioester carbonyl and the Glu376 backbone N-H. These two hydrogen bridges probably have the dual function of activating the thioester as well as of fixing the orientation of the substrate at the active center.

3) and 4). Interactions which modulate the redox potential of the flavin and affect the relative strength of binding of substrate and product. Steering of reactivity with acceptors.
Information on these topics is still scarce. The three-dimensional structure [17,18] does not show the presence of positive charges around the flavin pyrimidine ring, as is the case with flavoproteins having a high redox potential such as e.g. lactate oxidase and the related enzymes [28]. On the other hand, the pyrimidine functional groups appear to be inserted in a net of hydrogen bonds [17,18] and this is likely to contribute to the increase of the redox potential of MCADH. The factors which differentiate an oxidase from a dehydrogenase have not been identified although they are likely to reside in a control of accessibility of reduced flavin to oxygen. In fact tight binding of product prevents oxygen activity [29] and the exchange of Tyr375, which is bound to neighboring functions, with Phe increases the oxygen sensitivity of this reduced MCADH, probably due to increased mobility of the active center [3].

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