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SOME PROPERTIES OF GLU-376-GLN ACTIVE SITE MUTANT OF HUMAN MEDIUM-CHAIN ACYL-COA DEHYDROGENASE

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Introduction

We have reported recently on the expression of wild-type human medium chain acyl-CoA dehydrogenase (MCADH) and of an active site mutant [1]; both proteins having been purified to apparent homogeneity. Glu376 has been proposed by Powell and Thorpe [2] to be an essential residue and possibly the α-proton-abstracting base. This residue was mutated to Gln. The glu376-gln mutant was isolated in an essentially inactive form, which is green because of bound CoA-persulfide. The yellow form was prepared [3], it reduces substrate at a very low rate. We present spectroscopic and kinetic data on the substrate reaction of this mutant, which can be monitored with a conventional spectrophotometer. The interaction with several substrate analogues was studied.

Results and Discussion

Substrate reaction

Figure 1 shows the spectrum of the glu376-gln mutant immediately after addition of 25 equivalents of octanoyl-CoA. A characteristic red shift of the flavin spectrum has

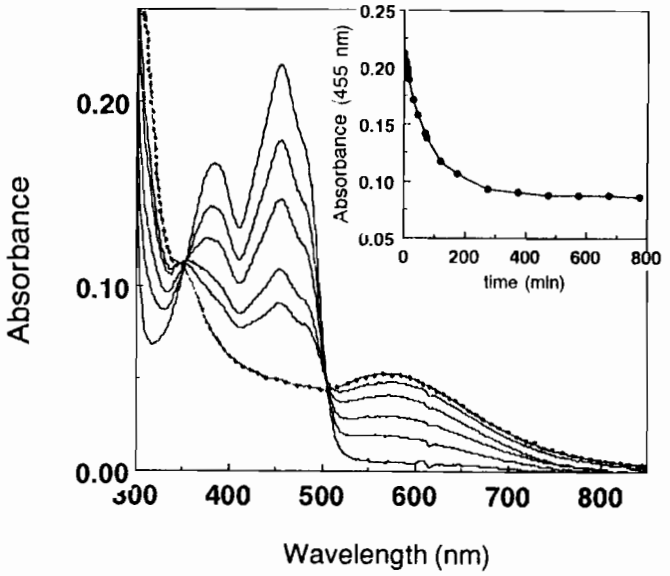

Fig.1: Anaerobic reduction of gln376 MCADH [$1.61 \cdot 10^{-5}$ M] upon addition of 24.9 equivalents octanoyl-CoA, spectra have been recorded immediately after addition and after 28, 70, 175 and 975 min (solid lines).Conditions: 100 mM potassium phosphate , pH 8, 0.3 mM EDTA at 5° C. Maximal bleaching of gln 376 MCADH after addition of 5 equivalents octanoyl-CoA at 25° C (dashed line).

occured, which is indicative of formation of the Michaelis complex.Similar spectral perturbations have been reported upon binding of α,ß deuterated butyryl-CoA [4]. In the latter case catalytic reduction of the flavin was slowed down extremely due to the low temperature and the α,ß-deuteration of the substrate. As shown in figure 1 the mutant does undergo reduction with concomitant formation of the typical longwavelength band. However the rate of reduction is drastically slowed down. The process is biphasic with half lives of 84 min and 303 min. The fast phase corresponds to about 90% of the total spectral changes at 455 nm. Note, that reduction of native MCADH occurs within milliseconds [4]. The extent of bleaching has been shown to reflect the equilibrium between

$E(FAD_{ox})\cdot SH_2$ and $E(FADH_2)\cdot S$. The values observed in our experiments (fig 1) are similar to those reported by Thorpe et al.[5]. This suggests, that the internal equilibrium is not affected by the glu-gln exchange.

Binding of substrate analogues

The interaction of acetoacetyl-CoA and 4-nitrophenylacetyl-CoA with glu376-gln MCADH have been of particular interest, since both compounds are α-C-H acids, their pKa is 8.5 and 13-15 (see separate paper in this volume). Binding of acetoacetyl-CoA to glu376-gln MCADH (data not shown) induces formation of a charge transfer absorption at 520 nm and a strong increase in absorption at 300 nm, which is indicative of the presence of the α-deprotonated analogue at the active site. All spectral changes, which are closely similar to those observed with wildtype MCADH, were complete before spectra could be recorded. Spectral changes occuring upon binding of 4-nitrophenylacetyl-CoA are shown in figure 2a and 2b; they are similar to those observed with wildtype MCADH (see separate paper, in this volume). A longwavelength absorption at 710 nm and a strong increase in absorbance at 468 nm clearly indicate that deprotonation has occured. However these spectral changes occur very slowly (figure 2b) in contrast to wildtype enzyme. This reaction is biphasic with a rapid phase of $t_{1/2}$~3.3 min which comprises 76% of the total spectral changes. These data clearly indicate that the glu376-gln mutant is competent in α-deprotonation. On the other hand the slowness of the reaction clearly shows that residue 376 does play an important role in catalysis.

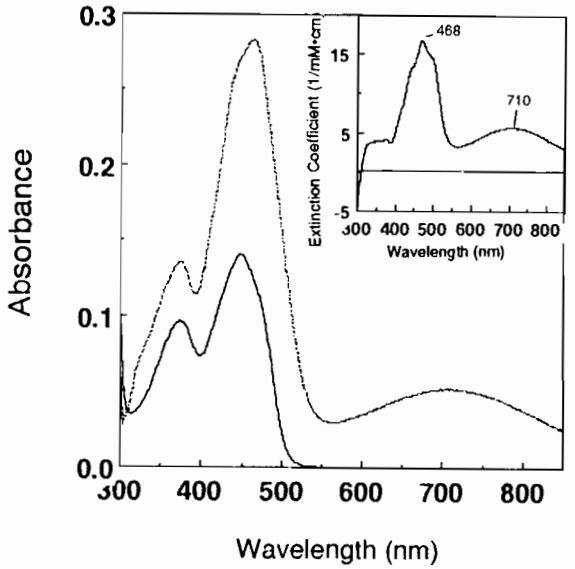

Fig.2a: Spectrum of gln376 MCADH [$9.34 \cdot 10^{-6}$ M] (solid line) and spectrum 2h after addition of 52.4 equivalents of 4-nitrophenylacetyl-CoA (dashed line, corrected for dilution). Conditions: potassium phosphate 100 mM, pH 8, 0.3 mM EDTA. The inset shows the difference between the two spectra.

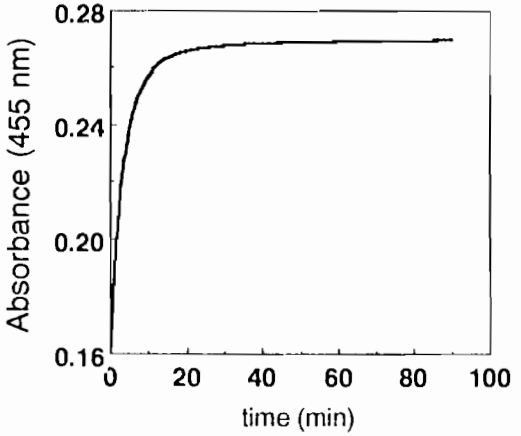

Fig.2b: Spectral changes after addition of 24.8 equivalents of 4-nitrophenylacetyl-CoA to gln376 MCADH [$1.1 \cdot 10^{-5}$ M] in 100 mM potassium phosphate , pH 8, 0.3 mM EDTA at 25° C.

Binding parameters of the analogues to the mutant are summarized in table 1 and compared to recombinant wildtype MCADH. Binding to the gln376 mutant is approximately one order of magnitude tighter than to wildtype enzyme. This suggests that the glu376-gln exchange specifically affects substrate binding. This finding may explain why the gln376 mutant was isolated as a complex with CoA-persulfide. The charge transfer absorption maxima observed upon binding of CoA-persulfide, acetoacetyl-CoA and 4-nitrophenylacetyl-CoA to the mutant are ~30nm blue-shifted as compared to wildtype enzyme.

Table 1	Wildtype MCADH	Glu376-gln MCADH	$\frac{K_d glu}{K_d gln}$
Acetoacetyl-CoA K_d λ_{max}	9.1x 10-6 M 550 nm	1.0x 10-6 M 520 nm	9.1
4-Nitrophenylacetyl-CoA K_d λ_{max}	2.3x 10-5 M 740 nm	2.4x 10-6 M 710 nm	9.6
4-Aminobenzoyl-CoA K_d	5.2x 10-5 M	5.5x 10-6 M	9.5
CoA-persulfide λ_{max}	710 nm	675 nm	

Conclusions

The gln 376 mutant is competent in substrate oxidation, in deprotonating α-C-H acidic analogues and in stabilizing the corresponding carbanions. However the rates of substrate oxidation and deprotonation of 4-nitrophenylacetyl-CoA are drastically slowed down (substrate oxidation approx. 1/1000 -1/10000) and suggest that glu376 plays a crucial role in the early events of α,β-dehydrogenation. Were glu376 the proton-abstracting

base, it is hard to imagine that gln 376 could play this role; thus deprotonation on the gln376 mutant possibly involves a different proton acceptor, e.g. a water molecule or an appropriate functional residue in the active center. Alternatively, glu376 may serve as a final proton acceptor in a charge relay system and thus may not act as the proton-abstracting base itself.

References

1. Bross, P., Engst, S., Strauss, A. W., Kelly, D. P., Rasched, I. & Ghisla, S. (1990) J. Biol. Chem. 265, 7116-7119
2. Powell, P. J. & Thorpe, C. (1988) Biochemistry 27, 8022-8028
3. Williamson, G., Engel, P., Mizzer, J. P., Thorpe, C. & Massey, V. (1982) J. Biol. Chem. 257, 4314-4320
4. Pohl, B., Raichle, T. & Ghisla, S. (1986) Eur. J. Biochem. 160, 109-115
5. Thorpe, C., Matthews, R. G. & Williams, C. H. (1979) Biochemistry 18, (2), 331-337