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Medium-Chain Acyl CoA Dehydrogenase: Evidence for Phosphorylation

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Mature medium chain acyl-CoA dehydrogenase isolated from pig kidney (pkMCADH) and originating from mitochondria carries a phosphate group as demonstrated by ^{31}P-NMR-spectroscopy and chemical analysis. Two broad resonances at –6.3 and –8 ppm are observed and are assigned to the pyrophosphate group of the cofactor FAD. A third, narrow resonance at 4.65 ppm indicates the presence of a phosphomonoester residue. Chemical analysis of intact pkMCADH shows the presence of 3 ± 0.3 phosphates, those of FAD and of an additional covalently attached phosphate. With recombinant, human wild type MCADH expressed in and purified from *E. coli* only the two FAD phosphates (2 ± 0.35) are found. Similarly, pkMCADH which has been converted to the apoenzyme and reconstituted to holoenzyme also contains 2 ± 0.4 phosphates. The covalently bound phosphate can be hydrolyzed by phosphatase and subsequently removed by dialysis. The phosphate group has no detectable effect on the catalytic activity of the MCADH measured with artificial and natural electron acceptors such as pig electron transferring flavoprotein. However, phosphorylation has a marked effect on protein solubility which is ⊕5-fold lower for the dephosphorylated protein.
Key words: Flavin / β-oxidation / ^{31}P-NMR.

Introduction

Mammalian fatty acid acyl-CoA dehydrogenases are members of a family of flavoproteins (Nandy et al., 1996; Tanaka and Indo, 1992) that catalyze the dehydrogenation step of fatty acid CoA conjugates, the first, and rate limiting step in β-oxidation. With the exception of very long chain acyl-CoA dehydrogenase (Aoyama *et al.,* 1995) all these dehydrogenases are soluble and consist of four identical subunits containing one FAD, and have a molecular mass ≈ 45 kDa. In recent years considerable effort has been put into the investigation of the mechanisms of these enzymes (Ghisla and Massey, 1989; Thorpe and Kim, 1995). Several of them have been cloned, expressed, and purified in their recombinant forms (Kieweg *et al.,* 1997; Nandy *et al.,* 1996; Peterson *et al.,* 1995; Mohsen and Vockley, 1995), and the latter have been used extensively for biochemical studies also involving active site directed mutagenesis. While the differences in the primary sequence of recombinant MCADH's compared to those present in mitochondria might be restricted to the first amino acid(s) at the N-terminus, further post-translational changes may occur upon import into mitochondria. Such chemical modifications can affect the general as well as the catalytic properties of enzymes, and thus an assessment of their effect can be of basic importance, in particular when conclusions drawn from studies with recombinant enzymes are to be extrapolated to native forms.

This is of importance also since acyl-CoA dehydrogenases have been recognized to be the source of medically relevant genetic defects (Gregersen, 1985, Roe and Coates, 1995). In particular the K304E-MCADH mutation (Kieweg *et al.,* 1997) is among the most common found in humans (Ziadeh *et al*., 1995). During our studies of various mutants of human MCADH, and specifically of the one above, we have observed differences in the electrophoretic migration behavior between native MCADH and recombinant proteins expressed heterologously (Bross *et al*., 1995). These differences could not be attributed solely to the change in charge resulting from the mutation itself since they were also observed between wild-type MCADH expressed in *Escherichia coli* and fibroblasts, respectively (Bross *et al*., 1995). Such differences complicate the interpretation of effects resulting from point mutations (Bross *et al*., 1995). They have been described as possibly resulting from a (pH dependent) difference in charge, which in turn originates from a difference in phosphorylation, since the wild-type enzyme is not subjected to post-translational modification when expressed in *E. coli* (Bross *et al*., 1995). This was a stimulus to verify whether mature MCADH as isolated from mitochondria is phosphorylated and to assess the possible effects of this modification.

Results

^{31}P-NMR Studies

The ^{31}P-spectra of MCADH from pig kidney shows three resonances (Figure 1A). The two broad upfield ones at

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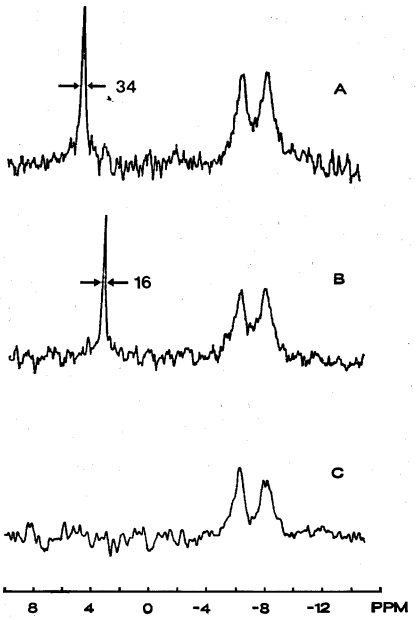

Fig. 1 31P-Spectra of Pig Kidney MCADH.
All spectra were obtained with a 270 MHz instrument, with a pulse recycle time of 2 s and a pulse width of 12 μs which equals a flip angle of 30. For each spectrum 30 000 transients were accumulated and further processed using an exponential multiplication with a line broadening factor of 20 Hz. (A) Spectrum of native pkMCADH (0.4 mM in standard buffer, pH 8.5, measured at 305 K). The two broad resonances at −6.3 ppm and −8 ppm are assigned to the pyrophosphate bridge of FAD. The phosphorus resonance at 4.65 ppm has a half line width of 34 Hz. (B) Effect of alkaline phosphatase. (See Materials and Methods for details). Note that the two resonances attributed to the FAD pyrophosphate are unaltered, while the signal at 4.65 ppm has shifted to ≈ 3.1 ppm (half line width 16 Hz). (C) Effect of dialysis against standard buffer, pH 7.6.

−6.3 ppm and −8.08 ppm are assigned to the two phosphorus atoms of the pyrophosphate moiety of FAD, which in free solution resonate at −9.8 and −10.3 ppm. They are thus shifted downfield upon binding to apo-MCADH. According to Kainosho and Kyogoku (1972) the upfield resonance of the pyrophosphate group (−10.3 ppm) in free FAD is due to the phosphorus atom of AMP and the downfield one (−9.8 ppm) from that of the FMN moiety. Such an unambiguous assignment of the phosphorus resonances is not feasible in our case since the relative magnitude of the downfield shift is not known. The third signal at −4.6 ppm has a much narrower line width and stems from a phosphate monoester which is probably at least partially exposed to solvent. Treatment of a sample of pkMCADH with alkaline phosphatase leads to disappearance of the 4.6 ppm resonance, which is replaced by a new one at 3.2 ppm (Figure 1B). The new signal at 3.2 ppm (16 Hz) is considerably narrower compared to the parent one (34 Hz). The two FAD phosphorus resonances are not affected. The upfield shift in question can be interpreted in terms of phosphomonoester cleavage resulting in the generation of free phosphate. However, after exhaustive dialysis of the sample against standard buffer, pH 8.5, the resonance at 3.2 ppm did not disappear indicating a very tight binding of the phosphate group to the protein. Removal was achieved by dialysis at a lower pH, i.e. pH 7.6 (Figure 1C). Dialysis of untreated pkMCADH at pH 8.5 or 7.6 did not shift or remove the resonance of the phosphorus signal.

With recombinant E376D-MCADH expressed in *E. coli* the signal at 4.6 ppm was absent, the phosphorus signals deriving from the FAD moiety, however, appeared at the same resonance frequency. This suggests a post-translational phosphorylation of pkMCADH which cannot occur in bacteria.

Reduction of an anaerobic sample of pkMCADH with a threefold excess of dithionite did not alter the chemical shift of the three phosphorus resonances (spectra not shown) suggesting that reduction of the flavin does not cause significant changes in the environment of the phosphorus atoms.

In order to probe the accessibility of the phosphorus atoms $MnCl_2$ was added to a sample of pkMCADH. This resulted in a considerable line broadening of the signal at 4.65 ppm while the two resonances assigned to the pyrophosphate moiety of FAD are not affected by addition of manganese. This clearly demonstrates that FAD is buried in the protein rendering the phosphorus atoms inaccessible to solvent borne reagents. The phosphorus resonating at 4.65 ppm, however, appears to be readily accessible to solvent in accordance to the observation that alkaline phosphatase is capable of cleaving the covalent linkage.

The chemical shift of the downfield resonance showed a considerable dependence on pH: with increasing pH the resonance is shifted downfield (pH 7.6 = 4.24; pH 8.5 = 4.65).

Determination of Phosphorus Content of pkMCADH

Chemical analysis of phosphorus was performed according to the procedure described in Materials and Methods and revealed the presence of 3 ± 0.3 phosphorus atoms per subunit of pkMCADH. Since the protein has tigthly bound FAD ($K_d < 10^{-7}$ M, Mayer and Thorpe, 1981) two phosphorus atoms are due to the FAD content. In contrast to the pig kidney enzyme, analysis of recombinant Y375G-MCADH expressed in and isolated from *E. coli* showed the presence of 2 ± 0.35 phosphorus atoms per enzyme subunit. Similarly, when apo-protein derived from pkMCADH was reconstituted with FAD, the resulting holo-pkMCADH enzyme showed only the presence of 2 ± 0.4 phosphorus atoms per enzyme subunit indicating that one was lost during the apo-protein preparation/reconstitution.

Catalytic Activity of MCADH and of Dephosphorylated MCADH

Using phenazine methosulfate (PMS) as electron acceptor no difference in the specific activity is apparent between phosphatase-treated and untreated pkMCADH. However, PMS is an artificial electron acceptor and it is therefore conceivable that only the natural occurring elec-

tron acceptor, electron transferring flavoprotein (ETF), is able to distinguish between the phosphorylated and dephosphorylated forms of pkMCADH. Regulation of enzymes by reversible phosphorylation is well documented and in the case of pkMCADH the bound phosphate could be involved in the binding-interaction of ETF and pkMCADH. At saturating concentrations of the substrate octanoyl-CoA (20 μM) and various concentrations of pkMCADH (65 nM to 3 μM) the activity was measured at a fixed concentration of ETF (10 μM). This experiment yields apparent K_m-values of 3.7×10^{-7} M for both the holo-pkMCADH and the phosphatase-treated enzyme. This indicates a strong and similar interaction of both forms of pkMCADH and ETF.

The turnover number of ≈ 28 mol 2,6-dichloroindophenol (DCI) reduced per minute and per mol ETF bound FAD found under these conditions was also the same for both proteins. Therefore it is concluded that the status of phosphorylation of pkMCADH has no influence on the catalytic parameters.

Influence of the Phosphomonoester on the Solubility and Apo-Preparation of MCADH

Native pkMCADH (containing the phosphomonoester) can be concentrated up to 1 mM in 1 M Tris-buffer, pH 7.6. In contrast, dephosphorylated pkMCADH shows a decreased solubility (0.2–0.3 mM under the same conditions). When preparations of apo-pkMCADH are carried out according to the method of Mayer and Thorpe (1981) high yields (> 75%) are obtained when starting with native enzyme. However, with dephosphorylated pkMCADH the yields were < 10%. Furthermore, pkMCADH that has undergone one cycle of apo-enzyme preparation and reconstitution with FAD shows essentially the solubility of dephosphorylated pkMCADH. This reconstituted pkMCADH lacks the phosphorus resonance at 4.65 ppm and shows poor yields when subjected to a second cycle for the preparation of apo-MCADH. We therefore conclude that this treatment leads to substantial dephosphorylation.

Viscosity of a MCADH Solution

Using an Ostwald capillary viscometer a radius of ≈ 41 Å (4.1×10^{-7} cm) was estimated for the protein molecule (assuming a spherical shape).

Discussion

The ^{31}P NMR-spectra of pkMCADH document a considerable shift to lower field for both FAD-pyrophosphate P-resonances. According to model studies of Gorenstein (1975) the chemical shift of phosphate esters is mainly governed by the P-O-P bond angle and the torsional angle. As judged from the divergence in the chemical shifts, the FAD binding site of MCADH is different than that in glucose oxidase (James *et al.*, 1981) with respect to the conformation of the phosphodiester bridge of FAD. As in glucose oxidase, addition of Mn^{++} did not result in an observable line broadening of the pyrophosphate P-resonances indicating that the groups are buried in the binding site and not accessible to solvent-borne reagents. Moreover, reduction of MCADH did not lead to any change in the chemical shift of the phosphorus resonances. This is in line with the three-dimensional structure of MCADH (Kim *et al.*, 1993) from which it appears that the bridge linking the isoalloxazine and adenosine moieties is packed inside the domain interfacing two subunits.

Phosphomonoesters bound to amino acid residues show a chemical shift in the range of 8.5 to 3.8 for phosphothreonine and phosphoserine respectively, and their chemical shift depends strongly upon pH (the range covers 4 ppm; Vogel, 1984). This value is based on an external standard of 85% phosphoric acid (= 0 ppm) and is 1 ppm shifted to higher field compared to our values. Accordingly, the phosphorus resonance observed in pkMCADH is in the range of a phosphomonoester formed with either serine or threonine. On the other hand, a phosphodiester, diphosphodiester, phosphoramidate or an acyl phosphate group can be ruled out on account of the different chemical shifts reported for those compounds ranging from 0 to –11.3 ppm (Vogel, 1984).

The fact that the chemical shift of the phosphorus resonance depends on the pH also supports our interpretation. Unfortunately, the exact pK_a-value of the phosphomonoester group could not be determined because of the low stability and activity range of MCADH (pH 7–9) impeding a reliable titration. Further evidence in favour of a phosphomonoester group was provided by the result that the bound phosphorus was cleaved upon incubation with alkaline phosphatase, which results in a new phosphorus resonance at the resonance position expected for free phosphate. At high field ($H_0 > 6$ Tesla) the chemical shift anisotropy becomes the dominant relaxation mechanism for phosphorus nuclei. Therefore the line width is mainly governed by T_2-chemical shift anisotropy-relaxation times, which in turn depend on the correlation time (τ_c) of the observed molecule. T_2-chemical shift anisotropy decreases linearly with increasing correlation times and causes a gradual line broadening with increasing molecular weight. The correlation time for MCADH can be estimated from the measured line width (= 34 Hz) of the phosphorus resonance (Figure 1A) and yields a value of ≈ 177 ns. This is in agreement with the correlation time reported for succinyl-CoA synthetase, a protein with a similar molecular mass (140 kDa; Vogel *et al.*, 1982). The correlation time can also be estimated from the Stokes-Einstein relationship. Using a radius ≈ 41 Å (4.1×10^{-7} cm) for MCADH that was obtained from viscosity measurements, a τ_c = 92 ns is obtained. It should be emphasized that this estimation is based on the assumption that MCADH is a spherical molecule, which is correct only at a first approximation, the shape of the MCADH tetramer resembling a tetrahedron. Also, hydration of the protein,

which has been estimated to increase the radius up to 3 Å was not taken into consideration; this might be the origin of the difference in the correlation times obtained using the two methods.

Catalytic activity of many enzymes is controlled by means of reversible phosphorylation (mainly at a serine rather than threonine), which, *in vivo*, is under hormonal control and is mediated by kinases and phosphatases (Vogel, 1984). In general, the site of reversible phosphorylation is not located close to the active site. Surprisingly the state of phosphorylation does not show any effect on the catalytic reductive or oxidative half reactions of MCADH. The good accessibility of the phosphorylation site to alkaline phosphatase provides strong evidence that the phosphomonoester is near the surface of the protein. This agrees with the lack of electron density attributable to a phosphate group in the structure of pig MCADH (J.J. Kim, personal communication), which also indicates that the residue must be highly mobile. Therefore it is conceivable that the phosphomonoester group mediates the interaction with other proteins that might be involved in the metabolism of fatty acids and/or protein degradation.

Materials and Methods

Chemicals and Enzymes

Octanoyl-CoA was purchased from Sigma or was synthesized according to Bernert and Sprecher (1977). 1-amino-2-naphtol-4-sulfonic acid was from Merck, alkaline phosphatase, PMS from Sigma and DCI from Fluka, ferricenium hexafluorophosphate was from Aldrich. pkMCADH was purified essentially as described by Lau *et al*. (1986); however, Q-Sepharose (instead of Whatman DEAE-cellulose) and commercial calcium phosphate gel cellulose (Fluka) were used. The $A_{278/446}$ ratio of the purified enzyme was ≈ 5 and its activity comparable to that reported by Thorpe *et al*. (1979). Human wild type MCADH was obtained as described elsewhere (Kieweg *et al*., 1997). ETF was purified as described by Gorelick *et al*. (1982).

Catalytic Activity

Assays were done at 25 °C according to Thorpe *et al*. (1979) in 0.7 ml incubations containing 50 mM KP_i, pH 7.6, 60 μM EDTA, 20 μM octanoyl-CoA, 1 mM PMS, and 30 μM DCI. The reaction was started by the addition of 5 μl MCADH. The ETF assays were performed as described by Frerman *et al*. (1985), at a concentration of octanoyl-CoA = 100 μM. Ferricenium assays were carried out according to Lehman *et al*. (1990).

Determination of Phosphate and Sample Preparation

Phosphate analysis was done according to Lanzetta *et al*. (1979). All procedures were carried out in polypropylene plastic laboratory ware. Incubations were stirred and left in the dark at room temperature for 30 min. Mixtures were then filtered through a 0.45 μm membrane (washed with at least 20 ml of double distilled water). Phosphate was determined by mixing 750 μl of this reaction mixture with 100 μl aliquots of the test solutions. After incubation for 20 min at room temperature the absorbance at 630 nm was read. KP_i-buffer was used as a standard. Protein samples were made phosphate-free by repeated washing (8 cycles) with 50 mM Tris-buffer, pH 7.6 containing 10 mM potassium sulphate in Centricon 30 microconcentrators. The protein concentration of these samples was determined spectrophotometrically from the $OD_{445\,nm}$ ($\varepsilon_{max} = 15000\,M^{-1}\,cm^{-1}$, Thorpe *et al*., 1979). For the release of the phosphate group from the protein the sample was treated with 10% trichloroacetic acid (or sulphuric acid) for 3 h at 90 °C. The samples were then left for 20 h at room temperature before the phosphate content was determined.

NMR-Spectroscopy

^{31}P-NMR-spectroscopy was carried out with a Bruker AM 270 instrument equipped with an Aspect 3000 digital computer, operating at 109.35 MHz. All spectra were obtained in 15 mm Wilmad precision NMR tubes containing 5–6 ml samples in a 100 mM Tris/400 mM NaCl, pH 8.5 buffer (called standard buffer) at 300 K. The chemical shifts were determined relative to 85% phosphoric acid in a capillary. Field-frequency locking was achieved by using the deuterium resonance of D_2O in the sample. Cleavage of phosphomonoester groups was achieved by adding 7 mg $MgCl_2$ and 60 units of alkaline phosphatase to a sample containing about 80 mg of MCADH (ca. 0.4 mM). After this treatment MCADH was dialyzed against standard buffer, pH 7.6. The spectra of reduced MCADH were obtained upon flushing the sample tube with argon and addition of a threefold molar excess of a concentrated dithionite solution. The experiments to study the solvent accessibility of the phosphorus atom were carried out in the presence/absence of 1/10 equivalent of $MgCl_2$. For comparison a 1.4 mM solution of FAD in standard buffer was measured under the same conditions.

Viscosity Measurements

Viscosity measurements of a MCADH solution were carried out with an Ostwald capillary viscometer in standard buffer at 25 °C.

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