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FT-IR Spectroscopic Studies on the Molecular Mechanism for Substrate Specificity/Activation of Medium-Chain Acyl-CoA Dehydrogenase

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The interactions of acyl-CoA with medium-chain acyl-CoA dehydrogenases (MCADs) reconstituted with artificial FADs—i.e. 8-CN-, 7,8-Cl_2-, 8-Cl-, 8-OCH_3- and 8-NH_2-FAD—were investigated by UV-visible absorption and FT-IR measurements. Although 8-NH_2-FAD-MCAD did not oxidize acyl-CoA the wavelength of the absorption maximum of the flavin was altered by acyl-CoAs binding. Thus, 8-NH_2-FAD-MCAD is one of the attractive materials for investigation of enzyme–substrate (ES) interaction in ES complex (the complex of oxidized MCAD with acyl-CoA). FT-IR difference spectra between non-labelled and [1-^{13}C]-labelled acyl-CoA free in solution and bound to oxidized 8-NH_2-FAD-MCAD were obtained. The broad 1668-cm^{-1} band of free octanoyl-CoA assigned to the C(1) = O stretching vibration appeared as a sharp signal at 1626 cm^{-1} in the case of the complex. The downward shift indicates a large polarization of C(1) = O, and the sharpness suggests that the orientation of the C(1) = O in the active-site cavity is fairly limited. The hydrogen-bond enthalpy change responsible for the polarization on the transfer of the substrate from aqueous solution to the active site of MCAD was estimated to be ~15 kcal/mol. The 1626-cm^{-1} band is noticeably weakened in the case of acyl-CoA with acyl chains longer than C12 which are poor substrates for MCAD, suggesting that C(1) = O is likely to exist in multiple orientations in the active-site cavity, whence the band becomes obscured. A band identical to that of bound C8-CoA was observed in the case of C4-CoA which is a poor substrate, indicating the strong hydrogen bond at C(1) = O.

Key words: artificial flavin, enzyme–substrate complex, FT-IR spectroscopy, hydrogen bond, medium-chain acyl-CoA dehydrogenase.

Abbreviations: CT, charge-transfer; IVD, isovaleryl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase.

Medium-chain acyl-CoA dehydrogenase (MCAD) is a member of the flavoprotein acyl-CoA dehydrogenases (ACDs) family that catalyses the dehydrogenation of acyl-CoA thioesters to the corresponding *trans*-2-enoyl-CoAs. Nine members are known to comprise the family, five of which are involved in fatty acid oxidation and four in amino-acid oxidation (*1*). The catalytic mechanisms of these enzymes share common features (*2*), namely, the reductive half-reaction has been established to occur by removal of the pro-2*R* hydrogen as a proton by a general base (Glu-COO^-) and pro-3*R* hydrogen as a hydride to the N(5) locus of the isoalloxazine ring. The crystal structure of porcine MCAD complexed with octanoyl-CoA revealed that C2–C3 bond of the bound ligand molecule is sandwiched between Glu376-COO^- and the isoalloxazine ring, and the carbonyl oxygen of the substrate is hydrogen bonded to the ribityl-2′-OH of FAD as well as to the main-chain amide N-H of Glu376 (*3*). The presumed mechanism of MCAD reductive half-reaction is shown in Scheme 1. These hydrogen bonds are not only responsible for proper orientation of the substrate, but also crucial for polarization of the substrate and the lowering of the p*K*a of the C-2 proton for abstraction by the catalytic base, Glu376 (*2*). By the same token, removal of the 2′-ribityl hydroxyl induced downward redox-potential shift, as revealed by binding study with product analogs (*4*).

Vibrational spectroscopy is one of the most useful methods for investigating molecular interactions such as hydrogen bonding. Raman and FT-IR spectroscopic studies have provided information on the hydrogen bond at the C = O groups of flavin in flavoproteins (*5–11*). In addition, the polarization of carbonyl group of ligands such as 2-enoyl-CoA (product) and hexadienoyl-CoA (product analog) upon binding to MCAD was also demonstrated by Raman spectroscopic studies (*4*, *12*, *13*). The information on C = O stretching vibration

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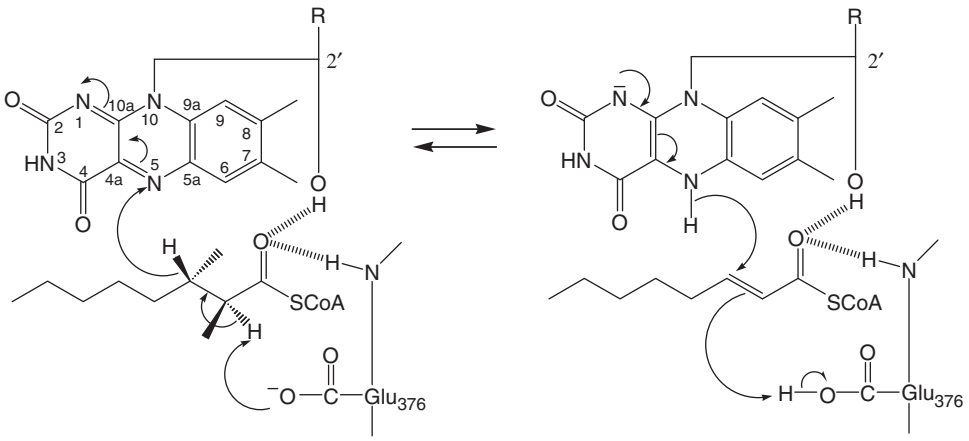

Scheme 1. **Presumed mechanism of MCAD reductive half-reaction.**

of ligand is enormously useful for estimating the hydrogen-bonding strength of the ligand at the C = O in the active site of MCAD. However, the aforementioned ligands are not the best probes for the substrate-active-site interaction in investigating the molecular mechanism of MCAD reductive half-reaction. This is because these ligands lack the hydrogen atom that is to be abstracted in the reductive half-reaction, and have a double bond at the C(2) = C(3) unlike a substrate acyl-CoA (Sch. 1). It is desirable that a normal substrate be used as an active-site probe for detailed studies on the reductive half-reaction.

The midpoint potential of FAD in free MCAD is −118 mV (*14*) against standard hydrogen electrode while that of the octanoyl-CoA/octenoyl-CoA couple is −6 mV (*14*). (In the reference (*14*), the midpoint potential values at pH 7.6 were reported. In this article, we use corrected values to pH 7.0 for clarity of the discussion.) Thus, the electron transfer from acyl-CoA to flavin is thermodynamically unfavourable. The binding of substrate/product significantly alters the midpoint potential of the enzyme, i.e. the midpoint potential of FAD bound to MCAD shifted positively by 110 mV upon binding substrate (the midpoint potential of FAD of MCAD complexed with substrate is −8 mV), in favour of electron transfer from acyl-CoA to flavin (*14*, *15*). This indicates that both potentials of substrate/product and MCAD complexed with substrate/product are comparable in magnitude. When we take these notions into consideration, we expect that MCAD reconstituted with 8-NH_2-FAD, which has a more negative midpoint potential (−330 mV) (*16*) than natural FAD (–208 mV) (*17*), forms a stable ES complex. In this case, the enzyme can bind a substrate but is not capable of oxidizing the substrate.

In the previous work we investigated the molecular mechanism underlying the drop in the p*K*a of a substrate analog 3-thiaoctanoyl-CoA bound to MCAD by taking advantage of artificial FADs—i.e. 8-CN-, 7,8-Cl_2-, 8-Cl, 8-OCH_3 and 8-NH_2-FADs—reconstituted into MCAD (*18*). A distinct linear correlation between the transition energy of the long-wavelength band of MCAD-3-thiaoctanoyl-CoA complex and the Hammett σ parameter of the substituent was recognized, suggesting that the modification of FAD does not affect the mode of CT interaction between the flavin and the ligand (*18*). Therefore, it is expected that the binding mode of a substrate to MCAD remains intact in the reconstituted MCADs.

We can presume from Scheme 1 that appropriate orientation of the pro-2*R* and pro-3*R* hydrogens in the active site and the strong hydrogen bonds of the substrate at the C(1) = O oxygen are essential for smooth reaction. We have a strong interest in the molecular mechanism of substrate specificity of MCAD, which accommodates substrates with a broad range of fatty acyl chain lengths with C8- and C10-CoA showing the highest rate (*19–21*). It may be useful for elucidating the molecular mechanism to determine the strength of the hydrogen bond with each substrate. Previously, we reported the resonance Raman study on the hydrogen bond of the ligand at C(1) = O in the 'purple complex' (the complex of reduced MCAD with 2-enoyl-CoA) of MCAD (*12*). The purple complex can be considered to be an ES complex in the backward reaction, where E is the reduced form of dehydrogenase and S is an enoyl-CoA. Recently, we reported the investigation on the electronic state of FAD by FT-IR spectroscopy by using the C(2) = O and C(4) = O stretching vibrations as probes; FT-IR difference spectra between non-labelled and isotope-labelled FAD were very useful for band assignments (*11*). In this article, we compared the strength of hydrogen bonds of substrate at the C(1) = O in both complexes of MCAD with good and poor substrates by using the C = O stretching vibration as probe by FT-IR spectroscopy. The complex prepared by using MCAD reconstituted with 8-NH_2-FAD can be considered to be an ES complex in the forward reaction.

MATERIALS AND METHODS

Enzymes—MCAD was purified from porcine kidney as described by Gorelick *et al*. (*22*), and Lau *et al*. (*23*). The apoenzyme was prepared by the method of Mayer and Thorpe (*24*) and was reconstituted with artificial FAD following the method of Mayer and Thorpe (*24*) or Engst *et al*. (*25*).

Chemicals—Artificial FADs were prepared by the method previously reported (*11*, *18*). Non-labelled and [1-^{13}C]-labelled acyl-CoAs were prepared as reported previously (*12*). The corresponding labelled aliphatic acids (>99 atom%) were purchased from Isotec, USA. The molar absorption coefficients ($mM^{-1}cm^{-1}$) used for FAD and artificial FADs are: ε_{450}(FAD) = 11.3, ε_{450}(8-CN) = 11.4, ε_{448}(7,8-Cl_2) = 10.4, ε_{448}(8-Cl) = 10.6, ε_{448}(8-OCH_3) = 22.0 and ε_{482}(8-NH_2) = 44.0—(*18*) and the references cited therein.

Spectrophotometric Measurements—Absorption spectra were measured with a Hitachi U-3310 spectrophotometer thermostated at 25°C. FT-IR spectra were obtained by a Nicolet NEXUS 670 FT-IR spectrometer. The FT-IR instrument was purged with Ar gas to avoid the presence of atmospheric water vapour. Spectra were recorded at room temperature (*ca*. 25°C) at 4-cm^{-1} resolution by averaging 100 scans. The samples were prepared in D_2O solution to avoid the interference from H_2O signal. The 8-NH_2-FAD-MCAD for FT-IR experiments was prepared with an enzyme concentration of approximately 1 mM in 100 mM potassium phosphate buffer in D_2O, pD 7.6. This was achieved by washing the enzyme several times with the above buffer through a Centricon YM-30 centrifugal filter. Each non-labelled or labelled acyl-CoA in D_2O was added in the MCAD solution. For FT-IR measurements, the sample solution was sandwiched by CaF_2 disks which were spaced with a 50-μm thick Teflon spacer.

RESULTS AND DISCUSSION

Interaction between Acyl-CoA and MCAD Reconstituted with Artificial FADs—Figure 1 shows the visible absorption spectra of natural MCAD and MCAD reconstituted with artificial FAD in the absence and presence of substrate octanoyl-CoA. The 'purple complex', an intermediary state characterized by a CT interaction between a product and reduced flavin, is formed in the reaction of some artificial FAD-MCAD; the spectra of natural and 8-Cl-FAD-MCAD are identical to those previously reported (*26*). The enzyme was almost fully reduced in the case of MCAD reconstituted with artificial FAD that has at the 8-position an electron-withdrawing group (CN or Cl) as well as in the case of natural MCAD. Meanwhile, in the case of artificial FAD that has an electron-donating group (OCH_3 or NH_2), the extent of flavin reduction was quite different: flavin was reduced only partially in 8-OCH_3-FAD-MCAD and the flavin was not reduced at all in 8-NH_2-FAD-MCAD. These findings demonstrate unequivocally that the oxidizing property of the enzyme depends on the electron-drawing or electron-donating property of the substituent and hence on the midpoint potential of FAD bound to the enzyme. The split of the absorption band of 8-OCH_3-FAD-MCAD in the presence of octanoyl-CoA (Fig. 1) is probably due to the vibronic structure of flavin, suggesting that the environment surrounding flavin is hydrophobic.

Because the midpoint potentials of free MCAD and free acyl-CoA are −118 and −6 mV—corrected to pH 7 from (*14*)—respectively, the reduction of free MCAD by free acyl-CoA is thermodynamically unfavourable. However, binding of substrate/product to MCAD induces a large positive shift in the midpoint potential of MCAD (−8 mV) and allows MCAD to oxidize substrate acyl-CoA (*14*). Since the midpoint potential of FAD free in aqueous solution is −208 mV (*17*), the potential is shifted positively by 200 mV upon FAD binding to apoMCAD and the binding of substrate/product to MCAD. If the potential of each artificial FAD would be shifted by the same

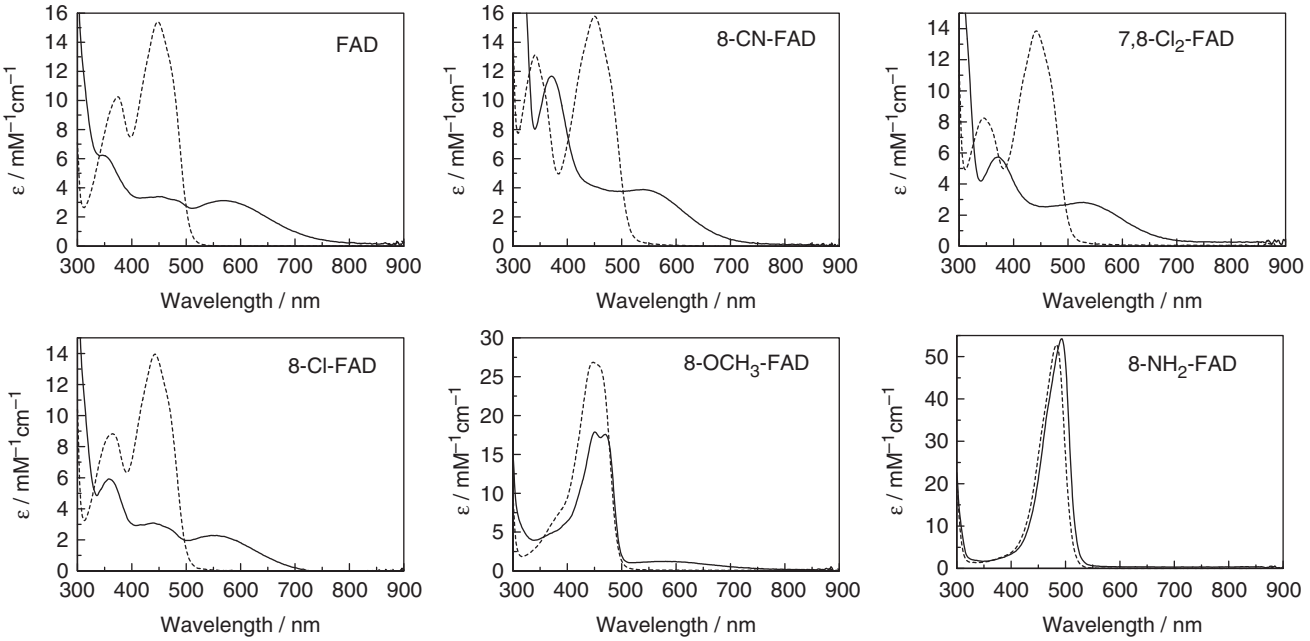

Fig. 1. **Absorption spectra of the reconstituted MCADs with artificial FADs in the absence (dotted lines) and the presence (solid lines) of octanoyl-CoA.** Spectra were measured in 50 mM potassium phosphate (pH 7.6) at 25°C. The concentrations of FADs, apoenzyme and octanoyl-CoA were 20–30, 57, and 120 μM, respectively.

amplitude (200 mV) in the reconstituted MCAD complexed with substrate/product, the potential of each artificial FAD (*27*) would be shifted as follows: 8-CN-FAD, from −50 mV of the FAD free in solution to +150 mV in the reconstituted MCAD complexed with substrate/product; 7,8-Cl_2-FAD, from −126 to +74 mV; 8-Cl-FAD, −152 to +48 mV; FAD, −208 to −8 mV; 8-OCH_3-FAD, −260 to −60 mV and 8-NH_2-FAD, −330 to −130 mV. If this holds true, 8-NH_2-FAD-MCAD is anticipated to be reduced only scarcely by octanoyl-CoA (Fig. 1). Even though 8-NH_2-FAD-MCAD does not oxidize octanoyl-CoA, it does bind octanoyl-CoA, because the absorption spectrum is changed in the presence of octanoyl-CoA (Fig. 1). Spectroscopic studies using MCAD reconstituted with artificial FADs suggested that the modification of FAD at 8-position does not affect the mode of CT interaction between flavin and substrate analog 3-thiaoctanoyl-CoA (*18*). The same is anticipated to be true in the case of ES interaction. Thus, 8-NH_2-FAD-MCAD is expected to be a favourable material for investigation of the interaction between oxidized MCAD and a substrate.

Figure 2 shows the absorption maximum wavelengths of the complexes of 8-NH_2-FAD-MCAD with various acyl-CoAs. The chain-length dependence of the maximum wavelength (Fig. 2) is similar to the dependence of catalytic activity of natural MCAD (*19–21*), suggesting that NH_2 group exerts no influence on the substrate binding mode. The 3D structures of porcine MCAD have been solved in binary complexes with substrates or inhibitors having C8-acyl chain, as well as in the uncomplexed form (*3*, *28*). The structures of human MCAD complexed with C8-CoA, C12- and C14-CoA have also been determined (*29*). The 3D structures of the porcine and the human MCADs are essentially the same (*29*). The fatty acyl portion of the thioester substrate is bound at the *re*-face of the flavin, and the cavity is deep enough to accommodate substrate with an acyl-chain length of up to 12 carbons (*3*). The enzyme flavin in the complex is a mixture of the fully reduced form produced in the catalytic reaction, and semiquinone and oxidized forms produced by secondary oxidation and the predominant form of the bound ligand is probably enoyl-CoA (*3*). The 8-NH_2-FAD-MCAD only scarcely oxidizes acyl-CoA to corresponding enoyl-CoA due to its low oxidizing power, thus the reconstituted MCAD has the potential to be one of the attractive materials for investigation of ES interaction in ES complex (the complex of oxidized MCAD with acyl-CoA). Figure 3 shows the chain-length dependence of the 8-NH_2-FAD-MCAD dissociation constant with acyl-CoA having various acyl-chain lengths. A plot of log K_d versus acyl-chain length of acyl-CoA is linear from C3 to C8 carbons, and binding becomes tight by approximately 550 cal/mol for each methylene group added to the chain. The dissociation constants between MCAD and several alkyl-SCoA derivatives, which lack a carbonyl group at the C(1), have been determined, and the free energy of binding is estimated to be 390 cal/mol for each methylene group (*30*). These results suggest that the hydrogen bonds of acyl-CoA at the C(1) = O favourably assist the hydrophobic interaction of the acyl-chain in the active site.

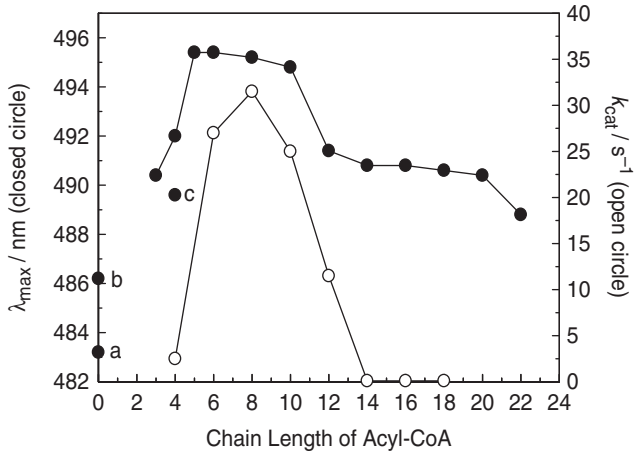

Fig. 2. **Absorption maximum wavelengths (closed circle) of 8-NH_2-FAD-MCAD-acyl-CoA complexes with acyl-chain lengths from 3 to 22.** Free 8-NH_2-FAD (a), free 8-NH_2-FAD-MCAD (b), and 8-NH_2-FAD-MCAD-isovaleryl-CoA complex (c) are also plotted. Turnover numbers (open circle) of rat liver MCAD for various acyl-CoAs are cited from Ikeda *et al.* (*21*).

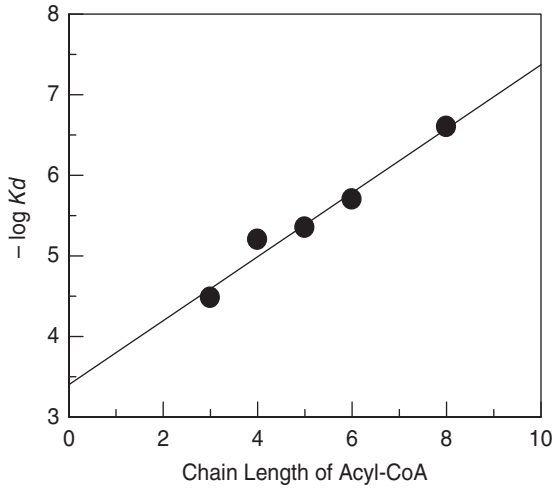

Fig. 3. **Chain-length dependency of dissociation constants between 8-NH_2-FAD-MCAD and acyl-CoAs.** The dissociation constants were obtained by spectroscopic titration in 50 mM potassium phosphate, pH 7.6, at 25°C.

FT-IR Difference Spectra between Non-Labelled and [1-^{13}C]-Labelled Octanoyl-CoA Free in Solution and Bound to Oxidized 8-NH_2-FAD-MCAD—FT-IR absorption spectra of the complexes of 8-NH_2-FAD-MCAD with non-labelled and [1-^{13}C]-labelled octanoyl-CoA and the difference spectra are shown in Fig. 4A and B; the difference spectrum of free octanoyl-CoA is also shown in Fig. 4B. The broad 1668-cm^{-1} band of free octanoyl-CoA shifted to 1633 cm^{-1} upon [1-^{13}C]-labelling, being assigned to the C(1) = O stretching mode. The corresponding bands in the case of the complexes appear at 1626 and 1586 cm^{-1}, respectively, with 42 and 47 cm^{-1} downward shift upon complexation. The bandwidth of the stretching absorption band of octanoyl-CoA bound to

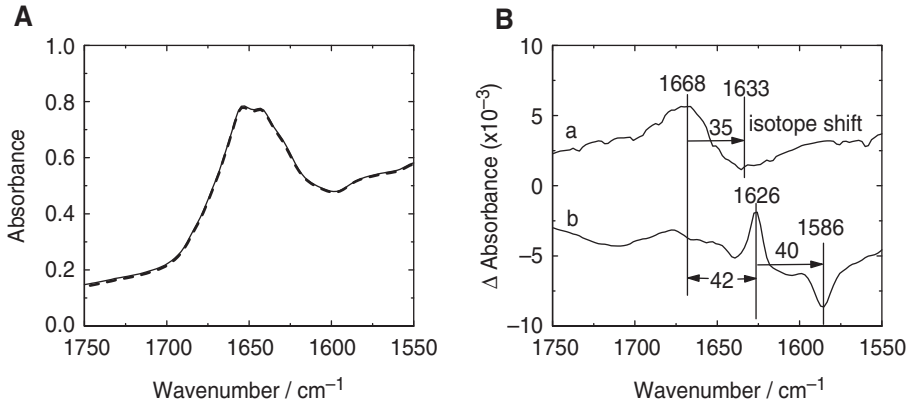

Fig. 4. **FT-IR difference spectra between the complexes of 8-NH_2-FAD-MCAD with non-labelled and [1-^{13}C]-labelled octanoyl-CoAs.** A: Absolute absorption spectra of the complexes of 8-NH_2-FAD-MCAD with octanoyl-CoA (solid line) and [1-^{13}C]octanoyl-CoA (dotted line). B: FT-IR difference spectra, between non-labelled and [1-^{13}C]-labelled octanoyl-CoAs (a); the complexes of 8-NH_2-FAD-MCAD with non-labelled and [1-^{13}C]-labelled octanoyl-CoAs (b). The concentrations were: non-labelled and the labelled octanoyl-CoA, 20 mM (a), 0.91 mM (b); 8-NH_2-FAD-MCAD, 1.0 mM (b).

the MCAD is narrow, compared to those of octanoyl-CoA free in solution. The sharpness suggests that the orientation of the C(1)=O in the active-site cavity is fairly limited. This phenomenon is explained by strong hydrogen bonds at the C(1)=O. The hydrogen-bond enthalpy change for the transfer of the substrate from aqueous solution to the active site of MCAD was estimated to be ~15 kcal/mol (~63 kJ/mol) by using the band shift (42 cm^{-1}), on the basis of the correlation data between C=O stretch and enthalpy change upon hydrogen-bond formation of various carbonyl compounds with alcohols (*31–33*). Similar strong hydrogen bondings were demonstrated in some ACD complexes with some ligands by Raman spectroscopy, i.e. enoyl-CoA in purple complexes of MCAD, short-chain acyl-CoA dehydrogenase and IVD (*12*) and hexadienoyl-CoA bound to MCAD (*4*, *13*). The strong hydrogen bonding is due to strong electrostatic interaction in the hydrophobic environment of the active site as shown by crystallographic studies (*3*). In the absence of bound substrate, the active-site cavity is occupied by a well-ordered string of water molecules that are successfully displaced as the length of the fatty acyl chain increases up to C12, at which point all the water molecules are expelled (*1*, *3*). Thus the active site of MCAD–substrate complex is in a hydrophobic environment and the electrostatic interaction responsible for hydrogen bond is considered to be strong.

Hydrogen Bonds at C(1)=O of Various Acyl-CoAs Bound to Oxidized 8-NH_2-FAD-MCAD—Figure 5 shows FT-IR difference spectra between the MCAD complexes with non-labelled and [1-^{13}C]-labelled acyl-CoAs with various acyl-chain lengths. The difference spectra of free acyl-CoAs are identical to those with octanoyl-CoA (data not shown). The 1626- and 1586-cm^{-1} bands are noticeably weakened in the case of acyl-CoA with longer acyl-chain than C12. This observation is consistent with the chain-length dependence of visible absorption maximum and the catalytic activity shown in Fig. 2 and the dependence of the CT absorption band of MCAD complex with 3-thiaacyl-CoA (*34*). In each acyl-chain-length dependency, be it on absorption maximum or

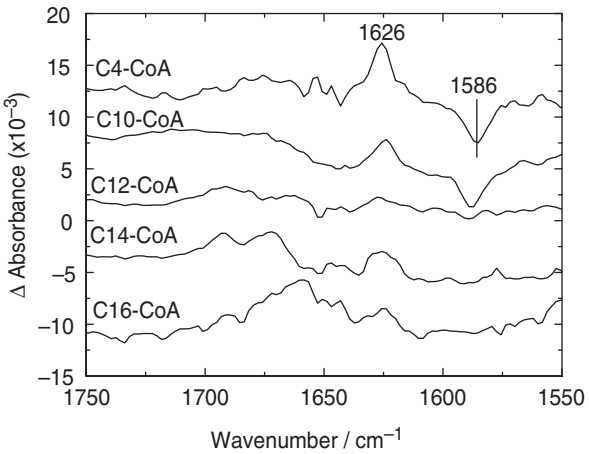

Fig. 5. **FT-IR difference spectra of the complexes of 8-NH_2-FAD-MCAD with non-labelled and [1-^{13}C]-labelled acyl-CoAs (C4, C10, C12, C14 and C16).**

catalytic activity, a substantial change is found at around C12. In the cases of acyl-CoAs with acyl-chain longer than the depth of the active-site cavity of MCAD—the cavity is deep enough to accommodate substrate with an acyl-chain length of up to C12 (*3*)—the hydrogen bonds at C(1)=O are probably disrupted by a steric repulsion of acyl-chain experienced in the cavity. Consequently C(1)=O of these acyl-CoAs probably exists in multiple orientations in the active-site cavity and the band becomes obscured. Meanwhile, a strong and sharp band is observed in the case of C4-CoA, contrary to the expectation driven from the chain-length dependences (Fig. 2). The frequency (1626 cm^{-1}) is the same as that observed with C8-CoA. These observations indicate the existence of strong hydrogen bonds at C(1)=O. When the acyl-chain is short, the hydrogen bond at C(1)=O exerts no steric influence, while hydrophobic interaction of acyl-chain in the cavity is weak and the existence probability of orientation other than the one suitable

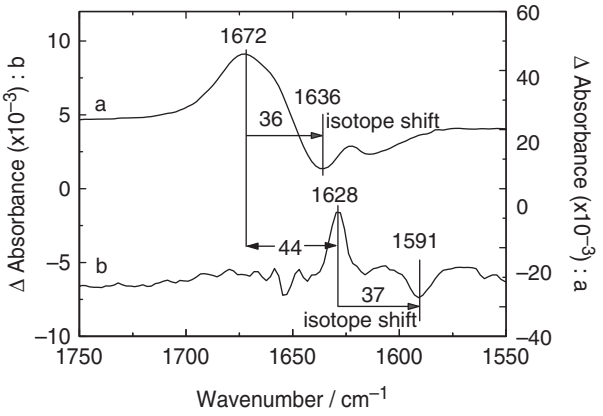

Fig. 6. **FT-IR difference spectra between the complexes of 8-NH_2-FAD-MCAD with non-labelled and [1-^{13}C]-labelled isovaleryl-CoA.** FT-IR difference spectra, between non-labelled and [1-^{13}C]-labelled isovaleryl-CoAs (a); the complexes of 8-NH_2-FAD-MCAD with non-labelled and [1-^{13}C]-labelled isovaleryl-CoAs (b).

for the catalytic reaction probably increases. A likely explanation is that the rotations around the axes of C(1)–C(2) and/or C(2)–C(3) occur easily in the case of C4-CoA. Then, the configuration in which pro-2*R* and pro-3*R* hydrogens are directed to carboxylate of Glu376 and N(5) of flavin ring, respectively, is energetically unfavourable. This explains the inefficiency of C4-CoA as a substrate for MCAD.

Theoretical studies on the electronic spectroscopic properties of 8-NH_2-flavin have shown that the band around 480 nm corresponds to the transition from HOMO to LUMO (*35*, *36*). The absorption maximum wavelength (492 nm) in the case of C4-CoA is shorter than those (495 nm) in the cases of the acyl-CoAs with chain length from C5 to C10 (Fig. 2). The difference occurs independently of the existence of the hydrogen bonds at the C(1) = O, because the hydrogen bond is also strong in C4-CoA. When the interaction between flavin N(5) and acyl-CoA pro-3*R* hydrogen, which is important for the reductive half-reaction, is strong, the absorption maximum may shift to longer wavelengths.

The difference spectra in the cases of isovaleryl-CoA (IV-CoA) and the complex with 8-NH_2-FAD-MCAD are shown in Fig. 6. The broad band at 1672 cm^{-1} of free IV-CoA C(1) = O stretch shifted to 1636 cm^{-1} upon [1-^{13}C]-labelling. The corresponding bands in the complex with the enzyme are obtained as sharp peaks at 1628 and 1591 cm^{-1}, respectively. IV-CoA, which has a branched chain at the C(3), is a poor substrate for MCAD, but the band of C(1) = O stretch shifted downward by 44 cm^{-1} in the bound form. The large shift indicates the existence of strong hydrogen bonds at C(1) = O in the active site, as in the case of octanoyl-CoA (42 cm^{-1} downward shift). This implies that the low activity of MCAD for IV-CoA is not due to the destruction of the hydrogen bonds by steric hindrance of IV group in the active site, but is explained by an inappropriate orientation of IV-CoA to the catalytic base and/or flavin in the active site. The absorption maximum wavelength of the 8-NH_2-FAD-MCAD complex was short with IV-CoA as well as with other poor substrates (Fig. 2). This may reflect the interference of the interaction between flavin N(5) and IV-CoA 3-hydrogen by the steric repulsion due to the 3-methyl group. A negative peak around 1615 cm^{-1} in the difference spectrum of IV-CoA (spectrum a in Fig. 6) is also observed, but we are not certain as to the origin of this peak at this stage.

We previously reported that the 1626-cm^{-1} band of C(1) = O stretch of 3-methylcrotonyl-CoA (the oxidation product of IV-CoA) shifted to 1598 and 1581 cm^{-1} upon binding to reduced MCAD and IV-CoA dehydrogenase (IVD), respectively (*12*). The shift (28 cm^{-1}) in MCAD is smaller than that (45 cm^{-1}) in IVD, which is explained by a steric repulsion between phenyl group of Tyr375 and 3-CH_3 located *cis* to C(1) = O in 3-methylcrotonyl-CoA in the case of MCAD. The group corresponding to Tyr375 is replaced by Gly in the case of IVD (*37*), allowing the 3-methyl group of the substrate to fit snugly in the cavity (*38*). As 3-methylcrotonyl-CoA has conjugated double bonds of C(1) = O and C(2) = C(3), the rotations around both the axes of C(1)–C(2) and C(2)–C(3) are prohibited. The dislocation of C(1) = O by the steric repulsion can be alleviated by the rotation in the case of IV-CoA, but the resulting arrangement is not favourable for the catalytic reaction. A steric repulsion between the phenyl group of Tyr375 and 3-CH_3 in IV-CoA probably hinders the interaction between flavin N(5) and IV-CoA 3-hydrogen.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Kim, J.-J.P. and Miura, R. (2004) Acyl-CoA dehydrogenases and acyl-CoA oxidases: Structural basis for mechanistic similarities and differences. *Eur. J. Biochem.* **271**, 483–493
2. Ghisla, S. and Thorpe, C. (2004) Acyl-CoA dehydrogenases: a mechanistic overview. *Eur. J. Biochem.* **271**, 494–508
3. Kim, J.-J.P., 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. USA.* **90**, 7523–7527
4. Wu, J., Bell, A.F., Luo, L., Stephens, A.W., Stankovich, M.T., and Tonge, P.J. (2003) Probing hydrogen-bonding interactions in the active site of medium-chain acyl-CoA dehydrogenase using Raman spectroscopy. *Biochemistry* **42**, 11846–11856
5. Kim, M. and Carey, P.C. (1993) Observation of a carbonyl feature for riboflavin bound to riboflavin binding protein in red-excited Raman spectrum. *J. Am. Chem. Soc.* **115**, 7015–7016
6. Hazekawa, I., Nishina, Y., Sato, K., Shichiri, M., Miura, R., and Shiga, K. (1997) A Raman study on the C(4) = O stretching mode of flavins in flavoenzymes: hydrogen bonding at the C(4) = O moiety. *J. Biochem.* **121**, 1147–1154
7. Yang, K.-Y. and Swenson, R.P. (2007) Nonresonance Raman study of the flavin cofactor and its interactions in the methylotrophic bacterium W3A1 electron-transfer flavoprotein. *Biochemistry* **46**, 2298–2305
8. Hellwig, P., Scheide, D., Bungert, S., Maentele, W., and Friedrich, T. (2000) FT-IR spectroscopic characterization of NADH:ubiquinone oxidoreductase (complex I) from *Escherichia coli*: oxidation of FeS Cluster N2 is coupled

with the protonation of an aspartate or glutamate side chain. *Biochemistry* **39**, 10884–10891
9. Wille, G., Ritter, M., Friedemann, R., Maentele, W., and Huebner, G. (2003) Redox-triggered FTIR difference spectra of FAD in aqueous solution and bound to flavoproteins. *Biochemistry* **42**, 14814–14821
10. Iwata, T., Nozaki, D., Sato, Y., Sato, K., Nishina, Y., Shiga, K., Tokutomi, S., and Kandori, H. (2006) Identification of the C=O stretching vibrations of FMN and peptide backbone by ^{13}C-labeling of the LOV2 domain of adiantum phytochrome3. *Biochemistry* **45**, 15384–15391
11. Nishina, Y., Sato, K., Tamaoki, H., Setoyama, C., Miura, R., and Shiga, K. (2007) Intramolecular and intermolecular perturbation on electronic state of FAD free in solution and bound to flavoproteins: FTIR spectroscopic study by using the C=O stretching vibration as probes. *J. Biochem.* **142**, 265–272
12. Nishina, Y., Sato, K., Hazekawa, I., and Shiga, K. (1995) Structural modulation of 2-enoyl-CoA bound to reduced acyl-CoA dehydrogenases: a resonance Raman study of a catalytic interaction. *J. Biochem.* **117**, 800–808
13. Pellett, J.D., Sabaj, K.M., Stephens, A.W., Bell, A.F., Wu, J., Tonge, P.J., and Stankovich, M.T. (2000) Medium-Chain Acyl-Coenzyme A Dehydrogenase bound to a product analogue, hexadienoyl-coenzyme A: effects on reduction potential, pKa, and polarization. *Biochemistry* **39**, 13982–13992
14. Lenn, N.D., Stankovich, M.T., and Liu, H.W. (1990) Regulation of the redox potential of general acyl-CoA dehydrogenase by substrate binding. *Biochemistry* **29**, 3709–15
15. Johnson, B.D., Mancini-Samuelson, G.J., and Stankovich, M.T. (1995) Effect of transition-state analogs on the redox properties of medium-chain acyl-CoA dehydrogenase. *Biochemistry* **34**, 7047–55
16. Eckstein, J.W., Hasting, J.W., and Ghisla, S. (1993) Mechanism of bacterial luminescence: 4a, 5-Dihydroflavin analogs as models for luciferase hydroperoxide intermediates and the effect of substituents at the 8-position on luciferase kinetics. *Biochemistry* **32**, 404–411
17. Hasford, J.J. and Rizzo, C.J. (1998) Linear free energy substituent effect on flavin redox chemistry. *J. Am. Chem. Soc.* **120**, 2251–2255
18. Nishina, Y., Sato, K., Tamaoki, H., Tanaka, T., Setoyama, C., Miura, R., and Shiga, K. (2003) Molecular mechanism of the drop in the p*K*a of a substrate analog bound to medium-chain acyl-CoA dehydrogenase: implications for substrate activation. *J. Biochem.* **134**, 835–842
19. Beinert, H. (1963) *Acyl-CoA dehydrogenases in* The Enzymes (Boyer, P.D., Lardy, H., and Myrback, K., eds.), 2nd edn, pp. 447–466, Academic Pres, New York
20. Nandy, A., Kieweg, V., Krautle, F.G., Vock, P., Kuchler, 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**, 12402–11
21. Ikeda, Y., Okamura-Ikeda, K., and Tanaka, K. (1985) Spectroscopic analysis of the interaction of rat liver short-chain, medium-chain, and long-chain acyl coenzyme A dehydrogenases with acyl coenzyme A substrates. *Biochemistry* **24**, 7192–7199
22. Gorelick, R.J., Mizzer, J.P., and Thorpe, C. (1982) Purification and properties of electron-transferring flavoprotein from pig kidney. *Biochemistry* **21**, 6936–6942
23. Lau, S.-M., Powell, P., Buettner, H., Ghisla, S., and Thorpe, C. (1986) Medium-chain acyl coenzyme A dehydrogenase from pig kidney has intrinsic enoyl coenzyme A hydratase activity. *Biochemistry* **25**, 4184–418
24. Mayer, E.J. and Thorpe, C. (1981) A method for resolution of general acyl-coenzyme A dehydrogenase apoprotein. *Anal. Biochem.* **116**, 227–229
25. 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
26. Thorpe, C. and Massey, V. (1983) Flavin analogue studies of pig kidney general acy-CoA dehydrogenase. *Biochemistry* **22**, 2972–2978
27. Edmondson, D.E. (1999) *Electronic effects of 7 and 8 ring substituents as predictors of flavin oxidation-reduction potentials in* Flavins and Flavoproteins *1999* (Ghisla, S., Kroneck, P., Macheroux, P., and Sund, H., eds.), pp. 71–76, Rudolf Weber, Berlin
28. Satoh, A., Nakajima, Y., Miyahara, I., Hirotsu, K., Tanaka, T., Nishina, Y., Shiga, K., Tamaoki, H., Setoyama, C., and Miura, R. (2003) Structure of the transition state analog of medium-chain acyl-CoA dehydrogenase. Crystallographic and molecular orbital studies on the charge-transfer complex of medium-chain acyl-CoA dehydrogenase with 3-thiaoctanoyl-CoA. *J. Biochem.* **134**, 297–304
29. Lee, H.J., Wang, M., Paschke, R., Nandy, A., Ghisla, S., and Kim., J.-J. (1996) Crystal structures of the wild type and the Glu376Gly/Thr255Glu mutant of human medium-chain acyl-CoA dehydrogenase: influence of the location of the catalytic base on substrate specificity. *Biochemistry* **35**, 12412–12420
30. Powell, P.J., Lau, S.M., Killian, D., and Thorpe, C. (1987) Interaction of acyl coenzyme A substrates and analogs with pig kidney medium-chain acyl-CoA dehydrogenase. *Biochemistry* **26**, 3704–3710
31. Thijs, R. and Zeegers-Huyskens, T. (1984) Infrared and Raman studies of hydrogen bonded complexes involving acetone, acetophenone, and benzophenone. I. Thermodynamic constants and frequency shifts of the ν_{OH} and ν_{C}=O stretching vibrations. *Spectrochimica Acta* **40A**, 307–313
32. Tonge, P.J. and Carey, P.R. (1992) Forces, bond lengths, and reactivity: fundamental insight into the mechanism of enzyme catalysis. *Biochemistry* **31**, 9122–9225
33. Tonge, P.J., Fausto, R., and Carey, P. R. (1996) FTIR studies of hydrogen bonding between α,β-unsaturated esters and alcohols. *J. Mol. Struc.* **379**, 135–142
34. Tamaoki, H., Nishina, Y., Shiga, K., and Miura, R. (1999) Mechanism for the recognition and activation of substrate in medium-chain acyl-CoA dehydrogenase. *J. Biochem.* **125**, 285–296
35. Shiga, K., Nishina, Y., Ohmine, I., Horiike, K., Kasai, S., Matsui, K., Watari, H., and Yamano, T. (1980) A study of the absorption, circular dichroism and magnetic circular dichroism spectra of a flavin derivative. *J. Biochem.* **87**, 281–287
36. Tanaka, T., Tamaoki, H., Nishina, Y., Shiga, K., Ohno, T., and Miura, R. (2006) Theoretical study on charge-transfer interaction between acyl-CoA dehydrogenase and 3-thiaacyl-CoA using density functional method. *J. Biochem.* **139**, 847–855
37. Matsubara, Y., Indo, Y., Naito, E., Ozasa, H., Glassberg, R., Vockley, J., Ikeda, Y., Kraus, J., and Tanaka, K. (1989) Molecular cloning and nucleotide sequence of cDNAs encoding the precursors of rat long chain acyl-coenzyme A, short chain acyl-coenzyme A, and isovaleryl-coenzyme A dehydrogenases. Sequence homology of four enzymes of the acyl-CoA dehydrogenase family. *J. Biol. Chem.* **264**, 16321–16331
38. Tiffany, K.A., Roberts, D.L., Wang, M., Paschke, R., Mohsen, A.-W.A., Vockley, J., and Kim, J.-J.P. (1997) Structure of human isovaleryl-CoA dehydrogenase at 2.6 Å resolution: structural basis for substrate specificity. *Biochemistry* **36**, 8455–8464