J. Biochem. **134**, 835–842 (2003)
DOI: 10.1093/jb/mvg209

Molecular Mechanism of the Drop in the pK_a of a Substrate Analog Bound to Medium-Chain Acyl-CoA Dehydrogenase: Implications for Substrate Activation

Yasuzo Nishina[1,*], Kyosuke Sato[1], Haruhiko Tamaoki[2], Takeyuki Tanaka[3], Chiaki Setoyama[2], Retsu Miura[2] and Kiyoshi Shiga[1]

[1]Department of Molecular Physiology and [2]Department of Molecular Enzymology, Graduate School of Medical Sciences, Kumamoto University, Honjo, Kumamoto 860-8556; and [3]Division of Biological Chemistry, Department of Life Science, Graduate School of Science and Technology, Kobe University, Nada-ku, Kobe 657-8501

Received September 2, 2003; accepted September 26, 2003

The pK_a value of a substrate analogue 3-thiaoctanoyl-CoA at αC-H is known to drop from *ca*. 16 in the free state to 5–6 upon binding to medium-chain acyl-CoA dehydrogenase (MCAD). The molecular mechanism underlying this phenomenon was investigated by taking advantage of artificial FADs, *i.e*., 8-CN-, 7,8-Cl_2-, 8-Cl-, 8-OCH_3-, 8-NH_2-, ribityl-2′-deoxy-8-CN-, and ribityl-2′-deoxy-8-Cl-FADs, reconstituted into MCAD. The stronger the electron-withdrawing ability of the substituent, the smaller the pK_a value became [*e.g*., 7.4 (8-NH_2-FAD) and 4.0 (8-CN-FAD)], suggesting that the flavin ring itself affects the pK_a value of the ligand *via* a charge-transfer interaction with the ligand. The destruction of the hydrogen bond between the thioester C(1)=O and the ribityl-2′-OH of FAD raised the pK_a by *ca*. 2.5 units. These results indicate that the interaction between the ligand and the flavin ring also serves to lower the pK_a of the ligand, in addition to the hydrogen bonds at C(1)=O of the ligand.

Key words: acyl-CoA dehydrogenase, artificial flavin, charge-transfer complex, flavoenzyme, hydrogen bond.

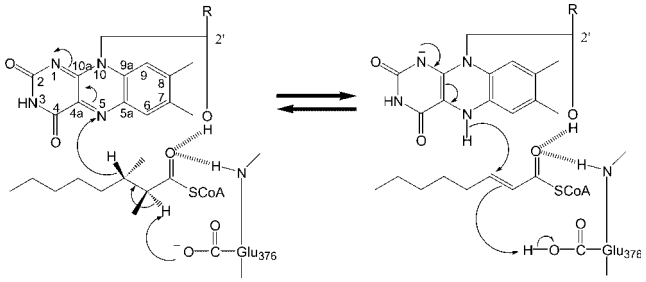
Abbreviations: CT, charge-transfer; DFT, density functional theory; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; MCAD, medium-chain acyl-CoA dehydrogenase.

Acyl-CoA dehydrogenases constitute a family of flavoenzymes that catalyze the dehydrogenation of acyl-CoA thioesters to the corresponding trans-2-enoyl-CoA products in the first step of the mitochondrial fatty acid β-oxidation cycle (*1*, *2*). Scheme 1 illustrates the mechanism of the reductive half-reaction (dehydrogenation of substrate and reduction of flavin): the α-hydrogen of acyl-CoA is abstracted as a proton by a protein base, *i.e*., Glu-COO^-, and the β-hydrogen is transferred as a hydride to the N(5) locus of flavin (*2*). Ghisla *et al*. proposed that the two hydrogen-removal processes are concerted at the α- and β-carbons in the reductive half-reaction of medium-chain acyl-CoA dehydrogenase (MCAD) on the basis of isotope effects with acyl-CoA deuterated at the α- and β-carbons (*3*). However, the two processes proceed either in a concerted manner or in a stepwise mode with α-proton abstraction preceding β-hydride transfer depending on the member of the family, type of substrate, or pH of the medium (*2*). The more stable the carbanion is, *i.e*., the lower the pK_a of substrate at αC-H, the more the stepwise mechanism is favored over the concerted mechanism.

A substrate-activating mechanism has been proposed, according to which a positive charge or a dipole adjacent to the carbonyl of an acyl-CoA thioester substrate lowers the pK_a value at C(2)-H (*4*–*7*). The polarization of the substrate/product upon binding to MCAD has been ascertained (*8*–*12*). Kim *et al*. (*13*) reported the crystal structures of MCAD with and without a substrate. The carbonyl oxygen of the thioester is hydrogen bonded to the ribityl-2′-hydroxyl group (2.9 Å) of FAD as well as to the main-chain amide N-H of Glu376 (3.1 Å), and these interactions serve to lower the p*K*a of the α-proton of the substrate, and also are responsible for the precise alignment of flavin, the substrate, and Glu376 for the α,β-dehydrogenation by anchoring the substrate at an appropriate depth and orientation in the deep active-site cavity (*13*). Investigations involving reconstituted enzyme with 2′- or 3′-deoxy-FAD have confirmed the importance of the hydrogen-bonding interaction between the 2′-hydroxyl and the carbonyl oxygen of the substrate (*14*, *15*).

Substrate analogs such as 3-ketoacyl-CoA and 3-thiaacyl-CoA are known to bind to MCAD, exhibiting charge-transfer (CT) absorption bands in a long-wavelength region (*16*, *17*). The CT-bands appear in complexes with anionic ligands deprotonated at αC-H (*16*, *17*). The pK_a values of the analogs at αC-H drop upon binding to MCAD; the pK_a of 3-thiaoctanoyl-CoA is reduced from *ca*. 16 in the free state to 5–6 when bound to the active site (*11*, *18*). The pK_a of 3-thiaoctanoyl-CoA shifts to *ca*. 11 when bound to 2′-deoxy-FAD-MCAD (*15*), indicating that the hydrogen bond between the ligand C(1)=O and FAD-2′-OH is important for the reduction in the pK_a. The three-dimensional structures of natural MCAD and 2′-deoxy-FAD-MCAD in complex with octanoyl-CoA/octenoyl-CoA show unambiguously that the FAD cofactor and the substrate/product bind in an identical fashion, imply-

*To whom correspondence should be addressed. Fax: +81-96-373-5052, E-mail: nishina@medic.kumamoto-u.ac.jp



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

ing that the observed effects are mainly due to the FAD-ribityl-2′-OH hydrogen bond (*15*).

As recognized in Scheme 1, multiple interactions between substrate and the MCAD active site cooperate for catalytic efficiency. In the present article, we focus on the role of flavin ring itself and the hydrogen bond between C(1)=O of a substrate analog and ribityl-2′-OH in FAD in lowering the pK_a at the αC-H in the enzyme active site. We carried out this study using reconstituted MCAD with FADs substituted at the phenyl ring and 2′-deoxy-8-substituted-FADs examined by electronic absorption spectroscopy.

MATERIALS AND METHODS

Enzymes—MCAD was purified from porcine kidney as described by Gorelick *et al*. (*19*), and Lau *et al*. (*20*). The apoenzyme was prepared by the method of Mayer and Thorpe (*21*) and was reconstituted with artificial FAD following the method of Mayer and Thorpe (*21*) or Engst *et al*. (*15*). Porcine kidney D-amino acid oxidase and the apoenzyme were purified as described elsewhere (*22*).

Chemicals—7,8-Cl_2-riboflavin was prepared by the method of Kuhn *et al*. (*23*) with some modifications (*24*). 8-NH_2-Riboflavin was synthesized according to Berezovskii *et al*. (*25*). Synthesis of 2′-deoxy-8-NH_2-riboflavin was carried out following the same procedure for the preparation of 8-NH_2-riboflavin using 2-deoxy-D-ribose instead of D-ribose. 8-CN-Riboflavin was obtained by the procedure of Murthy *et al*. (*26*). 8-Cl-Riboflavin was prepared by Sandmeyer's reaction from 8-NH_2-riboflavin. Preparations of 2′-deoxy-8-CN- and 2′-deoxy-8-Cl-riboflavin were similar to that of the corresponding 2′-OH-riboflavin. Artificial FADs were prepared from the corresponding riboflavins as described previously (*27*). The molar absorption coefficients (mM^{-1} cm^{-1}) used for FAD and artificial FADs are: ε_{450}(FAD) = 11.3 (*28*), ε_{448}(8-Cl) = 10.6 (*29*), ε_{450}(8-CN) = 11.4 (*26*), ε_{482}(8-NH_2) = 44.0 (*30*), ε_{448}(8-OCH_3) = 22.0, ε_{448}(7,8-Cl_2) = 10.4, ε_{448}(2′-deoxy-8-Cl) = 10.6, ε_{450}(2′-deoxy-8-CN) = 11.4. ε_{448}(8-OCH_3) obtained by a spectrophotometric titration with apoMCAD. The value of ε_{448}(7,8-Cl_2) was obtained on the basis of the spectral change of the flavin when it binds to apo-D-amino acid oxidase using ε_{460} of the enzyme reconstituted with 7,8-Cl_2-FAD (*31*). The values of ε_{448}(2′-deoxy-8-Cl) and ε_{450}(2′-deoxy-8-CN) were assumed to be the same as those of the corresponding 2′-OH-FAD.

The procedures for preparing acetoacetyl-CoA, 3-ketooctanoyl-CoA, and 3-thiaoctanoyl-CoA have been described elsewhere (*9*, *11*).

Spectrophotometric Measurements—Absorption spectra were measured with a Hitachi U-3310 spectrophotometer thermostated at 25°C.

Density Functional Theory Calculations—Lumiflavin was adopted as a model for flavin, and an anionic ligand was designed by the removal of the α-proton of 3-thiabutyrate ethylthiolester (2,5-dithia-heptane-4-one). The complex model was constructed by fitting the two

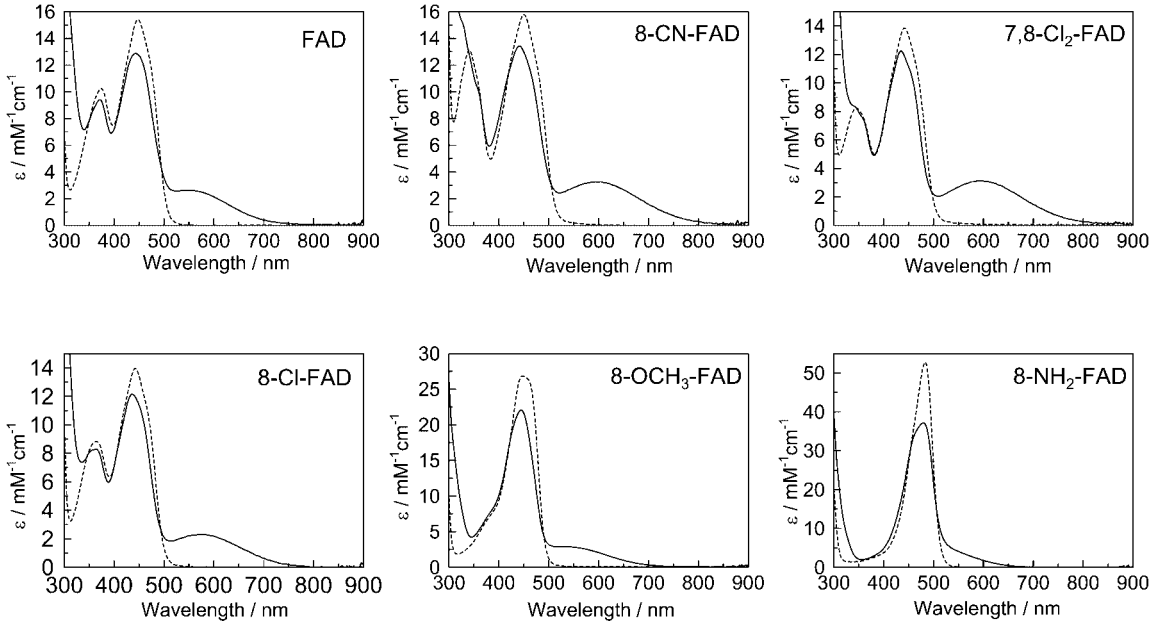


Fig. 1. **Absorption spectra of reconstituted MCADs with artificial FADs (dotted lines) and the complexes with acetoacetyl-CoA (solid lines).** Spectra were measured in 50 mM potassium phosphate, pH 7.6, at 25°C .The concentrations of FADs, apoenzyme, and acetoacetyl-CoA were 20–30, 57, and 170 μM, respectively.

molecular models to each other according to the arrangement obtained from the X-ray crystallographic structure of the MCAD-3-thiaoctanoyl-CoA complex (*32*). The geometry of the complex model was energetically optimized without any constraints using the density functional theory (DFT) method. On the basis of the complex model thus optimized, the energy and oscillator strength of the electronic excitation were calculated by means of the time-dependent DFT method (*33*). The calculations of the optimized geometries and the excitation energies were repeated for the complex models of 8-substitued lumiflavins with the anionic ligand. All DFT calculations were performed with the Gaussian98 program (*34*) on a HP Exemplar Technical Server V2250K and Dell Precision Workstation 450 machines using the B3LYP functional and 6–311G(d) basis set (*35*, *36*) with the closed-shell system.

RESULTS

Complexes of Substrate Analogs with MCAD Reconstituted with FADs Substituted at the Phenyl Ring—Figure 1 shows the visible absorption spectra of natural MCAD and MCAD reconstituted with ring-substituted FAD in the absence and presence of substrate analog acetoacetyl-CoA: the spectra of natural and 8-Cl-FAD-MCAD are identical to those previously reported (*16*). The molar absorption coefficient at the absorption maximum around 450 nm of the reconstituted enzyme was about 1.3 times larger than that of free flavin in each case, suggesting that the substituents have negligible, if any, steric effect on the environment surrounding the flavin ring in the enzyme active site. The long-wavelength band, which was not observed in uncomplexed MCAD, was observed for each complex with acetoacetyl-CoA; the stronger the electron-withdrawing ability (CN>Cl>CH_3>OCH_3>NH_2) of the substituent, the longer the wavelength region in which the band appears. It has been confirmed that the long-wavelength absorption band is associated with the CT interaction between oxidized flavin and the enolate form of acetoacetyl-CoA, and that the interaction between the C(4*a*)=N(5) moiety of flavin and the O=C(3)-C(2)H=C(1)-O$^-$ moiety of acetoacetyl-CoA plays a critical role in the CT interaction (*7*). We examined the correlation between the transition energy of the long-wavelength band and the Hammett σ parameter (*37*) (a in Fig. 2). The 7 and 8 positions are *meta*- and *para*-positions, respectively, to the C(4*a*)=N(5) moiety. Thus, in the analysis of the influence of a substituent at the 7- or 8-position of the flavin ring on the electronic properties of the C(4a)=N(5) moiety, it is reasonable to use Hammett σ_{meta} or σ_{para} parameters; here we used $\sigma=\sigma_{meta}(7) + \sigma_{para}(8)$. A strong linear correlation was recognized, suggesting that the present modification of FAD does not affect the mode of CT interaction between the flavin and the ligand.

Figure 3 shows the absorption spectra of the MCAD complexes with substrate analog 3-thiaoctanoyl-CoA. The spectrum of natural MCAD in complex with 3-thiaoctanoyl-CoA is identical to that previously reported (*11*, *17*). The long-wavelength absorption bands can be ascribed to the CT interaction between oxidized flavin and anionic 3-thiaoctanoyl-CoA (*11*, *17*). The correlation between the transition energy and the Hammett σ parameter (b in Fig. 2) was also examined: a strong correlation was recognized, but the correlation (positive slope) is opposite to that of the complex with acetoacetyl-CoA (a in Fig. 2).

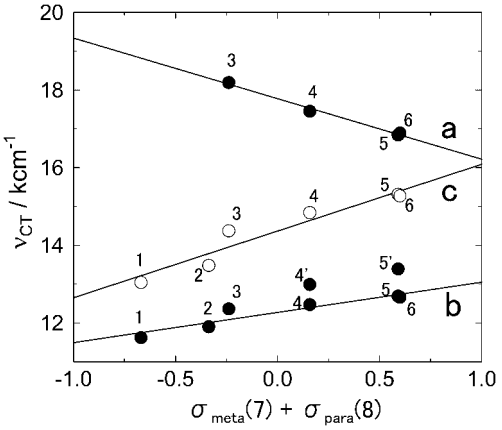


Fig. 2. **Correlation between the energy of the CT absorption band of the complexes between ligand and reconstituted MCAD with artificial FADs and the sum of the Hammett σ parameters for the substituents at the 7- and 8-positions of the artificial FAD.** Data numbering: 1, 8-NH_2-FAD; 2, 8-OCH_3-FAD; 3, FAD; 4, 8-Cl-FAD; 5, 8-CN-FAD; 6, 7,8-Cl_2-FAD; 4′, 2′-deoxy-8-Cl-FAD; 5′, 2′-deoxy-8-CN-FAD. The graph shows the correlation of the complex of MCAD with acetoacetyl-CoA (a) or 3-thiaoctanoyl-CoA (b). The values of ν_{CT} are the reciprocals of wavelengths of absorption maximum of the long wavelength bands observed in Figs. 1 and 3. The values (c) of the complex with 3-thiaoctanoyl-CoA were obtained from DFT calculation.

The pK_a value of 3-thiaoctanoyl-CoA at αC-H falls from *ca.* 16 in the free form to 5–6 upon binding to native MCAD (*11*, *18*). The pK_a values of the ligands bound to the reconstituted MCADs were estimated by the procedure described elsewhere (*11*) and the correlation between pK_a and the Hammett σ parameter is shown in Fig. 4. The stronger the electron-withdrawing ability of the substituent, or the greater the σ parameter**,** the smaller the pK_a value becomes, indicating that the flavin ring itself affects the pK_a value of 3-thiaoctanoyl-CoA at αC-H.

Complexes of Substrate Analogs with MCAD Reconstituted with Ribityl-2′-deoxy-8–Substituted-FAD—It is known that the ribityl-2′-OH group of FAD plays a pivotal role in the catalysis of MCAD; 2′-deoxy-FAD-MCAD is scarcely reduced by those substrates that are easily oxidized by natural MCAD (*14*).

FADs of the 2′-deoxy type affect the features of the complexes of oxidized MCAD. The molar absorption coefficients of the long-wavelength absorption bands of the complexes of 2′-deoxy-8-CN-FAD-MCAD and 2′-deoxy-8-Cl-FAD-MCAD with substrate analog acetoacetyl-CoA (Fig. 5) are smaller compared with those of FADs of the ribityl-2′-OH type (Fig. 1). In the case of the complexes of 2′-deoxy-8-CN/Cl-FAD-MCAD with substrate analog 3-ketooctanoyl-CoA, the molar absorption coefficients are larger than that of the complex with acetoacetyl-CoA, but smaller than that of the complex between 8-Cl-FAD-MCAD and 3-ketooctanoyl-CoA (not shown).

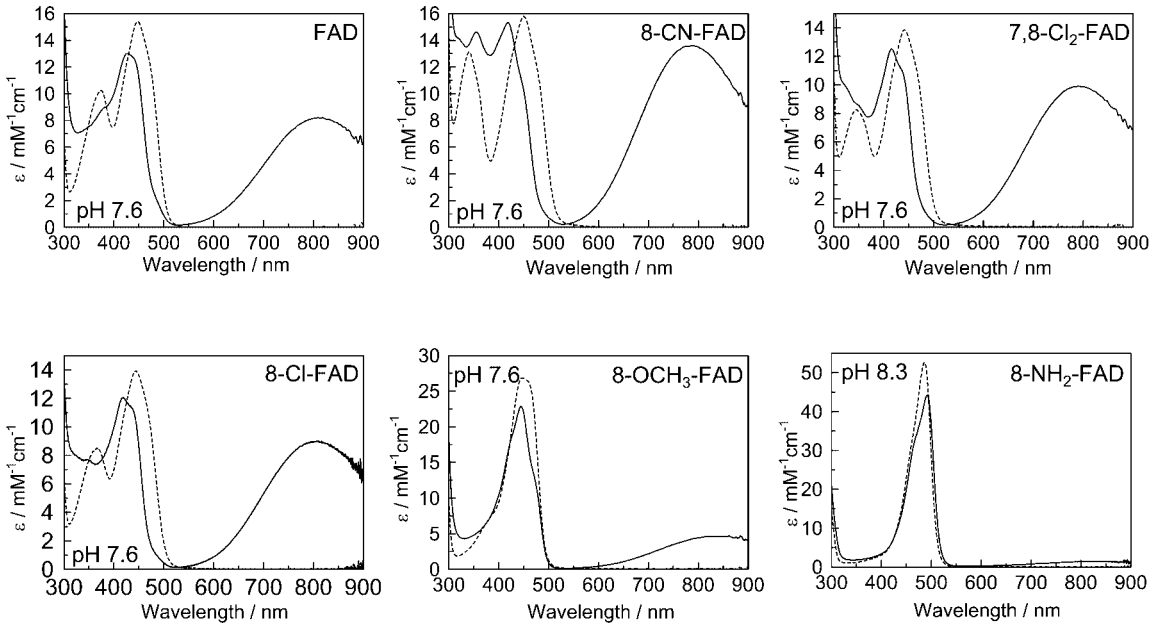


Fig. 3. **Absorption spectra of the reconstituted MCADs with artificial FADs (dotted lines) and the complexes with 3-thiaoctanoyl-CoA (solid lines).** Spectra were measured in 50 mM potassium phosphate (pH 7.6) or 50 mM sodium pyrophosphate (pH 8.3), at 25°C. The concentrations of FADs, apoenzyme, and 3-thiaoctanoyl-CoA were 20–30, 57, and 770 μM, respectively.

The absorption spectra of the complexes of 2′-deoxy-8-CN-FAD-MCAD and 2′-deoxy-8-Cl-FAD-MCAD with substrate analog 3-thiaoctanoyl-CoA are also shown in Fig. 5. Long-wavelength absorption bands were observed in both cases, but the molar absorption coefficients are smaller than those of 8-CN-FAD-MCAD and 8-Cl-FAD-MCAD (Fig. 3), and the long-wavelength absorption maxima are at a shorter wavelength (4′ and 5′ in Fig. 2). This phenomenon is also due to the destruction of the hydrogen bond between ribityl 2′-OH and C(1)-O^- of 3-thiaoctanoyl-CoA as observed with acetoacetyl-CoA or 3-ketooctanoyl-CoA. The pK_a (αC-H) values of 3-thiaoctanoyl-CoA bound to 2′-deoxy-8-CN-FAD-MCAD and 2′-deoxy-8-Cl-FAD-MCAD were found to be 6.3 and 7.3, respectively (Fig. 4). The pK_a values in the case of complexes with 2′-deoxy type-FADs are *ca.* 2.5 units larger than those of complexes with the 2′-OH type (*i.e.*, natural)-FADs (Fig. 4). This indicates that the hydrogen bond between the ligand C(1)=O and ribityl 2′-OH contributes to the drop in pK_a at the αC-H. Engst *et al.* (*15*) estimated the shift in the pK_a of 3-thiaoctanoyl-CoA from 5–6 in the bound state with natural MCAD to *ca.* 11 in the bound state with 2′-deoxy-FAD-MCAD, a difference of *ca.* 5. The large difference (*ca.* 5 units) compared to our observation (*ca.* 2.5 units) may be an overestimation due to their assumption that the molar absorption coefficient of the long-wavelength absorption band of the CT-complex in 2′-deoxy-FAD-MCAD is identical to that of the complex with natural human MCAD. As shown in the present study, the coefficient of the CT-complex in 2′-deoxy-8-CN/Cl-FAD-MCAD is smaller than that in the case of 8-CN/Cl-FAD-MCAD. If the coefficient of the CT-complex in 2′-deoxy-FAD-MCAD is also smaller than that in the complex of natural human MCAD, contrary to their assumption, the estimated pK_a should be smaller than 11.

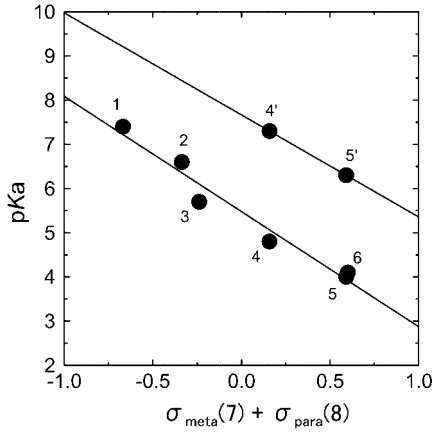


Fig. 4. **Correlation between the pK_a (αC-H) of 3-thiaoctanoyl-CoA bound to reconstituted MCAD with artificial FADs and the sum of the Hammett σ parameters for the substituents at the 7- and 8-positions of the FADs.** Data numbering: 1, 8-NH_2-FAD; 2, 8-OCH_3-FAD; 3, FAD; 4, 8-Cl-FAD; 5, 8-CN-FAD; 6, 7,8-Cl_2-FAD; 4′, ribityl-2′-deoxy-8-Cl-FAD; 5′, ribityl-2′-deoxy-8-CN-FAD.

DISCUSSION

We focus in this report mainly on the roles of the flavin ring and the hydrogen bond between C(1)=O of substrate analogs and ribityl 2′-OH in FAD in the reduction of the pK_a value of the ligand at αC-H.

Charge-Transfer Complexes of MCAD Reconstituted with Non-Natural FADs—The three-dimensional structures of the MCAD-3-thiaoctanoyl-CoA complex (*32*), butyryl-CoA dehydrogenase-acetoacetyl-CoA complex (*38*), and short-chain acyl-CoA dehydrogenase-acetoacetyl-CoA complex (*39*) have been resolved; the ani-

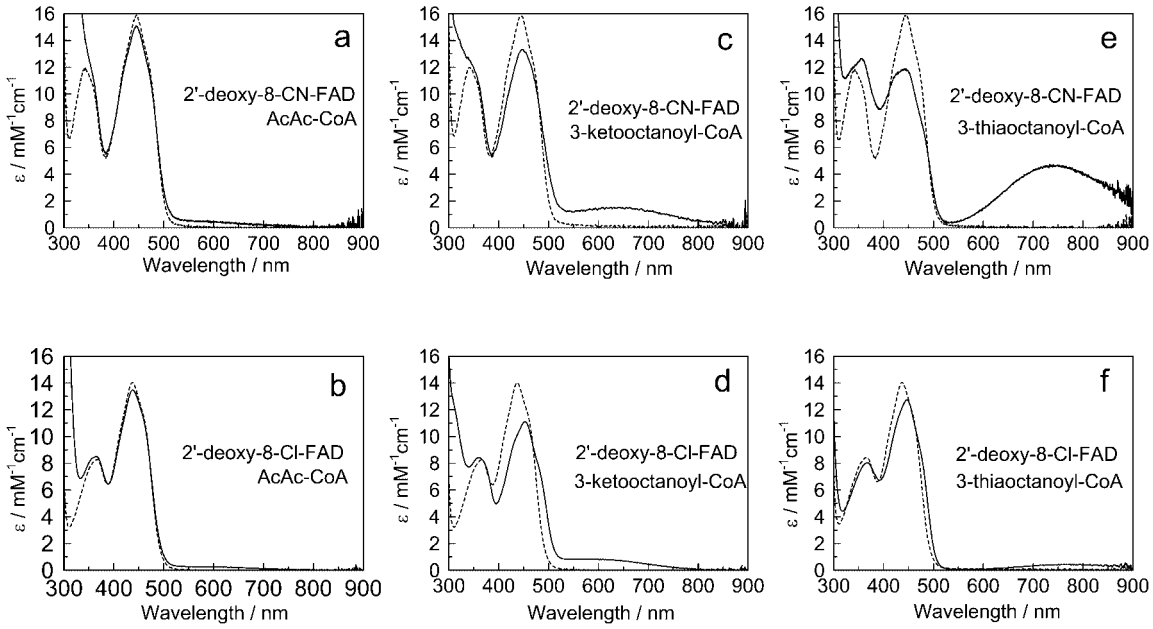


Fig. 5. **Absorption spectra of 2′-deoxy-8-Cl-FAD- and 2′-deoxy-8-CN-FAD-MCAD in the absence (dotted lines) and presence (solid lines) of acetoacetyl-CoA, 3-ketooctanoyl-CoA or 3-thiaoctanoyl-CoA.** Spectra were measured in 50 mM potassium phosphate, pH 7.6, at 25°C. The concentrations were: enzyme, 11 μM (a, c, e), 37 μM (b, d, f); acetoacetyl-CoA, 230 μM (a), 1.8 mM (b); 3-ketooctanoyl-CoA, 20 μM (c), 99 μM (d); 3-thiaoctanoyl-CoA, 1.1 mM (e, f).

onic ligand is stacked with the flavin ring in each complex. The MCAD-acetoacetyl-CoA complex has also been investigated by means of resonance Raman and NMR spectroscopy and molecular orbital calculations (*40–42*): the C(1)=O of anionic acetoacetyl-CoA is substantially polarized in the bound form and the C(4a)=N(5) moiety of flavin participates in the CT interaction with the ligands. A charge-transfer interaction is a chemical bond formed by the flow of part of the electron-cloud in the highest occupied molecular orbital (HOMO) of a molecule into the lowest unoccupied molecular orbital (LUMO) of a partner molecule. The ^{13}C-NMR spectra of the acetoacetyl-CoA bound to MCAD have been interpreted with reference to the CT model based on the optimum overlap between the LUMO of flavin and the HOMO of the enolate form of the acetoacetyl moiety of acetoacetyl-CoA (*43*). From these investigations, it is concluded that oxidized flavin acts as an electron acceptor and the anionic ligands act as an electron donor in the CT complexes. Thus, the CT transition energy is expected to decrease by substitution of the flavin ring with an electron-withdrawing group, because such substitution lowers the LUMO energy level of flavin and hence decreases the HOMO-LUMO energy gap. These phenomena are indeed observed in the case of the MCAD-acetoacetyl-CoA complex and the complexes of old yellow enzyme with phenols (*43*), where the slopes of the Hammett plot are negative. But the slope is positive in the case of the MCAD-3-thiaoctanoyl-CoA complex (Fig. 2).

The opposite direction of the slopes in the Hammett plot of the acetoacetyl-CoA complex and the 3-thiaoctanoyl-CoA complex (Fig. 2) is interpreted in terms of the difference in intensity of the CT interaction in these complexes. A conceptual diagram for this notion is shown in Fig. 6. The carbanion of acetoacetyl-CoA is more stabilized than that of 3-thiaoctanoyl-CoA due to the presence of two C=O groups adjacent to the deprotonation site, leading to the lower HOMO energy level (compare HOMO energy levels in I and II). In the case of a weak CT interaction, such as that in the acetoacetyl-CoA-MCAD complex (I), changes in the stabilizing energy (b – a) of the HOMO and destabilizing energy (d – c) of the LUMO by orbital-orbital interaction, as the result of replacement by a stronger electron acceptor (A_2), are smaller than the decrement (e) of the LUMO energy in the replacement; hence, the CT band energy decreases ($h\nu_1 > h\nu_2$). On the contrary, in the case of a strong CT interaction such as that in the 3-thiaoctanoyl-CoA complex (II), changes in the stabilizing (g–f) and destabilizing (i – h) energies are larger and superior to the change (j) in the electron affinity of flavin; hence, the CT transition energy increases ($h\nu_3 < h\nu_4$). A charge-transfer interaction is strong when the overlap integral between the HOMO of the electron donor and the LUMO of the electron acceptor is large and the energy gap ($E_{HOMO} - E_{LUMO}$) between the donor HOMO and the acceptor LUMO is small. Therefore, in addition to the energy gap as shown in Fig. 6, the overlap integral may also be responsible for the strong CT interaction in the 3-thiaoctanoyl-CoA complex. The strong CT interaction in the complex has been shown by DFT calculation: the amount of charge transferred from anionic 3-thiaoctanoyl-CoA to flavin was estimated to be 37% of one electron (*32*). On the basis of the alignment between the flavin ring and 3-thiaoctanoyl-CoA in the crystal structure of the MCAD-3-thiaoctanoyl-CoA complex (*32*), the electronic spectra of the complexes of various artificial FAD-MCAD with 3-thiaoctanoyl-CoA were examined by the same DFT method employed for the natural MCAD-3-thiaoctanoyl-CoA complex. The CT transition energy obtained by the calculation predicts the sign and

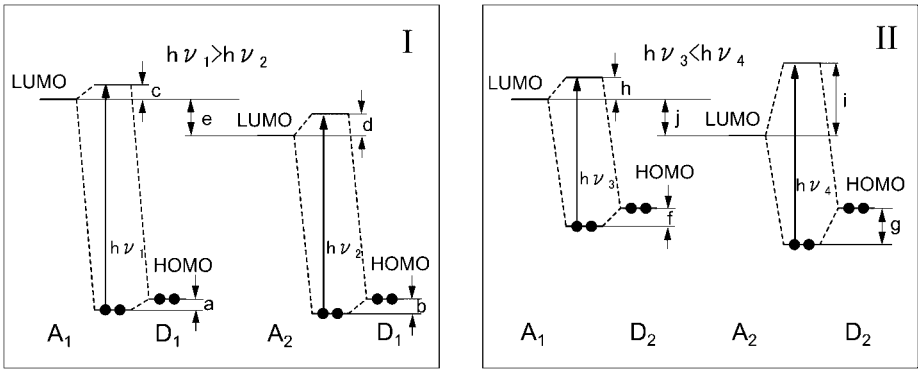


Fig. 6. **Explanatory diagram for the difference between MCAD-acetoacetyl-CoA (I) and MCAD-3-thiaoctanoyl-CoA (II) complexes in the correlations between the transition energy of the charge-transfer band and Hammett σ value.** A_1 (*e.g.*, FAD) and A_2 (*e.g.*, 8-CN-FAD) are weak and strong electron acceptors, respectively. D_1 (*e.g.*, acetoacetyl-CoA) and D_2 (*e.g.*, 3-thiaoctanoyl-CoA) are weak and strong electron donors, respectively.

steepness of the slope of the correlation obtained experimentally (b and c in Fig. 2).

Hydrogen Bond between Ribityl-2′-OH and C(1)=O of Substrate Analogs—A possible mechanism by which the hydrogen bond between 3-thiaoctanoyl-CoA C(1)=O and ribityl-2′-OH lowers the pK_a value of 3-thiaoctanoyl-CoA at αC-H in the MCAD complex is as follows. The hydrogen bond will be strengthened when the polarization of C=O is large, as predicted from a Coulombic model for electrostatic interactions between hydrogen bond donors and acceptors (*44*, *45*). The charge density at the oxygen of C(1)=O is larger in the anionic form than in the neutral form of 3-thiaoctanoyl-CoA, and thus the hydrogen bond energy between the ligand C(1)=O and ribityl-2′-OH is larger in the anionic form of the ligand than in the neutral form. Hence, the anionic form is stabilized by the hydrogen bond.

The CT band of the complex of 3-thiaoctanoyl-CoA with 2′-deoxy-8-CN/Cl-FAD-MCAD (Fig. 5) shifts to a shorter wavelength than that of 8-CN/Cl-FAD-MCAD (Fig. 3). The anionic form of 3-thiaoctanoyl-CoA acts as an electron donor in the CT complexes. Thus, the results show that the destruction of the hydrogen bond between C(1)-O^- of 3-thiaoctanoyl-CoA and ribityl-2′-OH strengthens the electron-donating ability of 3-thiaoctanoyl-CoA by increasing the HOMO energy, so that the CT band appears at a shorter wavelength.

The hydrogen bond also acts as the anchor for the ligand in the enzyme active site. In the case of the complex with acetoacetyl-CoA, the anchoring ability without the hydrogen bond is probably too small to arrest the wobble of the ligand, so that the probability of the optimum conformation for CT interaction becomes small, leading to a weakening of the CT-absorption band. In contrast, in the case of 3-ketooctanoyl-CoA, the hydrophobic interaction of the acyl-chain with the active site is probably strong enough to anchor the ligand and, hence, sustain the CT interaction. The three-dimensional structure of 2′-deoxy-FAD-MCAD in complex with octanoyl/octenoyl-CoA reveals on identical binding mode of the substrate/product in complex with natural FAD-MCAD (*15*).

The Role of the Flavin Ring—The pK_a value of 3-thiaoctanoyl-CoA at αC-H falls from *ca*. 16 in the free state to 5–6 upon binding to MCAD. The large shift of 10 units cannot be explained by only the two hydrogen bonds at C(1)=O, since the destruction of the hydrogen bond with ribityl-2′-OH causes the pK_a value to fall by only *ca*. 2.5. Therefore, other factors in addition to the hydrogen bonds must contribute to the large pK_a shift. The pK_a value varies depending on the electronic properties of the substituent introduced to the flavin (Fig. 4), indicating that the interaction between the ligand and the flavin ring also lowers the pK_a of the ligand. Namely, the flavin ring itself plays an important role in lowering the pK_a value. When the ligand is in the neutral state, the interaction with the flavin ring is weak and the differences in the strengths of the interactions among artificial FADs are expected to be small. In contrast, when the ligand is anionic, the electron donating ability of the ligand is large and the interaction with flavin is strengthened through the CT interaction. Therefore, the stronger the electron-accepting ability of the flavin, the smaller becomes the pK_a of the ligand.

We have unveiled the two factors underlying the drop of the pK_a value of the substrate analog, 3-thiaoctanoyl-CoA, upon binding to the active site of MCAD: namely, the hydrogen-bonding interaction between C(1)=O of the ligand and ribityl-2′-OH of FAD, as well as the electron-withdrawing power of the flavin ring, participate cooperatively in lowering the pK_a value of 3-thiaoctanoyl-CoA. We will assess these two factors separately. The participation of the hydrogen-bonding interaction between C(1)=O and the ribityl-2′-OH of FAD in the drop of the pK_a value of substrate can be explained by the lack of activity in MCAD reconstituted with ribityl-2′-deoxy-FAD (*15*). Whether the electronic properties of the flavin ring affect the pK_a value of the substrate depends on the substrate-flavin interaction in the reaction with normal substrate. We have proved in this report that the effect of the flavin ring on lowering the pK_a value of 3-thiaoctanoyl-CoA is closely associated with the charge-transfer interaction between the deprotonated ligand and flavin. The α-β dehydrogenation of the normal substrate catalyzed by the enzyme should fall into one of three extreme cases, *i.e.*, (1) a stepwise abstraction of the α-proton by the catalytic protein base prior to β-hydride transfer to flavin N(5), (2) a stepwise transfer of β-hydride prior to α-proton abstraction, and (3) a concerted process of α-proton abstraction and β-hydride transfer with equal weights between the two separate processes. There can also be concerted processes with different weights between α-proton abstraction and β-hydride transfer. These can be envisaged as being intermediate between (1) and (3) and between (2) and (3). In process (1), the overlap between the hydride portion of the β-position and flavin at N(5) precedes β-hydride transfer.

This overlap can be simulated by the CT interaction between the anionic ligand and flavin. In cases (2) and (3), cases intermediate between (1) and (3) and between (2) and (3), the transition state involves at least a partial overlap between the hydride portion of the β-position and flavin N(5). This overlap can also be simulated by the CT interaction between anionic 3-thiaoctanoyl-CoA and the flavin ring (*32*). Thus, whether the reaction proceeds by way of any of the three extreme cases or intermediary cases, the interaction between the hydride portion of the substrate β-position and flavin at N(5) is expected to occur. Therefore, the flavin ring itself is expected to play some role in lowering the pK_a of the substrate. Although the direct involvement of the flavin in substrate activation awaits further investigation, our present hypothesis offers a unique subject in substrate activation in flavoprotein catalysis.

We thank Dr. Sabu Kasai, Osaka City University, for the generous gift of 8-OCH_3-riboflavin. We also are grateful to Dr. Yasuo Musashi, Kumamoto University Center for Multimedia and Information, for access to the HP Exemplar Technical Server V2250K computer. This study supported in part by Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Science, Sports, and Culture of Japan [1312506 (R.M.)].

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