"Dehydrogenase" and "Oxidase" Reactions of Medium-Chain Fatty Acyl-CoA Dehydrogenase Utilizing Chromogenic Substrates: Role of the 3′,5′-Adenosine Diphosphate Moiety of the Coenzyme A Thioester in Catalysis†

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ABSTRACT: We undertook a comparative investigation of the medium-chain fatty acyl-CoA dehydrogenase (MCAD)-catalyzed reaction utilizing indole-, furyl-, and 4-(dimethylamino)phenyl-substituted propionyl- and acryloyl-CoAs as potential substrate/product pairs. All these propionyl-CoA derivatives undergo MCAD-catalyzed conversion into their corresponding acryloyl-CoAs via both "dehydrogenase" (in the presence of "organic" electron acceptors) and "oxidase" (buffer-dissolved oxygen serving as the electron acceptor) pathways [Johnson, J. K., Wang, Z. X., & Srivastava, D. K. (1992) *Biochemistry 31*, 10564−10575]. The steady-state kinetic parameters for the enzyme utilizing these substrates reveal that the K_ms (for the CoA substrates) and k_{cat}s for the dehydrogenase reaction are at least an order of magnitude higher than those for the oxidase reaction. As with the CoA substrates, the enzyme catalyzes the conversion of indolepropionyl pantetheine phosphate (IPPP) into indoleacryloyl pantetheine phosphate (IAPP) via these two pathways. However, with IPPP as substrate, the K_m (for IPPP) and k_{cat} values of the dehydrogenase and oxidase reactions are the same. These, coupled with the spectral changes of the enzyme−product complexes as well as the binding affinities of the enzyme−substrate/product complexes, lead to the following conclusions: (1) The aromatic/heterocyclic group-containing substrates are converted into their corresponding products via both the dehydrogenase and the oxidase pathways. (2) The 3′,5′-ADP moiety of the CoA thioester provides a significant fraction of the total binding energy in stabilizing the enzyme−substrate/product complexes. This moiety is also involved in lowering the energy barrier toward the formation of an intermediary species, "X" [Johnson, J. K., & Srivastava, D. K. (1993) *Biochemistry 32*, 8004−8013], which is responsible for the dehydrogenase reaction of the enzyme. (3) The kinetic instability of the intermediary species X, formed with IPPP as substrate, results in identical K_m (for IPPP) and k_{cat} values for the MCAD-catalyzed dehydrogenase and oxidase reactions, respectively. The structural and functional roles of the 3′,5′-ADP moiety of the CoA thioester in MCAD catalysis are discussed.

The mammalian β-oxidative pathway of fatty acids proceeds via a sequence of enzymatic steps involving several fatty acyl-CoA dehydrogenases of varied chain length specificities [for reviews, see Schulz (1991) and Engel (1990)]. Among them, the medium-chain fatty acyl-CoA dehydrogenase (MCAD)[1] is the most prominent form of the enzyme found in the mammalian kidney cortex (Thorpe et al., 1979). Although this enzyme exhibits a preferential selectivity for the medium-chain fatty acid substrates (optimum being for C_8), it utilizes a broad range of substrates ranging from palmitoyl-CoA to butyryl-CoA (Crane et al., 1956; Beinert, 1963). The fatty acyl-CoA dehydrogenase (E)-catalyzed reaction (irrespective of substrate specificity) is envisaged to proceed in two steps. The first step involves the abstraction of a proton and a hydride ion from the α and β carbons of the acyl-CoA substrates, respectively, yielding E-$FADH_2$-enoyl-CoA product complexes (Beinert, 1963). The repetitive turnover of this reaction is maintained by oxidation of the E-$FADH_2$-enoyl-CoA complex by transfer of electrons to suitable electron acceptors in the second step; under physiological conditions, the immediate electron acceptor is the electron-transferring flavoprotein (ETF) (Crane & Beinert, 1956) (eq 1).

$$\text{E-FAD + Acyl-CoA} \rightleftharpoons \text{E-FADH}_2\text{-Enoyl-CoA}$$

$$\text{E-FADH}_2\text{-Enoyl-CoA + ETF-FAD} \rightleftharpoons \text{E-FAD-Enoyl-CoA + ETF-FADH}_2 \quad (1)$$

From the classic experiment of Knoop, it has been clear that the substitution of a bulky (e.g., phenyl) group at the ω position of the fatty acid chains is easily tolerated by the enzymes of the β-oxidative pathway (Knoop, 1904). Given that this pathway involves a spiral sequence of cleavage reactions, it is likely that MCAD participates in the oxidation of the aromatic ring-substituted fatty acids. This expectation

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[1] Abbreviations: MCAD, medium-chain fatty acyl-CoA dehydrogenase; IPCoA, 3-indolepropionyl coenzyme A; IACoA, *trans*-3-indoleacryloyl coenzyme A; FPCoA, furylpropionyl coenzyme A; FACoA, furylacryloyl coenzyme A; DPCoA, 4-(dimethylamino)-phenylpropionyl coenzyme A; DCCoA, 4-(dimethylamino)cinnamoyl coenzyme A; IPPP, indolepropionyl pantetheine phosphate; IAPP, indoleacryloyl pantetheine phosphate; $FcPF_6$, ferricenium hexafluorophosphate; PMS, phenazine methosulfate; DCPIP, 2,6-dichlorophenolindophenol; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ETF, electron-transferring flavoprotein.

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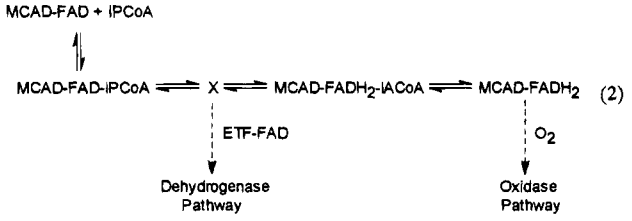
is supported by the demonstration that such fatty acids are metabolized by intact mitochondria under in vitro conditions (Mao et al., 1994). Prior to our use of indolepropionyl/acryloyl-CoA, two other chromophoric substrate/product pairs have been utilized for investigating the mechanistic details of this enzyme (McFarland et al., 1982; Murfin, 1974). Of these, the studies pertaining to the utilization of hydrocinnamoyl-CoA as the enzyme substrate have so far remained confined to the Ph.D. dissertation of Murfin (1974). McFarland and his collaborators elaborated on several interesting aspects of the enzyme-catalyzed reactions (including the discovery of the oxidase activity), utilizing furylpropionyl/acryloyl-CoA as a chromophoric substrate/product pair (McFarland et al., 1982; Reinsch et al., 1980; Gustafson, 1986).

While utilizing IPCoA as substrate, we discerned that the reductive half-reaction of the enzyme proceeds via formation of an intermediary enzyme species (designated as "X"), characterized by an absorption maximum at 400 nm (about 33 nm red-shifted from IACoA) and a broad charge transfer complex band around 600 nm (Johnson & Srivastava, 1993). The red shift in the IACoA spectrum was also observed upon formation of the MCAD-FAD-IACoA complex (Johnson et al., 1992). The microscopic pathway leading to formation of the above complex was identical to that observed during the formation of the intermediary species X (Johnson et al., 1994). Due to the chromophoric nature of the reaction product, we could easily demonstrate that the MCAD catalyzes both a dehydrogenation (in the presence of a variety of organic electron acceptors) and an oxidation (O_2 being the terminal electron acceptor) reaction of IPCoA. We demonstrated that the k_{cat}s for the dehydrogenase and oxidase reactions are limited by the rates of the formation and decay of X, respectively (Johnson & Srivastava, 1993; Johnson et al., 1994; eq 2).



(2)

We wanted to ascertain whether all the chromophoric substrate/product pairs exhibit similar properties with this enzyme, or whether there are differences based on the chemical nature of the substrates. Toward this goal, we synthesized furylpropionyl/acryloyl-CoA (FPCoA/FACoA) and [4-(dimethylamino)phenyl]propionyl/acryloyl-CoA (DPCoA/DCCoA) as additional substrate/product pairs for the enzyme. The latter pair was selected in preference to hydrocinnamoyl/cinnamoyl-CoA [previously utilized by Murfin (1974)] on the basis that the spectral shifts would be more pronounced due to the added contribution of the π-electrons of the dimethylamino groups of DPCoA and DCCoA species (Rao, 1975). Besides these, we prepared indolepropionyl pantetheine phosphate (IPPP) and indoleacryloyl pantetheine phosphate (IAPP) as a potential substrate/product pair of the enzyme. The latter pair was introduced to infer the roles of the 3′,5′-ADP moiety of the CoA thioester in catalysis. As presented in the subsequent sections, the comparative studies involving these substrate/product pairs have provided significant insights into the mechanism of the MCAD-catalyzed dehydrogenase and oxidase reactions.

MATERIALS AND METHODS

Materials

Coenzyme A (sodium salt) and phosphodiesterase I (type VI from *Crotalus adamanteus*) were purchased from Sigma. 3-Indolepropionic acid, *trans*-(3-indole)acrylic acid, 4-(dimethylamino)cinnamic acid, furylacrylic acid, ferricenium hexafluorophosphate, 2,4-dichlorophenolindophenol, and palladium (10% on activated charcoal) were purchased from Aldrich. All other reagents were of analytical reagent grade.

Methods

All experiments were performed at 25 °C in 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA, unless stated otherwise. Steady-state kinetic and spectral acquisitions were performed on a Perkin Elmer Lambda 3B spectrophotometer with either a 1- or a 10-cm path length cuvette. Medium-chain fatty acyl-CoA dehydrogenase was purified from pig kidney and assayed as described by Johnson et al. (1992).

Preparations of Furylpropionic Acid and [4-(Dimethylamino)phenyl]propionic Acid. These compounds were prepared by catalytic reduction of their acrylic acids according to Boudjouk and Han (1983). A reaction mixture containing 0.83 g of furylacrylic acid or 0.5 g of 4-(dimethylamino)-cinnamic acid, 0.5 g of palladium (10% on activated charcoal), 0.9 mL of formic acid, and 8 mL of absolute ethanol (in a 100-mL round bottom flask) was first flushed with nitrogen. The reaction mixture was then subjected to ultrasonication (in an ultrasonic laboratory cleaner bath) for 1 h at 25 °C under a continuous flow of nitrogen. Following this, the contents of the flask were filtered through a fiberglass filter. The solid residue on the filter paper was washed with 20 mL of chloroform, and the filtrates were combined and evaporated to dryness under a stream of nitrogen (total yield = 70−85%). The NMR analysis of the resultant products versus their starting materials conformed to the reduction of the double bonds of the acryloyl moieties. The extent of reduction is inferred by comparison of the UV−vis spectra of the starting materials and the resultant products (see Figure 1, Results).

The CoA derivatives of propionic and acrylic acids were prepared using the mixed anhydride procedure of Bernert and Sprecher (1977) and purified as described by Johnson et al. (1992). The total yields of these products varied between 30 and 50% of the theoretical yields. The extinction coefficients of the thioesters (CoA as well as pantetheine phosphate derivatives; see below) were determined by measuring the concentrations of the thiol groups released upon hydrolysis in the presence of 1 M hydroxylamine, pH 7.0, for 30 min at 37 °C. The thiol groups released were estimated by the DTNB method (Ellman, 1959). This was correlated with the absorption of IPCoA, IACoA, IPPP, IAPP, DPCoA, DCCoA, and FPCoA at 259, 367, 279, 364, 250, 400, and 257 nm, respectively. The extinction coefficients of these compounds were determined to be 18.2, 26.5, 6.66, 26.5, 19.4, 20.6, and 16.0 mM^{-1} cm^{-1}, respectively. The extinction coefficient of FACoA was taken to be 22.9 mM^{-1} cm^{-1} at 335 nm (McFarland et al., 1982).

Preparations of Indolepropionyl/Indoleacryloyl Pantetheine Phosphate (IPPP/IAPP). These pantetheine phosphate derivatives were prepared by the phosphorolytic cleavage of the corresponding CoA thioesters utilizing phosphodiesterase I (from *Crotalus adamanteus*). In a typical cleavage reaction (total volume = 5 mL), 3 mM IPCoA/IACoA was incubated with 20 mg of phosphodiesterase I in 0.1 M Tris-HCl buffer, pH 8.5, for 12 h at 4 °C. After this period, the solution was filtered through a 0.45 μm membrane filter. The resultant solution was loaded onto a C_{18}-HPLC preparatory column (Alltech, 25 × 1 cm i.d.), which was preequilibrated with a 20 mM phosphate buffer, pH 7.0 (solvent A). The column was first washed with this buffer for 5 min, followed by a wash with 30% pure methanol (solvent B) for an additional 5 min. After this, a gradient of 30% to 100% in solvent B was applied in 40 min, maintaining a flow rate of 1.8 mL/min. The effluents from the column was monitored at 254 nm. The IPPP and IAPP fractions were identified by their absorption profiles, shown in Figure 1. The appropriate fractions were pooled and stored at −20 °C after removal of the solvent methanol (total yields = 50−70%).

Steady-State and Transient Kinetic Investigations. The initial rates of the MCAD-catalyzed reaction at varying concentrations of IPCoA, IPPP, FPCoA, and DPCoA were measured at 367, 364, 335, and 400 nm, respectively, in the presence and absence of $FcPF_6$. A 1-cm path length cuvette was used in all the experiments except for measurement of the enzyme-catalyzed oxidase activities utilizing IPCoA and FPCoA as substrates. In the latter cases, a 10-cm path length cuvette was used.

Single-wavelength transient kinetic experiments and data analysis were performed on an Applied Photophysics MV-14 sequential mixing stopped-flow system (optical path length = 10 mm, dead time = 1.34 ms).

Rapid-scanning spectra were acquired on a Beckman 7400 diode-array spectrophotometer. The solutions of MCAD and IPPP were made anaerobic by repeated degassing and purging with oxygen-free argon, followed by addition of glucose and glucose oxidase (Johnson & Srivastava, 1993). To a continuously stirred solution of MCAD (with the help of a magnetic stirrer, installed at the bottom of the cuvette holder) was added a small aliquot of IPPP. In this way, the mixing was complete within 3 s, and the first spectrum was acquired within 5 s. The subsequent spectra were acquired at every 3-s interval via the repetitive rapid-scanning mode of the instrument.

The experimental results were analyzed by a nonlinear regression analysis program, Enzfitter (Biosoft).

RESULTS

Figure 1 shows a comparison account of the spectral properties of indole-, furyl-, and 4-(dimethylamino)phenyl-substituted (at the γ position) propionyl- and acryloyl-CoAs in 50 mM phosphate buffer (pH 7.6), containing 0.3 mM EDTA. Note that all these CoA derivatives exhibit a common absorption peak around 260 nm, which is contributed both by the adenine portion of the CoA moiety and by the aromatic/heterocyclic rings of these compounds. Unlike acyl-CoAs, the corresponding enoyl-CoAs are marked by distinct spectral bands at higher wavelength regions, and their absorption maxima depend upon the chemical nature of the aromatic/heterocyclic groups. For example, indoleacryloyl-

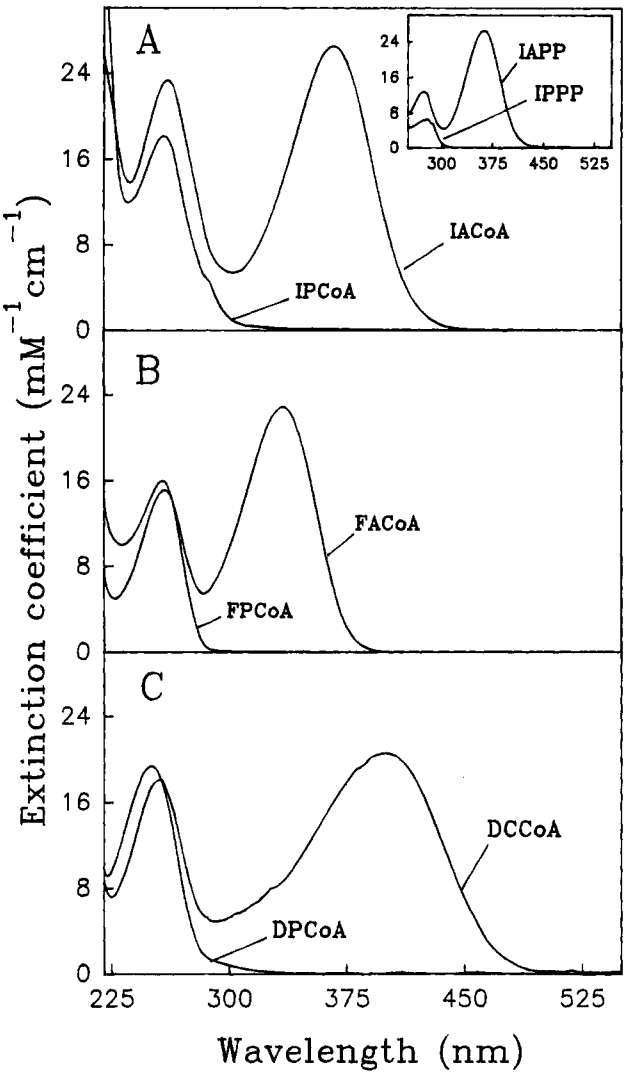


FIGURE 1: UV−vis absorption spectra of various substrate/product pairs (individually labeled in panels A, B, and C) of the medium-chain fatty acyl-CoA dehydrogenase in 50 mM potassium phosphate buffer, pH 7.6, containing 0.3 mM EDTA at 25 °C. The individual spectra are presented as extinction coefficients as functions of the wavelength.

CoA (IACoA), furylacryloyl-CoA (FACoA), and 4-(dimethylamino)cinnamoyl-CoA (DCCoA) are characterized by unique spectral bands at 367, 335, and 400 nm, respectively. The origin of these spectral bands lies in the extended conjugation of the π-electrons of the acryloyl moieties to the aromatic/heterocyclic rings (Rao, 1975).

Besides these CoA derivatives, we prepared indolepropionyl- and indoleacryloyl pantetheine phosphates (IPPP and IAPP) by phosphorolytic cleavage of indolepropionyl-CoA (IPCoA) and indoleacryloyl-CoA (IACoA), respectively, followed by removal of the 3′,5′-ADP moiety (see Materials and Methods). The inset of Figure 1A shows the spectra of these pantetheine derivatives. Note that, due to deletion of the adenine group from IPCoA/IACoA, the absorption peaks of IPPP/IAPP around 260 nm are considerably lower. However, the near-visible spectral band of IAPP (absorption maximum = 364 nm) is comparable to that found in the case of IACoA (absorption maximum = 367 nm; Johnson et al., 1992). Hence, the deletion of the 3′,5′-ADP moiety from IACoA has practically no influence on the electronic structure of the indoleacryloyl conjugation.

Table 1: Steady-State Kinetic Parameters for the "Dehydrogenase" and "Oxidase" Reactions of the Enzyme Utilizing Different Chromogenic Substrates[a]

kinetic parameters	substrates			
	IPCoA	FPCoA	DPCoA	IPPP
dehydrogenase reaction[b]				
K_m (μM)	10.3 ± 0.58	14.4 ± 0.65	61.0 ± 4.7	160 ± 39.1
k_{cat} (s^{-1})	0.46 ± 0.0052	13.2 ± 0.18	0.61 ± 0.012	0.0064 ± 0.0007
k_{cat}/K_m (M^{-1} s^{-1})	4.5×10^4	9.2×10^5	1.0×10^4	40
oxidase reaction[c]				
K_m (μM)	0.49 ± 0.035	<0.05	8.6 ± 1.01	158 ± 10.4
k_{cat} (s^{-1})	0.016 ± 0.0011	~0.0054	0.088 ± 0.003	0.0071 ± 0.0002
k_{cat}/K_m (M^{-1} s^{-1})	3.3×10^4		1.0×10^4	45.8

[a] Determined in 50 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA at 25 °C. K_m represents the apparent value for the individual acyl-CoA substrate under the experimental condition employed herein. [b] Measured in the presence of 180−300 μM $FcPF_6$. [c] Buffer dissolved oxygen (240 μM) served as the electron acceptor.

Taking advantage of the spectral differences between the chromophoric propionyl- and acryloyl-CoAs, we monitored the enzyme-catalyzed reaction rates (at absorption maxima of different enoyl-CoAs) without recourse to the absorption signals of the "colored" electron acceptors. A preliminary experiment suggested to us that all these CoA substrates are converted into their corresponding enoyl-CoA products via the MCAD-catalyzed "dehydrogenase" and "oxidase" reactions (data not shown). By maintaining *identical reaction* conditions, we measured the time courses for the enzyme-catalyzed conversions of these substrates into their corresponding products in the presence of $FcPF_6$ (180−300 μM) or in its absence; under the latter condition, the buffer-dissolved O_2 (240 μM) served as an electron acceptor (i.e., the oxidase reaction). The initial rates of the enzyme-catalyzed dehydrogenase reactions (utilizing different substrates) showed hyperbolic dependence on the individual propionyl-CoA substrate concentrations. The apparent K_ms (for the CoA substrates) and the k_{cat}s, derived from the best fit of the experimental data according to the Michaelis−Menten equation, as well as the k_{cat}/K_m values are summarized in Table 1. Although the K_m values for IPCoA (10.3 μM) and FPCoA (14.4 μM) in the dehydrogenase reaction are comparable, they are about 5−6-fold lower than that for DPCoA (K_m = 61 μM). On the other hand, although the k_{cat}s for the enzyme with IPCoA (0.46 s^{-1}) and DPCoA (0.61 s^{-1}) are comparable, they are about 20-fold lower than that obtained with FPCoA (13.2 s^{-1}) as substrate. On the basis of the k_{cat}/K_m values, the specificities (Fersht, 1985, page 105) of these substrates during the MCAD-catalyzed dehydrogenase reaction were judged to be in the following order: FPCoA > IPCoA > DPCoA.

We also measured the apparent K_m values (for the individual propionyl-CoA substrates) and the k_{cat} values for the enzyme-catalyzed oxidase reactions. For these reactions, the buffer-dissolved oxygen (240 μM) served as the electron acceptor. These values are also summarized in Table 1. Upon comparison of these vis a vis the results obtained during the dehydrogenase reaction, it is apparent that both the K_m values for the individual substrates and the k_{cat} values are lower during the oxidase than during the dehydrogenase reaction (see Discussion). Among different substrates, although the k_{cat} values for the oxidase reactions are more or less the same, the K_m values for FPCoA (<0.05 μM) and DPCoA (8.6 μM) are the lowest and highest, respectively, among the substrates utilized herein. The k_{cat}/K_m values calculated for the individual substrates during the oxidase and dehydrogenase reactions are found to be the same, suggesting that both of these reactions proceed via formation of a common enzyme−substrate complex (see Discussion). Furthermore, the specificities for different substrates (during the MCAD-catalyzed oxidase reaction) followed the same order as observed during the dehydrogenase reaction, i.e., FPCoA > IPCoA > DPCoA.

Reactivity of Indolepropionyl Pantetheine Phosphate (IPPP) with MCAD-FAD. Given that indoleacryloyl pantetheine phosphate (IAPP) absorbs light at 364 nm (see the inset of Figure 1A), we could easily measure the dehydrogenase and oxidase activities of the enzyme involving indolepropionyl pantetheine phosphate (IPPP) as substrate. In this endeavor, we employed the experimental conditions utilized with the other chromogenic substrates (see above). Figure 2 shows the time course for the formation of IAPP (measured by an increase in absorption at 364 nm) upon incubation of 100 μM IPPP with 1 μM MCAD-FAD in the absence and presence of 200 μM $FcPF_6$. Note that the reaction progress under both these conditions has more or less a common slope, implying that the dehydrogenase and oxidase reactions proceed with equal efficiencies. This was surprising since with all the other substrates, including IPCoA, the time profiles for the dehydrogenase reactions were at least 10-fold steeper than those for the complementary oxidase reactions (data not shown).

Assuming that the apparent similarity in the reaction profiles for the dehydrogenase and oxidase reactions is due to some unforeseen kinetic complexity, we measured the IPPP concentration dependence of the MCAD-catalyzed reaction in the absence and presence of 180 μM $FcPF_6$. As shown in Figure 2B, the initial rates of the enzyme-catalyzed reactions under these conditions exhibit a common hyperbolic dependence on IPPP concentration, with apparent K_m (for IPPP) and k_{cat} values of 158 μM and 0.007 s^{-1}, respectively. It is noteworthy that although the K_m for IPPP is 1−2 orders of magnitude higher than those obtained with IPCoA during the dehydrogenase and oxidase reactions, respectively (see Table 1), the k_{cat} for the latter reaction (utilizing IPPP as substrate) is remarkably similar to that obtained with IPCoA as substrate. In fact, to our surprise, the k_{cat}s for the oxidase reactions with all the chromogenic substrates, utilized herein, have been found to be within an order of magnitude of one another.

The experimental results of Figure 2 prompted us to inquire whether the dehydrogenase activity of the enzyme indeed persists with IPPP as substrate or not. To ascertain

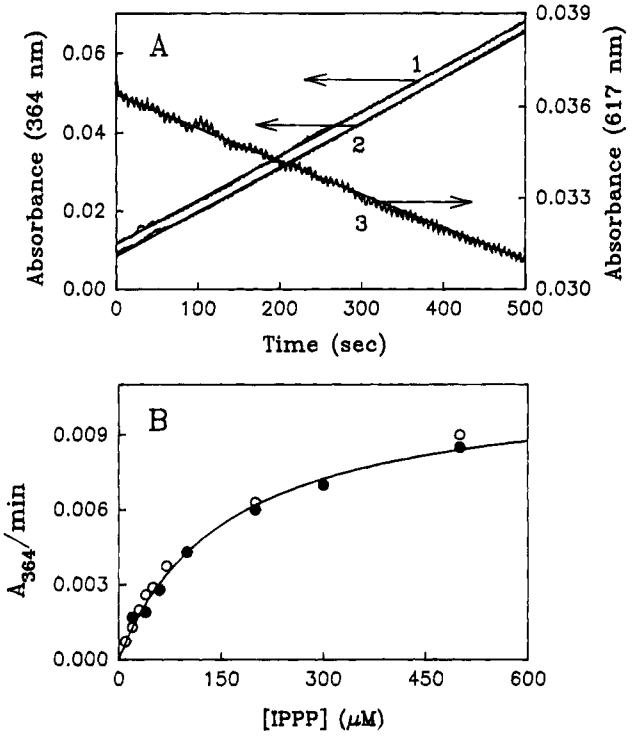


FIGURE 2: Medium-chain fatty acyl-CoA dehydrogenase-catalyzed conversion of indolepropionyl pantetheine phosphate (IPPP) to indoleacryloyl pantetheine phosphate (IAPP). Panel A represents the time courses for the increase in absorption at 364 nm due to formation of IAPP upon incubation of 100 μM IPPP with 1 μM MCAD in the absence and presence of 200 μM $FcPF_6$ (traces 1 and 2, respectively). Trace 3 represents the decrease in absorption at 617 nm due to the reduction of $FcPF_6$ upon incubation of 100 μM IPPP, 100 μM $FcPF_6$, and 3 μM MCAD. Other conditions are similar to those of Figure 1. Panel B represents the IPPP concentration dependent oxidase (○) and dehydrogenase (●) activities of the enzyme (1.03 μM) at varying concentrations of IPPP. During the dehydrogenase reaction, 180 μM $FcPF_6$ was used as electron acceptor. The solid line represents the best fit of the experimental data with a K_m of 158 μM and a V_{max} of 0.011 A_{364}/min. The corresponding k_{cat} was calculated to be 0.0067 s^{-1}.

this, we compared the time-dependent reduction of $FcPF_6$ (measured at 617 nm; Johnson et al., 1993) during the course of the enzyme-catalyzed conversion of IPPP to IAPP under aerobic conditions. Figure 2A (trace 3) shows the time-dependent decrease in absorption at 617 nm due to the reduction of $FcPF_6$, under a reaction condition similar to that employed to detect the conversion of IPPP to IAPP (Figure 2A, traces 1 and 2). The reduction of $FcPF_6$, concomitant with the oxidation of IPPP, confirmed that the dehydrogenase reaction indeed prevails with this substrate. However, the turnover rate of the enzyme calculated for the reduction of $FcPF_6$ was found to be about 2-fold higher than that calculated for the oxidation of IPPP to IAPP. This difference is presumably due to the fact that whereas the $FcPF_6$ reduction is a one-electron process, the IPPP oxidation involves abstraction of two electrons. A similar reaction stoichiometry has been seen during the ETF/DCPIP and PMS/DCPIP coupled assay systems (data not shown). Hence, in the presence of organic electron acceptors (e.g., $FcPF_6$, ETF/DCPIP, PMS/DCPIP), the enzyme-catalyzed conversion of IPPP to IAPP exclusively proceeds via the dehydrogenase pathway. This is presumably because the presence of $FcPF_6$ "shuts off" the oxidase pathway, either by stabilizing the intermediary species X or by facilitating a faster rate of electron transfer (from X to $FcPF_6$) vis a vis the rate of conversion of X to the MCAD-$FADH_2$-IAPP complex (see Discussion).

To assess the mechanistic basis of similarity between the K_m and k_{cat} values of the dehydrogenase and oxidase pathways, respectively, we measured the transient course of the reaction of MCAD-FAD and IPPP (at 450 nm) under both anaerobic and aerobic conditions (Figure 3). Note that under anaerobic conditions this reaction is characterized by a decrease in absorption at 450 nm (Figure 3, curve 1). The reaction profile is consistent with a first-order rate law, with a rate constant of 0.0091 s^{-1}. On the contrary, no absorption changes are detectable at 450 nm under aerobic condition (Figure 3, curve 2). However, under the latter condition, the oxidase activity of the enzyme is detectable as a zero-order increase in absorption at 364 (Figure 3, curve 3). The turnover rate of the enzyme calculated from this zero-order phase (0.0059 s^{-1}) is about 1.5-fold lower than the first-order rate constant for the reduction of the enzyme-bound flavin by IPPP under anaerobic conditions (see Discussion).

DISCUSSION

The experimental results presented in the previous section reveal that the K_ms for the acyl-CoA substrates during the oxidase reaction are about an order of magnitude lower than those obtained during the dehydrogenase reaction. In general, K_m is qualitatively given by the ratio of [E][S] to the sum of the intermediary species involved in the overall reaction pathway (Dixon & Webb, 1979). Accordingly, the lower K_ms for the acyl-CoA substrates, during the oxidase versus the dehydrogenase reaction, imply that more intermediary species are involved in the former than in the latter pathway. This expectation is attested by our previous demonstration that the dehydrogenase reaction is a result of transfer of electrons from the intermediary species X (formed upon reaction of MCAD with IPCoA) to the organic electron acceptors, whereas the oxidase reaction is a result of the transfer of electrons from MCAD-$FADH_2$ to O_2 (Johnson & Srivastava, 1993; Johnson et al., 1993, 1994).

Like K_ms, the k_{cat}s for the oxidase reaction are also about an order of magnitude lower than those for the corresponding dehydrogenase reactions with acyl-CoA substrates (Table 1). As a consequence, k_{cat}/K_m remains invariant between the oxidase and dehydrogenase reactions of the individual substrates. This is not unexpected since k_{cat}/K_m is a function of the free enzyme concentration and is not affected by the complexity of the overall microscopic pathway (Fersht, 1985, pp 103−106). Since both dehydrogenase and oxidase reactions proceed via formation of a common enzyme−substrate complex (Johnson & Srivastava, 1993; Johnson et al., 1994), this ratio remains constant for a particular substrate type. This ratio, however, changes from substrate to substrate (Table 1). Hence, although the k_{cat}/K_m values serve as the measure of the "specificity" of the enzyme for different substrates, they do not provide any information about the efficiency of the branching pathways.

Role of the 3′,5′-ADP Moiety of the CoA Thioester during Catalysis. The most interesting aspect of the IPPP-dependent enzyme catalysis is the fact that the K_m (for IPPP) and k_{cat} values obtained during the dehydrogenase reaction are equal to the corresponding values obtained during the oxidase reaction (see Figure 2). Obviously, k_{cat}/K_m remains the same

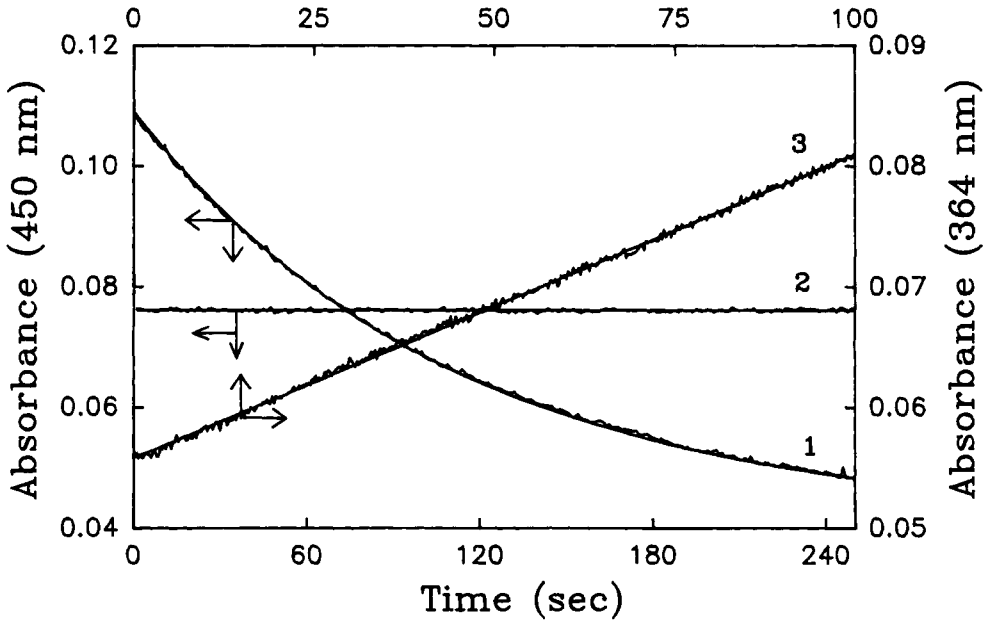


FIGURE 3: Reactions of MCAD-FAD with IPPP. Trace 1: Time-dependent decrease in absorption at 450 nm due to the reaction of MCAD-FAD (6 μM) with IPPP (400 μM) under anaerobic conditions. The solid smooth line is the best fit of the experimental data for a single-exponential decay in absorption with a rate constant of 0.0091 s^{-1}. Trace 2: Time course for the absorption changes at 450 nm (under aerobic conditions) for the reaction of 5 μM MCAD-FAD with 75 μM IPPP. Trace 3: Time course for the increase in absorbance at 364 nm under the experimental conditions of trace 2. The solid smooth lines for traces 2 and 3 are the best fits of the experimental data according to the linear regression analysis, with slopes equal to 0 (trace 2) and 2.52×10^{-4} ΔA_{364} s^{-1} (equivalent to a k_{cat} of 0.0059 s^{-1}; trace 3), respectively.

for these two (dehydrogenase and oxidase) reactions involving IPPP as substrate. This observation puzzled us initially, since all the (chromogenic) acyl-CoA substrates, including IPCoA, yielded higher K_m and k_{cat} values during the dehydrogenation than the oxidation reaction (see Table 1). Since IPPP differs from IPCoA by the 3′,5′-ADP moiety of the CoA thioester, the role of this moiety in demarcating the two activities of the enzyme was envisaged.

On the basis of the X-ray crystallographic structure of the enzyme−octenoyl-CoA complex, it is evident that the 3′,5′-ADP moiety constitutes a "short arm and loop" of the "J"-shaped coenzyme A thioester molecule (Kim et al., 1993). This moiety appears to interact rather loosely within the protein structure, and it is freely accessible to the outside solvent environment. Of several amino acid residues present in the vicinity of the 3′,5′-ADP moiety (e.g., Phe-245, Met-249, Ser-191, Arg-324), the side chains of Ser-191 and Arg-324 are known to form hydrogen bonds. This, in conjunction with the experimental results of Frerman et al. (1980), has led to the speculation that the 3′-AMP fragment of the coenzyme A might not be involved in catalysis (Kim et al., 1993). Since no additional hydrogen bonding has been deduced between the 5′-phosphate of the coenzyme A thioester and the protein molecule, it is unlikely that the affinity of the protein for the 3′,5′-ADP moiety would be drastically different from that expected for the 3′-AMP moiety. Unfortunately, this expectation is contrary to the experimental data presented herein. In fact, our experimental results suggest that the 3′,5′-ADP moiety plays a crucial role in stabilizing the intermediary species, X (see below), which is responsible for promoting the dehydrogenase reaction of the enzyme (Johnson & Srivastava, 1993; Johnson et al., 1994). Given that the binding region of the 3′,5′-ADP moiety (on the protein structure) is far removed from the catalytic site of the enzyme (Kim et al., 1993), the question arises as to how such a distal fragment of the substrate structure influences the catalytic properties (dehydrogenase versus oxidase reaction) of the enzyme. To answer this question, we first attempted to discern whether the enzyme-catalyzed reaction mechanism utilizing IPPP as substrate is the same as that obtained with IPCoA (Johnson & Srivastava, 1993; Johnson et al., 1993, 1994). In particular, we intended to establish whether the IPPP-dependent reductive half-reaction of the enzyme involves the formation of the intermediary species X (known to be formed with IPCoA) or not. Our initial attempts toward this goal were frustrating; we could not detect X during the transient course of reaction of MCAD-FAD with IPPP under normal (i.e., [IPPP] > [MCAD-FAD]) experimental condition, a situation contrary to that observed with IPCoA substrate (Johnson & Srivastava, 1993; Johnson et al., 1994). However, under strictly anaerobic conditions, and when [MCAD-FAD] > [IPPP], we could detect the formation of X during the (transient) course of the MCAD-FAD + IPPP reaction. Figure 4 shows the time-resolved spectral data (see Materials and Methods) for the reaction of 15 μM MCAD-FAD and 13 μM IPPP. The difference spectra (the spectra at a given time minus the zero time; Figure 4B) reveal that, during the initial time regimes (e.g., spectra 1−3), the chromophoric (intermediary) species are characterized by absorption bands around 386 nm. As the reaction progresses further, the 386 nm-absorption band shifts to 364 nm (the absorption maximum characteristic of IAPP). This is evident by a decrease in $\Delta A_{386}/\Delta A_{364}$ (Figure 4B) from 1.6 (trace 1) to 0.78 (trace 11). The shift in the spectral pattern of Figure 4B is qualitatively similar to that observed with the IPCoA-dependent reductive half-reaction of the enzyme (Johnson & Srivastava, 1993), suggesting that the overall reaction pathway of the enzyme involving these two substrates (i.e., IPPP and IPCoA) is the same. Hence, it is unlikely that MCAD-FAD-IPPP is directly converted into MCAD-$FADH_2$-IAPP, i.e., without the formation of the intermediary species

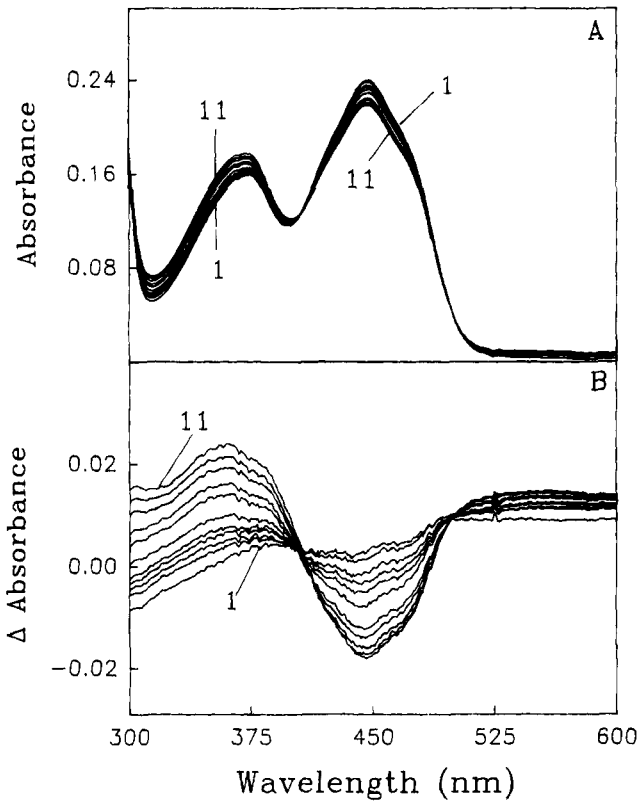


FIGURE 4: Rapid-scanning spectra for the reaction of MCAD-FAD and IPPP under anaerobic conditions. Spectra in panel A represent the time courses for the reaction of 15 μM MCAD-FAD and 13 μM IPPP. Spectra in panel B represent the difference spectra (i.e., the spectra at a given time minus the zero-time spectrum). The zero-time spectrum was constructed by addition of the independently acquired spectra of MCAD-FAD and IPPP under identical experimental conditions. The first spectrum was obtained within 5 s after reaction. The time sequence (in seconds) for spectra 2−11 is as follows: (2) 6, (3) 9, (4) 12, (5) 15, (6) 21, (7) 33, (8) 39, (9) 48, (10) 54, and (11) 60.

X. Therefore, we considered that the similar K_m (IPPP) and k_{cat} values during the enzyme-catalyzed dehydrogenase and oxidase reactions, respectively, might have arisen due to a common rate-limiting step of these two pathways. Furthermore, the following circumstantial evidence prompted us to realize that, unlike the IPCoA-generated X, the IPPP-generated X is considerably unstable: (1) a single (instead of double) relaxation process during the IPPP-dependent reduction of MCAD-FAD, (2) the lack of evidence for the formation and decay of X (at 386 nm) under anaerobic conditions where [IPPP] > [MCAD-FAD] (data not shown), and (3) the absence of absorption changes at 450 nm during the reaction of MCAD-FAD + IPPP under aerobic conditions (Figure 3, trace 2) and a zero-order increase in absorbance at 364 nm (Figure 3, trace 3). These observations led us to propose that the primary difference between the IPCoA- and IPPP-dependent reactions is the relative magnitudes of the equilibration rate constants of X between its preceding and succeeding enzyme species; in the case of IPCoA, the formation of X is faster than its decay, whereas in the case of IPPP, the formation of X is slower than its decay (eq 3).

$$\text{MCAD-FAD-IPCoA} \underset{}{\overset{\text{Fast}}{\rightleftharpoons}} \text{X} \overset{\text{Slow}}{\rightleftharpoons} \text{MCAD-FADH}_2\text{-IACoA}$$

$$\text{MCAD-FAD-IPPP} \overset{\text{Slow}}{\rightleftharpoons} \text{X} \overset{\text{Fast}}{\rightleftharpoons} \text{MCAD-FADH}_2\text{-IAPP} \qquad (3)$$

According to this model, with either of the two substrates, X and MCAD-FADH$_2$ are the specific redox enzyme species which transfer electrons to organic electron acceptors (dehydrogenase reaction) and O_2 (oxidase reaction), respectively (Johnson & Srivastava, 1993; Johnson et al., 1994). Since with IPCoA the rate of formation of X is faster than its decay, K_m (for IPCoA) and k_{cat} during the dehydrogenase reaction are higher than the corresponding parameters during the oxidase reaction (Johnson & Srivastava, 1993; Johnson et al., 1994). However, a completely different situation prevails for the IPPP-dependent reaction. The single-exponential process during the IPPP-dependent redox reaction of MCAD-FAD (resulting in the formation of a mixture of X, MCAD-FADH$_2$-IAPP, and MCAD-FADH$_2$ species) implies that the MCAD-FAD-IPPP $\rightleftharpoons$ X step limits the overall relaxation process. Consequently, the relaxation rate constant of 0.0091 s^{-1} serves as a qualitative measure of the sum of the forward and reverse steps between MCAD-FAD-IPPP and X. Since this rate constant is comparable to the k_{cat} for the enzyme (both dehydrogenase and oxidase reactions), it follows that the formation of X is the rate-limiting step during the IPPP-dependent reaction. Given that the electron transfers either from X to $FcPF_6$ (Johnson et al., 1993) or from MCAD-FADH$_2$ to O_2 (Johnson et al., 1994) are faster than 0.007 s^{-1}, the K_m (for IPPP) and k_{cat} values during dehydrogenase and oxidase reactions emerge to be the same. This explanation provides the mechanistic basis for why the steady-state kinetic parameters during the IPPP-dependent dehydrogenase and oxidase reactions are the same (Figure 2B).

Just how does the 3′,5′-ADP moiety of IPCoA enhance the catalytic turnover of the dehydrogenase reaction? Although a definitive answer to this question must await further structural−functional studies, some energetic consequences of the effects of the 3,5′-ADP moiety on enzyme catalysis are noteworthy. A comparative analysis of the magnitudes of the equilibrium and rate constants for the reactions of eq 3 (Johnson & Srivastava, 1993; see also Table 1) allows us to predict that, whereas the ground state of the ES is stabilized by 1.6 kcal/mol, the transition state (leading to the formation of X) is stabilized by 4.1 kcal/mol. Hence, the potential of the 3′,5′-ADP moiety is maximally realized toward stabilizing the transition state, ES‡. This, together with the stabilization of the ground state of X by 2.0 kcal/mol, suggests that the 3′,5′-ADP moiety (the seemingly "useless" fragment of the coenzyme A structure) is intimately involved in alleviating the energy barrier toward the formation of the intermediary species X, as well as in stabilizing the ground state of the latter species. These effects are translated into an enhancement of the k_{cat} of the dehydrogenase reaction by about 72-fold, with a decrease in the K_m for the substrate by 16-fold. However, to our surprise, the 3′,5′-ADP moiety does not seem to play any significant role in suppressing the oxidase activity (k_{cat}) of the enzyme. In fact, the oxidase activity remains unchanged among all the substrates utilized herein (Table 1).

We propose that the medium-chain fatty acyl-CoA dehydrogenase is primarily evolved to promote the dehydrogenase reaction of the enzyme, and this is achieved, in part, by utilizing the binding energy of the 3′,5′-ADP moiety of the coenzyme A thioester. The oxidase activity of the enzyme emerges due to a slow decay of X (in the absence of the organic electron acceptors) into MCAD-FADH$_2$, which is readily oxidized by the buffer-dissolved molecular oxygen

(Johnson et al., 1994). We are currently investigating the factors which are responsible for destabilizing X, kinetically and/or thermodynamically, and we will report on these findings subsequently.

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