Functional role of a distal (3'-phosphate) group of CoA in the recombinant human liver medium-chain acyl-CoA dehydrogenase-catalysed reaction

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The X-ray crystallographic structure of medium-chain acyl-CoA dehydrogenase (MCAD)–octenoyl-CoA complex reveals that the 3'-phosphate group of CoA is confined to the exterior of the protein structure [approx. 15 Å (1.5 nm) away from the enzyme active site], and is fully exposed to the outside solvent environment. To ascertain whether such a distal (3'-phosphate) fragment of CoA plays any significant role in the enzyme catalysis, we investigated the recombinant human liver MCAD (HMCAD)-catalysed reaction by using normal (phospho) and 3'-phosphate-truncated (dephospho) forms of octanoyl-CoA and butyryl-CoA substrates. The steady-state kinetic data revealed that deletion of the 3'-phosphate group from octanoyl-CoA substrate increased the turnover rate of the enzyme to about one-

quarter, whereas that from butyryl-CoA substrate decreased the turnover rate of the enzyme to about one-fifth; the K_m values of both these substrates were increased by 5–10-fold on deletion of the 3'-phosphate group from the corresponding acyl-CoA substrates. The transient kinetics for the reductive half-reaction, oxidative half-reaction and the dissociation 'off-rate' (of the reaction product from the oxidized enzyme site) were all found to be affected by deletions of the 3'-phosphate group from octanoyl-CoA and butyryl-CoA substrates. A cumulative account of these results reveals that, although the 3'-phosphate group of acyl-CoA substrates might seem 'useless' on the basis of the structural data, it has an essential functional role during HMCAD catalysis.

INTRODUCTION

In pursuit of delineating the kinetic mechanism of medium-chain acyl-CoA dehydrogenase (MCAD) [1–7], we became interested in the role(s) of distal ('seemingly useless') portions of the CoA structure (among acyl-CoA substrates) in the enzyme catalysis. The latter was prompted by our observation that the 3',5'-ADP fragment of CoA of a chromogenic substrate, 3-indolepropionyl-CoA (IPCoA), has a crucial role in the enzyme catalysis [8]. By undertaking comparative kinetic, thermodynamic and spectroscopic studies of pig kidney MCAD, with IPCoA and indolepropionyl pantetheine phosphate (IPPP, the 3',5'-ADP-truncated CoA derivative of IPCoA) as substrates, we concluded the following [8]. (1) The 3',5'-ADP fragment provides approx. 6.7 kJ/mol of binding energy for stabilizing the ground state of the ES complex. This fragment stabilizes the transition state between the ES complex and the intermediary charge-transfer (CT) complex (formerly referred to as 'X'; [4]) by approx. 17.1 kJ/mol. (2) This deletion of the 3',5'-ADP fragment of IPCoA destabilizes the intermediary CT complex. As a result, the CT complex is not readily detectable during the IPPP-dependent reductive half-reaction of the enzyme. (3) With IPCoA as substrate, whereas the rate-limiting step for the 'dehydrogenase' reaction is the conversion of the ES Michaelis complex into a CT complex, the rate-limiting step for the 'oxidase' reaction is the decay of CT (Scheme 1) [3,4]. In contrast, with IPPP as substrate, both the dehydrogenase and oxidase reactions of the enzyme are limited by a common microscopic step, namely the conversion of the ES Michaelis complex into a CT complex [8]. On the basis of these differences, we were prompted to conclude that the 3',5'-fragment of CoA has an essential role in promoting the physiologically desirable dehydrogenase reaction of this enzyme [8].

In Scheme 1, S and P refer to substrate (acyl-CoA) and product (enoyl-CoA) respectively. Although the above model was initially proposed for the IPCoA/IPPP-dependent reaction of the enzyme, further experimental data suggested that it was also applicable for the octanoyl-CoA-dependent reaction of the enzyme (as octanoyl-CoA is a physiological substrate of the enzyme) [6].

The X-ray crystallographic structure of the pig liver MCAD–FAD–octenoyl-CoA complex reveals that the 3'-AMP moiety of CoA forms a short arm and the pyrophosphate the right-angled turn; the fatty acyl-pantetheine fragment is extended as a long arm of the J-shaped octenoyl-CoA molecule. There are only two

Scheme 1 Mechanistic origin of the dehydrogenase and oxidase pathways of MCAD

ETF, electron-transferring flavoprotein; FcPF₆, ferrocenium hexafluorophosphate. The asterisk indicates change in the electronic structure.

Abbreviations used: CT complex, charge-transfer complex; ETF, electron-transferring flavoprotein; FcPF₆, ferrocenium hexafluorophosphate; HMCAD, human liver medium-chain acyl-CoA dehydrogenase; IPCoA, 3-indolepropionyl-CoA; IPPP, 3-indolepropionyl pantetheine phosphate; MCAD, medium-chain acyl-CoA dehydrogenase.

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Figure 1 Ribbon structure of pig liver MCAD–FAD–octenoyl-CoA complex

The monomeric protein backbone of the enzyme with the side chain residue Ser-191 is shown. The distance between the 3'-phosphate group of octenoyl-CoA and the OH-group of Ser-191 is 3.33 Å (0.333 nm). A water molecule in the vicinity of the 3'-phosphate group is shown.

potential hydrogen bonds between octenoyl-CoA and the enzyme protein. These are between the 3'-phosphate oxygen and the hydroxy group of Ser-191, and between the amino group of adenine and the guanidino group of Arg-324 [9]. The 3'-phosphate group of octenoyl-CoA constitutes the most distal fragment of the CoA structure [approx. 15 Å (1.5 nm) removed from the enzyme active site] and is fully exposed to the exterior solvent environment (Figure 1).

Given the above mechanistic deductions from the X-ray crystallographic data [9], we became interested in ascertaining the minimal structural requirement of CoA for maintaining the efficient dehydrogenase reaction of the enzyme. Towards this end, we note that Frerman et al. [10], in a preliminary manner, had compared the K_m and k_{cat} values of pig liver MCAD with octanoyl-CoA and 3'-dephospho-octanoyl-CoA as substrates, and claimed that the above parameters of the enzyme remained unaffected on deletion of the 3'-phosphate group from octanoyl-CoA substrate. Clearly, according to these investigators, the 3'-phosphate group of CoA had no functional role during MCAD catalysis. In contrast, Osumi et al. [11] noted, for the functionally analogous enzyme acyl-CoA oxidase, that the steady-state parameters of the latter enzyme were substantially affected on deletion of the 3'-phosphate group from its physiological substrate, palmitoyl-CoA. Although the origin of the above apparently contradictory results might be attributed to the difference in the enzyme types, we decided to reinvestigate the effect of the 3'-phosphate on the human liver MCAD (HMCAD)-catalysed reaction with butyryl-CoA and octanoyl-CoA as the enzyme substrates. These substrates were selected for the following reasons: (1) the microscopic pathways of the octanoyl-CoA- and butyryl-CoA-dependent reactions are different [5,6,12], and (2) the rate-limiting step of the enzyme is different with these substrates [13]. By using these substrates we sought to probe the specific role (if any) of the 3'-phosphate group of acyl-CoAs during different steps of the enzyme catalysis.

MATERIALS AND METHODS

Materials

CoA (sodium salt), octanoic acid, octanoyl-CoA, 3'-dephospho-CoA and EDTA were purchased from Sigma. Butyric anhydride and ferrocenium hexafluorophosphate (commercial name of ferrocenium hexafluorophosphate; $FcPF_6$) were purchased from Aldrich. Crotonoyl-CoA was purchased from Fluka. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim. All other reagents were of A. R. 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. HMCAD was purified and assayed essentially as described by Peterson et al. [7], except that *Escherichia coli* (BL21 DE3) cells were grown at 20 °C for expressing the enzyme. The latter modification increased the yield of crude HMCAD by 3-fold.

Butyryl-CoA was prepared by the reaction of butyric anhydride with CoA as described by Johnson et al. [1,14]. The 3'-dephosphorylated derivatives of octanoyl-CoA, octenoyl-CoA, butyryl-CoA and crotonoyl-CoA were prepared by alkaline phosphatase treatment of the corresponding CoA derivatives. The 3'-dephospho-octanoyl-CoA was alternatively prepared by direct thio-esterification of octanoic acid with 3'-dephospho-CoA by the mixed-anhydride method of Bernert and Sprecher [15]. For the preparation of the 3'-dephospho derivatives of octanoyl-CoA and butyryl-CoA by the alkaline phosphatase method, in a typical reaction mixture (total volume 5 ml), 4 mM octanoyl-CoA or butyryl-CoA was incubated with approx. 2 units (1 unit = enzyme amount required to hydrolyse 1 μ mol of 4-nitrophenyl phosphate to 4-nitrophenyl in 1 min at pH 8 and

37 °C) of alkaline phosphatase at 4 °C for 24 h in a 50 mM Tris/HCl buffer, pH 8.5, containing 1 mM EDTA. The solution was subsequently filtered through a 0.45 μ m membrane filter and then loaded on a C₁₈ HPLC preparatory column (Alltech; 25 cm $\times$ 1 cm internal diam.) that had been pre-equilibrated with 50 mM ammonium acetate buffer, pH 5.5. The column was first washed with the above buffer for 5 min. After this, a gradient of 50 mM ammonium acetate buffer (100 % in A) to pure methanol (100 % in B) was applied over 45 min, and the elution profile was monitored at 254 nm. The 3'-dephospho-CoA derivatives were found to be eluted just after the corresponding acyl-CoA substrates. To avoid contamination of parent acyl-CoA-derivatives in our 3'-dephospho preparations, we checked the individual fractions by re-chromatography on an analogous C₁₈ HPLC analytical column (Alltech; 25 cm $\times$ 0.46 cm internal diam.). Only fractions that were devoid of any contamination were pooled, then stored at -20 °C after removal of methanol on a rotatory evaporator.

The purity of crotonoyl-CoA and 3'-dephosphocrotonoyl-CoA preparations were checked by their conversions into corresponding β -hydroxy derivatives in the presence of enoyl-CoA hydratase [16].

The absorption coefficients of octanoyl-CoA, 3'-dephospho-octanoyl-CoA, butyryl-CoA, 3'-dephosphobutyryl-CoA, crotonoyl-CoA and 3'-dephosphocrotonoyl-CoA were all taken to be 15.6 mM⁻¹·cm⁻¹ at 259 nm. The absorption coefficient of octenoyl-CoA was taken to be 20.4 mM⁻¹·cm⁻¹ at 258 nm [5].

Steady-state and transient kinetic experiments

The steady-state kinetic experiments were performed on a Perkin-Elmer Lambda 3B spectrophotometer, equipped with a water-jacketed cuvette holder. The temperature of the reaction mixture was maintained by circulating water from a thermostatically controlled bath. The initial rates of the HMCAD-catalysed reactions were measured by following the reduction of FcPF₆ either at 300 nm ([FcPF₆] $\leq$ 350 μ M) or at 617 nm ([FcPF₆] > 350 μ M). The absorption coefficients of FcPF₆ were taken to be 0.41 and 4.3 mM⁻¹·cm⁻¹ at 617 and 300 nm respectively [17].

The transient kinetic experiments were performed with an Applied Photophysics SX-17MV sequential-mixing stopped-flow system (optical path length 10 mm, dead time 1.3–1.5 ms) in the single or sequential-mixing modes [2,5,14]. The stopped-flow kinetic traces were analysed by the data analysis package provided by Applied Photophysics.

Dissociation of reaction products from the oxidized enzyme site

The dissociation 'off-rates' of normal and dephosphoenoyl-CoAs from the oxidized enzyme site were measured by employing the stopped-flow technique in a sequential-mixing mode [2,6]. This allowed us to minimize the contributions of slow hydration and/or isomerization reactions of enoyl-CoAs. In this approach, HMCAD-FAD was first mixed with one of the reaction products (normal or dephospho forms of octenoyl-CoA and crotonoyl-CoA) via the two stopped-flow syringes. The reaction mixture was allowed to age for 0.5–5 s (depending on the rapidity of the reaction) in the aging loop. This was followed by mixing with an excess of octanoyl-CoA (as a competing ligand of reaction products) via the third stopped-flow syringe. The time course for the displacement of reaction products by octanoyl-CoA immediately followed a fast reduction of HMCAD-FAD by octanoyl-CoA [5,6], resulting in a decrease in absorption at 450 nm. The rate of the latter reaction was taken as the measure of the dissociation off-rates of the reaction products from the oxidized enzyme site.

Presentation of MCAD structure

The atomic co-ordinates of the pig liver MCAD-octenoyl-CoA complex (file name 3MDE) were downloaded from the Brookhaven Protein Data Bank and displayed on a Silicon Graphic Indigo XZ workstation. The protein structures were displayed and distance measurements were made with the aid of Insight II software (Biosym Technologies).

RESULTS

Steady-state kinetics

To ascertain the contribution of the 3'-phosphate group of CoA during HMCAD catalysis, we prepared 3'-dephospho derivatives of octanoyl-CoA and butyryl-CoA. This was accomplished by treatment of the latter substrates with alkaline phosphatase, followed by purification of the resultant products on a reverse-phase C₁₈ HPLC column (see the Materials and methods section). The syntheses of 3'-dephospho-octanoyl-CoA and 3'-dephosphobutyryl-CoA were confirmed by their distinct elution patterns (compared with the corresponding parent compounds) from the HPLC column (results not shown). To confirm the homogeneity of the 3'-dephospho derivatives we prepared 3'-dephospho-octanoyl-CoA (by the mixed-anhydride method; see the Materials and methods section) by using octanoic acid and a commercial preparation of 3'-dephospho-CoA. The HPLC elution profile of the latter preparation precisely matched that obtained with 3'-dephospho-octanoyl-CoA prepared by alkaline phosphatase treatment (results not shown). As will be indicated below, both these preparations exhibited identical properties during the steady-state kinetic experiments.

To ascertain the functional role of the 3'-phosphate group of acyl-CoA substrates, we compared the steady-state kinetic parameters of the HMCAD-catalysed reaction with octanoyl-CoA and butyryl-CoA (and their 3'-dephospho analogues) as substrates and FcPF₆ as an electron acceptor. However, while performing these experiments we realized that, owing to a high background absorption (at 300 nm) as well as the solubility problems, we could not use saturating concentrations of FcPF₆, particularly during the octanoyl-CoA- and 3'-dephospho-octanoyl-CoA-dependent reactions. With the latter substrates, the concentration of FcPF₆ was taken to be only five times their K_m values. However, under such conditions, the enzyme would be saturated by at least 82 % with respect to FcPF₆. No such problems were encountered while performing the butyryl-CoA- and 3'-dephosphobutyryl-CoA-dependent reactions (owing to low K_m values of FcPF₆ involving these substrates). Hence, with the latter substrates, the concentration of FcPF₆ could be easily maintained at 10 times their K_m values, leading to approx. 91 % saturation of the enzyme. Considering these, the steady-state

Table 1 Steady-state kinetic parameters for HMCAD

Assays were performed in 50 mM potassium phosphate buffer, pH 7.6, containing 0.3 mM EDTA. Abbreviations: OcaCoA, octanoyl-CoA; d-OcaCoA, 3'-dephospho-octanoyl-CoA; ButCoA, butyryl-CoA; d-ButCoA, 3'-dephosphobutyryl-CoA.

Variable substrate	Fixed substrate	Apparent K_m (μ M)	Apparent k_{cat} (s ⁻¹)
OcaCoA	FcPF ₆ (350 μ M)	2.6 $\pm$ 0.4	15.6 $\pm$ 0.5
d-OcaCoA	FcPF ₆ (1 mM)	25.1 $\pm$ 2.8	58.3 $\pm$ 1.9
ButCoA	FcPF ₆ (300 μ M)	356 $\pm$ 56	8.0 $\pm$ 0.6
d-ButCoA	FcPF ₆ (300 μ M)	1608 $\pm$ 134	1.55 $\pm$ 0.1

Figure 2 Reductive half-reaction of HMCAD with octanoyl-CoA, dephospho-octanoyl-CoA, butyryl-CoA and dephosphobutyryl-CoA substrates

(A, B) Stopped-flow traces (at 450 nm) for the reaction at 10 °C of HMCAD-FAD with octanoyl-CoA (A) and 3'-dephospho-octanoyl-CoA (B). The concentrations (after mixing) of HMCAD-FAD, octanoyl-CoA and 3'-dephospho-octanoyl-CoA were 1.25, 50 and 125 μM respectively. The dotted and solid smooth lines are the best fit to the experimental data by single- and double-exponential rate equations respectively. The residuals of these fits are given at the bottom of each panel. The fast (k_{fast}) and slow (k_{slow}) relaxation rate constants and their corresponding amplitudes, ΔA_{fast} and ΔA_{slow} , for the HMCAD-FAD + octanoyl-CoA reaction (derived from the best fit to the experimental data by the biphasic rate equation) were $493 \pm 16 \text{ s}^{-1}$, $42.4 \pm 3.0 \text{ s}^{-1}$, 0.0135 ± 0.004 and 0.0045 ± 0.0006 respectively. The corresponding parameters for the reaction with dephospho-octanoyl-CoA substrate, were $208 \pm 11 \text{ s}^{-1}$, $57.2 \pm 4.6 \text{ s}^{-1}$, 0.0081 ± 0.0007 and 0.007 ± 0.0006 respectively. (C, D) Stopped-flow traces (at 450 nm) for the reaction at 25 °C of HMCAD-FAD with butyryl-CoA (C) and 3'-dephosphobutyryl-CoA (D). The concentrations (after mixing) of HMCAD-FAD, butyryl-CoA and 3'-dephosphobutyryl-CoA were 4 μM , 5.5 mM and 5.5 mM respectively. The dotted and solid smooth lines are the best fit to the experimental data by the single- and double-exponential rate equations respectively. The fast (k_{fast}) and slow (k_{slow}) relaxation rate constants and their corresponding amplitudes, ΔA_{fast} and ΔA_{slow} , for the HMCAD-FAD + butyryl-CoA reaction (derived from the best fit to the experimental data by the biphasic rate equation) were $13.9 \pm 0.40 \text{ s}^{-1}$, $2.0 \pm 0.03 \text{ s}^{-1}$, 0.0343 ± 0.0002 and 0.0103 ± 0.0002 respectively. The above parameters for the reaction with dephosphobutyryl-CoA were $1.38 \pm 0.02 \text{ s}^{-1}$, $0.53 \pm 0.01 \text{ s}^{-1}$, 0.0264 ± 0.0006 and 0.0148 ± 0.0006 respectively.

kinetic parameters derived here are referred to be as 'apparent' rather than 'true'.

Table 1 summarizes the apparent K_m (for acyl-CoA substrates) and k_{cat} values of HMCAD in the presence of 82–91 % -saturated FcPF₆. The data of Table 1 reveal that the apparent k_{cat} of HMCAD is approx. 4-fold higher with 3'-dephospho-octanoyl-CoA than with octanoyl-CoA. In contrast, the apparent k_{cat} of the enzyme for 3'-dephosphobutyryl-CoA is about one-fifth of that for butyryl-CoA. However, for both these substrates, the apparent K_m values for the 3'-dephospho derivatives are 5–10-fold higher than those observed with the corresponding phosphorylated (normal) substrates.

Note that, before this investigation, Frerman et al. [10] compared the K_m and k_{cat} values of octanoyl-CoA and 3'-dephospho-octanoyl-CoA, and reported that the above parameters were nearly the same for both these substrates; this is obviously in direct contradiction of our results (see Table 1). In attempting to resolve the origin of this disparity, we noted that Frerman et al. [10] measured the initial rates of the pig liver

enzyme-catalysed reaction in a coupled assay system with 0.4 μM electron-transferring flavoprotein (ETF) and (presumably a saturating concentration of) 2,6-dichlorophenol-indophenol. When we compared the HMCAD-catalysed reaction (in our standard 50 mM phosphate buffer, pH 7.6, containing either 100 μM octanoyl-CoA or 100 μM 3'-dephospho-octanoyl-CoA), utilizing 0.4 μM (pig-kidney ETF) and 40 μM 2,6-dichlorophenol-indophenol, we found the initial rate of the 3'-dephospho-octanoyl-CoA-dependent reaction to be only 16 % faster than that of the octanoyl-CoA-dependent reaction. However, when we performed a similar experiment in the presence of 40 μM octanoyl-CoA or 250 μM 3'-dephospho-octanoyl-CoA with 20 μM ETF, we found that the 3'-dephospho-octanoyl-CoA-dependent reaction was 2.2-fold faster than the octanoyl-CoA reaction (results not shown). Here we note that the K_m values for pig kidney ETF during the octanoyl-CoA-dependent (pig kidney) MCAD-catalysed reaction are 9.7 and 1.7 μM in 50 mM phosphate buffer and 20 mM phosphate buffer respectively (N. Ravi Kumar and D. K. Srivastava, unpublished work). The

latter value is consistent with the reported values of $2.0 \mu\text{M}$ [18] and $0.79 \mu\text{M}$ [19]. Hence it is likely that the near similarity in the steady-state kinetic parameters (for the octanoyl-CoA- and 3'-dephospho-octanoyl-CoA-dependent reaction of pig liver MCAD) of Frerman et al. [10] is due to the employment of subsaturating concentrations of ETF and/or 2,6-dichlorophenol-indophenol during the enzyme catalysis. However, we cannot rule out the possibility that the above discrepancy is not due to the difference in the enzyme types, i.e. their biological origins.

The fact that deletion of the 3'-phosphate group from octanoyl-CoA increases the apparent turnover rate of the enzyme by 4-fold, whereas that from butyryl-CoA decreases the enzyme turnover to one-fifth, surprised us initially, particularly because the removal of the same 3'-phosphate group (of CoA) yielded opposite results with acyl-CoA substrates of different chain lengths. To ascertain the mechanistic basis of the above effects, we undertook a detailed transient kinetic investigation of the enzyme by using phospho and dephospho forms of octanoyl-CoA and butyryl-CoA substrates.

Reductive half-reaction of HMCAD

We compared the transient courses for the reduction of HMCAD-FAD by using each of the four substrates octanoyl-CoA, 3'-dephospho-octanoyl-CoA, butyryl-CoA and 3'-dephosphobutyryl-CoA, under pseudo-first-order conditions ($[\text{HMCAD-FAD}] \ll [\text{substrate}]$). The results are shown in Figure 2. As reported earlier [5,7,12,20–23], the reductive half-reaction of the enzyme with both butyryl-CoA and octanoyl-CoA as substrates shows a biphasic decrease in absorption at 450 nm, although such profiles originate from different microscopic mechanisms (see the Discussion section).

Because the octanoyl-CoA-dependent reaction of the enzyme is substantially faster than the butyryl-CoA-dependent reaction, the former was performed at 10°C , to allow an accurate determination of the individual rate constants. Figures 2(A) and 2(B) show the stopped-flow traces at 450 nm for the reaction of HMCAD-FAD ($1.25 \mu\text{M}$) with saturating concentrations of octanoyl-CoA ($50 \mu\text{M}$) and 3'-dephospho-octanoyl-CoA ($125 \mu\text{M}$) respectively. These traces were fitted by the single-exponential (dotted smooth lines) and biphasic (solid smooth lines) rate equations. The residuals of such fits are shown just below the reaction panels. An inspection of the fitted lines and their residuals reveals that the octanoyl-CoA- and 3'-dephospho-octanoyl-CoA-dependent reductive half-reactions of the enzyme are biphasic in nature. For octanoyl-CoA, the magnitudes of the fast (k_{fast}) and slow (k_{slow}) relaxation rate constants, and their corresponding amplitudes (at 450 nm), ΔA_{fast} and ΔA_{slow} (derived from the best fit of the experimental data), were $493 \pm 16 \text{ s}^{-1}$, $42.3 \pm 3.0 \text{ s}^{-1}$, 0.0135 ± 0.004 and 0.0045 ± 0.0006 respectively. The corresponding parameters for dephospho-octanoyl-CoA were $208 \pm 11 \text{ s}^{-1}$, $57.2 \pm 4.6 \text{ s}^{-1}$, 0.0081 ± 0.0007 and 0.007 ± 0.0006 respectively. These results suggest that the deletion of the 3'-phosphate group from octanoyl-CoA slows down the fast relaxation rate constant (k_{fast}) to about one-half, without exhibiting any significant effect on the slow relaxation rate constant (k_{slow}). We further performed the above reactions at increasing concentrations of octanoyl-CoA and dephospho-octanoyl-CoA (while maintaining pseudo-first-order conditions; $[\text{HMCAD-FAD}] \ll [\text{substrate}]$). As observed previously [5–7], for octanoyl-CoA, both fast and slow relaxation rate constants exhibited apparent zero-order dependence on the substrate concentration. However, for dephospho-octanoyl-CoA, the fast relaxation rate constant showed a hyperbolic dependence on the substrate concentration, with $K_{0.5}$ (the concentration of substrate required

for half-saturation of the enzyme), maximum relaxation rate constant and minimum relaxation rate constant being equal to $25.6 \mu\text{M}$, 98.2 s^{-1} and 27.7 s^{-1} respectively. These results suggest that the deletion of the 3'-phosphate group impairs the binding of octanoyl-CoA to HMCAD.

We performed an identical experiment, with 93% and 77% saturating concentrations of butyryl-CoA (Figure 2C) and dephosphobutyryl-CoA (Figure 2D) as substrates respectively. The reaction traces were fitted by the single-exponential (dotted smooth lines) and biphasic (solid smooth lines) rate equations. The residuals of such fits are shown below the reaction panels. The fitted lines and their residuals clearly suggest that both butyryl-CoA- and dephosphobutyryl-CoA-dependent reductive half-reactions are biphasic in nature. For butyryl-CoA, the magnitudes of fast (k_{fast}) and slow (k_{slow}) relaxation rate constants, and their corresponding amplitudes (at 450 nm), ΔA_{fast} and ΔA_{slow} (derived from the best fit of the experimental data), were $13.9 \pm 0.40 \text{ s}^{-1}$, $2.0 \pm 0.03 \text{ s}^{-1}$, 0.0343 ± 0.0002 and 0.0103 ± 0.0002 respectively. The corresponding parameters for dephosphobutyryl-CoA were $1.38 \pm 0.02 \text{ s}^{-1}$, $0.53 \pm 0.01 \text{ s}^{-1}$, 0.0264 ± 0.0006 and 0.0148 ± 0.0006 respectively. Hence, unlike the octanoyl-CoA-dependent reaction, the deletion of the 3'-phosphate group from butyryl-CoA results in the deceleration of both fast and slow relaxation time constants to one-tenth and one-quarter respectively. The deletion of the 3'-phosphate group from the butyryl-CoA substrate has a more pronounced effect on the reductive half-reaction of the enzyme than the deletion of that from octanoyl-CoA substrate. Owing to technical problems, we have not yet been able to study the concentration dependences of the relaxation rate constants involving 3'-dephosphobutyryl-CoA as substrate.

Oxidative half-reaction of HMCAD

We investigated the transient kinetics for the oxidative half-reaction of the enzyme with both normal and 3'-dephospho forms of octanoyl-CoA and butyryl-CoA as substrates and FcPF_6 as electron acceptor via the sequential-mixing stopped-flow method [2]. During these experiments the substrate-reduced enzyme was freshly generated by mixing HMCAD-FAD with either of the above substrates via the first two stopped-flow syringes. The reaction mixture was allowed to age for a brief period (1–5 s), followed by mixing with a high ($175 \mu\text{M}$) concentration of FcPF_6 via the third stopped-flow syringe. In this way the substrate-reduced enzyme was not significantly oxidized (by the buffer-dissolved oxygen, particularly involving butyryl-CoA/dephosphobutyryl-CoA substrates), before its reaction with FcPF_6 .

Figure 3 shows the stopped-flow traces (at 450 nm) for the oxidation of the substrate-reduced enzymes by FcPF_6 . Figure 3(A) compares the octanoyl-CoA-dependent (trace 1) and dephospho-octanoyl-CoA-dependent (trace 2) oxidative half-reactions of the enzyme involving FcPF_6 as an electron acceptor. The concentrations (after mixing) of MCAD-FAD, octanoyl-CoA, dephospho-octanoyl-CoA and FcPF_6 during these experiments were 4, 3.5, 3.5 and $175 \mu\text{M}$ respectively. Both these reaction traces were best fitted (solid smooth lines) by the single-exponential rate equation for the increase in absorption at 450 nm. The observed rate constant and the amplitude for the octanoyl-CoA-dependent reaction were $58.4 \pm 0.4 \text{ s}^{-1}$ and 0.0355 ± 0.0002 respectively. The corresponding parameters for the dephospho-octanoyl-CoA-dependent reaction were $52.2 \pm 0.6 \text{ s}^{-1}$ and 0.0234 ± 0.0002 respectively. Note that, whereas the deletion of the 3'-phosphate group from octanoyl-CoA does not influence the observed rate constant of the oxidative half-

Figure 3 Oxidative half-reaction of HMCAD

Transient kinetic traces (at 450 nm) for the oxidation of octanoyl-CoA/3'-dephospho-octanoyl-CoA-reduced (A) and butyryl-CoA/3'-dephosphobutyryl-CoA-reduced (B) enzyme by FcPF_6 at 25 °C are shown. (A) For the reaction involving octanoyl-CoA (trace 1) and 3'-dephospho-octanoyl-CoA (trace 2), the concentrations (after mixing) of HMCAD-FAD, octanoyl-CoA/dephospho-octanoyl-CoA and FcPF_6 were 4, 3.5 and 175 μM respectively. The solid smooth lines are the best fit to the experimental data with the single-exponential rate equation, with relaxation rate constants of $58.4 \pm 0.4 \text{ s}^{-1}$ (amplitude 0.036 ± 0.0002) and $52.2 \pm 0.6 \text{ s}^{-1}$ (amplitude 0.023 ± 0.0002), for reactions involving octanoyl-CoA and 3'-dephospho-octanoyl-CoA substrates respectively. (B) For reactions involving butyryl-CoA (trace 1) and 3'-dephosphobutyryl-CoA (trace 2), the concentrations (after mixing) of HMCAD-FAD, butyryl-CoA/3'-dephosphobutyryl-CoA and FcPF_6 were 5, 50 and 500 μM respectively. The solid smooth lines are the best fit to the experimental results with the single-exponential rate equation, with relaxation rate constants of $162 \pm 1 \text{ s}^{-1}$ (amplitude 0.054 ± 0.0003) and $62.8 \pm 0.7 \text{ s}^{-1}$ (amplitude 0.018 ± 0.0001) for the butyryl-CoA-dependent and 3'-dephosphobutyryl-CoA-dependent reactions respectively.

reaction, it decreases the amplitude by approx. 34%. We believe that the latter is due primarily to an impaired binding of dephospho-octanoyl-CoA (compared with octanoyl-CoA) to the HMCAD-FAD site. The latter results in a lower yield of the HMCAD-FADH₂-octanoyl-CoA complex, which is oxidized by FcPF_6 during the observed relaxation phase. Explicit evidence for a decrease to one-tenth in the binding affinity of octenoyl-CoA (the reaction product of octanoyl-CoA) to HMCAD, on deletion of the 3'-phosphate group, comes from titration microcalorimetric experiments (results not shown).

Figure 3(B) compares the butyryl-CoA-dependent (trace 1) and dephosphobutyryl-CoA-dependent (trace 2) oxidative half-reactions of the enzyme involving FcPF_6 as an electron acceptor. The concentrations (after mixing) of HMCAD-FAD, butyryl-CoA, dephosphobutyryl-CoA and FcPF_6 during these experiments were 5, 50, 50 and 500 μM respectively. The reaction traces were best fitted by the single-exponential rate equation (solid smooth lines) for increase in absorbance at 450 nm. The

Figure 4 FcPF_6 concentration dependence of the oxidative half-reaction of the enzyme

The relaxation rate constants for the oxidative half-reaction involving different substrates (as a function of the concentration of FcPF_6 after mixing) were determined as described in the legend to Figure 3. (A) FcPF_6 concentration-dependent increase in the relaxation rate constants ($1/\tau$) of the oxidative half-reaction involving octanoyl-CoA (Δ) and 3'-dephospho-octanoyl-CoA ($\circ$) substrates. The solid line is the linear regression analysis of the experimental data with a common slope and intercept of $0.297 \pm 0.005 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ and $2.84 \pm 2.2 \text{ s}^{-1}$ respectively. (B) FcPF_6 concentration-dependent increase in the relaxation rate constants ($1/\tau$) of the oxidative half-reaction involving butyryl-CoA ($\circ$) and 3'-dephosphobutyryl-CoA (Δ) as substrates. Note that the reactions involving butyryl-CoA and 3'-dephosphobutyryl-CoA as substrates exhibit linear and hyperbolic dependences respectively on FcPF_6 concentration. The slope and intercept derived from the linear regression analysis of the data (for butyryl-CoA) are $0.301 \pm 0.007 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ and $8.67 \pm 3.3 \text{ s}^{-1}$ respectively. The hyperbolic data (for 3'-dephosphobutyryl-CoA) are best fitted by $K_{0.5} = 660 \pm 86 \mu\text{M}$ and $1/\tau_{\text{max}} = 143 \pm 10 \text{ s}^{-1}$.

observed rate constant and amplitude (derived from the best fit of the experimental data) were $162 \pm 1 \text{ s}^{-1}$ and 0.0538 ± 0.0003 respectively for butyryl-CoA, and $62.8 \pm 0.7 \text{ s}^{-1}$ and 0.0181 ± 0.0001 respectively for dephosphobutyryl-CoA. Hence, unlike octanoyl-CoA, the deletion of the 3'-phosphate group from butyryl-CoA decreases both the observed rate constant and the amplitude of the oxidative half-reaction to 1/2.6 and 1/3 respectively. These effects are likely to be due to a combined effect of the impaired binding affinity of dephosphobutyryl-CoA (compared with butyryl-CoA) for HMCAD-FAD, and an unfavourable internal equilibrium between HMCAD-FAD-substrate and HMCAD-FADH₂-product complexes. Owing to technical problems, we have not yet been able to quantify the magnitudes of the above effects involving butyryl-CoA and dephosphobutyryl-CoA substrates.

We further examined the influence of FcPF_6 concentration on the observed (relaxation) rate constants for the oxidative half-reaction of the enzyme involving octanoyl-CoA, 3'-dephospho-octanoyl-CoA, butyryl-CoA and 3'-dephosphobutyryl-CoA substrates (Figure 4). The data in Figure 4(A) show that the observed rate constant for the oxidative half-reaction of the enzyme increases linearly with the increase in FcPF_6 concentration, with both octanoyl-CoA and 3'-dephospho-octanoyl-CoA as substrates. The observed rate constants (at different concentrations of FcPF_6) remain invariant irrespective of whether the enzyme was previously reduced by octanoyl-CoA or 3'-dephospho-octanoyl-CoA. Hence the deletion of the 3'-phosphate from octanoyl-CoA does not influence the oxidative half-

reaction of the enzyme. Lehman and Thorpe [24] performed a similar stopped-flow experiment for the oxidation of octanoyl-CoA-reduced pig kidney enzyme by FcPF₆, and reported that their kinetic reaction traces (including those at 450 nm) were biphasic in nature. Although we observed a biphasic reaction profile at 390 nm, the reaction profile at 450 nm was repeatedly found to be single-exponential in nature. We believe the latter observation to be due either to a mixing artifact or to a lower sensitivity of Thorpe's stopped-flow system.

Unlike octanoyl-CoA, a marked influence of the 3'-phosphate group on the oxidative half-reaction is evident with butyryl-CoA as substrate. The data in Figure 4(B) show that the observed rate constants for the oxidative half-reaction of the enzyme are linearly and hyperbolically dependent on FcPF₆ concentration with butyryl-CoA and 3'-dephosphobutyryl-CoA as substrates respectively. Of these, the hyperbolic dependence of the oxidative half-reaction (involving dephosphobutyryl-CoA-reduced enzyme) on FcPF₆ was unique among all the substrates utilized here. No such hyperbolic profile was shown by the reduced enzyme generated by octanoyl-CoA, dephospho-octanoyl-CoA or butyryl-CoA. The hyperbolic, as opposed to linear, dependence of the transient relaxation rate constants on ligand concentrations has been attributed to the formation of tighter enzyme-ligand complexes [25]. Accordingly, the dephosphobutyryl-CoA-reduced enzyme is expected to have a higher affinity for FcPF₆ than the reduced enzyme generated by the other three substrates. Although the molecular basis of this phenomenon must await further mechanistic studies, we do note that at lower pH (namely pH 6.0–6.7) the relaxation rate constant for the oxidative half-reaction (with octanoyl-CoA-reduced enzyme) shows a hyperbolic dependence, whereas the same reaction at higher pH (above 7.5) shows a linear dependence up to 1.0 mM FcPF₆ (N. Ravi Kumar, K. L. Peterson and D. K. Srivastava, unpublished work). Whether these observations have a common mechanistic origin is currently being investigated in our laboratory.

Dissociation 'off-rates' of the reaction products from the oxidized enzyme site

After the oxidative half-reaction, the reaction products must dissociate from the oxidized enzyme site to maintain a repetitive turnover. Of the two products, namely reduced FcPF₆ (electron acceptor) and enoyl-CoA, the latter has been found to be more ‘sticky’ (see below), and is thus likely to dissociate last from the oxidized enzyme site. To characterize further the influence of the 3′-phosphate group of acyl-CoA substrates on the overall turnover of the enzyme, we determined the dissociation ‘off-rate’ constants of octenoyl-CoA and dephospho-octenoyl-CoA (the reaction product of octanoyl-CoA) and crotonoyl-CoA (the reaction product of butyryl-CoA) from the oxidized enzyme site. Previously we designed the acetoacetyl-CoA displacement method for measuring the dissociation ‘off-rate’ constants of *trans*-3-indoleacryloyl-CoA and octenoyl-CoA from the enzyme site [1,6]. We realized that the above method was appropriate as long as the dissociation ‘off-rate’ constant of octenoyl-CoA was lower than the rate constant for the formation of enzyme acetoacetyl-CoA isomerized complex (the species responsible for the increase in absorption at 545 nm). Because the dissociation off-rate constants of some of the reaction products employed in the present study were found to be either comparable with or faster than the rate constant for the isomerization of the enzyme-acetoacetyl-CoA complex, we could not employ the latter procedure during the present studies. Recourse was therefore made to displace enoyl-CoAs from the oxidized enzyme site

Scheme 2 Sequence of steps in measurement of the dissociation ‘off-rates’ of enoyl-CoAs

Figure 5 Dissociation ‘off-rates’ of the reaction products from the oxidized enzyme site at 25 °C

(A) Transient stopped-flow kinetic traces for the dissociation of octenoyl-CoA (trace 1) and 3'-dephospho-octenoyl-CoA (trace 2) from the oxidized enzyme site. The concentrations (after mixing) of HMCAD-FAD, octenoyl-CoA or 3'-dephospho-octenoyl-CoA, and octenoyl-CoA were 2.5, 5 and 800 μM respectively. The solid smooth lines are the best fit to the experimental data with the single-exponential rate equation, with dissociation off-rate constants of octenoyl-CoA and 3'-dephospho-octenoyl-CoA of 12.6 ± 0.06 and $172 \pm 2 \text{ s}^{-1}$ respectively. **(B)** Transient stopped-flow kinetic traces for the dissociation of crotonoyl-CoA (trace 1) and 3'-dephosphocrotonoyl-CoA (trace 2) from the oxidized enzyme site. The concentrations (after mixing) of HMCAD-FAD, crotonoyl-CoA or 3'-dephosphocrotonoyl-CoA, and octenoyl-CoA were 2.5 μM , 250 μM and 2 mM respectively. The smooth lines are the best fit to the experimental data with the single-exponential rate equation, with dissociation off-rate constants of crotonoyl-CoA and 3'-dephosphocrotonoyl-CoA of 261 ± 3 and $582 \pm 18 \text{ s}^{-1}$ respectively.

in the presence of an excessive concentration of octanoyl-CoA. Being an efficient substrate, octanoyl-CoA (after displacing enoyl-CoA products from the oxidized enzyme site) reduces the enzyme-bound FAD with a rate constant of 494 s^{-1} at 10°C (Figure 2). Given that the energy of activation of this step is 28.8 kJ/mol (K. L. Peterson and D. K. Srivastava, unpublished work), the above rate constant at 25°C can be calculated as 916 s^{-1} . The latter value is higher than the dissociation ‘off-rate’ constants of all the products (from the oxidized enzyme site) used

in this study. Hence, by employing the octanoyl-CoA displacement method, the dissociation 'off-rate' constants of the enoyl-CoA products could be measured by monitoring the time-dependent decrease in absorption at 450 nm (due to reduction of E-FAD by octanoyl-CoA; Scheme 2).

Figure 5 shows the comparative stopped-flow traces for the dissociation of various enoyl-CoA products from the oxidized enzyme site. The solid smooth lines are the best fit of the experimental data to a single-exponential decrease in absorption at 450 nm. Note that the deletion of the 3'-phosphate group from both octenoyl-CoA and crotonoyl-CoA products results in an increase in the dissociation off-rate constants. Whereas the deletion of the 3'-phosphate group increases the dissociation off-rate constant of octenoyl-CoA from 13 to 172 s⁻¹, it increases the dissociation off-rate constant of crotonoyl-CoA from 261 to 582 s⁻¹. It is evident that the deletion of the 3'-phosphate group from octenoyl-CoA increases the dissociation 'off-rate' constant by approx. 14-fold. However, for crotonoyl-CoA, the deletion of the 3'-phosphate group enhances the dissociation off-rate constant by only 2-fold. Hence, unlike octenoyl-CoA, the stickiness of crotonoyl-CoA for the oxidized enzyme site is not much alleviated on deletion of the 3'-phosphate group.

DISCUSSION

The experimental results presented here provide unequivocal evidence for the involvement of the 3'-phosphate group of CoA (in acyl-CoA substrates) during the HMCAD-catalysed reaction. These pieces of evidence include the following. (1) The deletion of the 3'-phosphate group from both octanoyl-CoA and butyryl-CoA substrates alters the steady-state kinetic parameters of the enzyme. Whereas the apparent K_m values of both these substrates are increased, the apparent k_{cat} values of the enzyme are increased and decreased with octanoyl-CoA and butyryl-CoA as substrates respectively (Table 1). (2) The reductive half-reaction of the enzyme is impaired on deletion of the 3'-phosphate group from both octanoyl-CoA and butyryl-CoA substrates (Figure 2). (3) The kinetics of the oxidative half-reaction is altered on deletion of the 3'-phosphate group from butyryl-CoA substrate (Figure 4). (4) The dissociation 'off-rate' constants of the reaction products octenoyl-CoA and crotonoyl-CoA are increased on deletion of their 3'-phosphate groups (Figure 5). (5) These, coupled with the titration microcalorimetric studies suggesting that the deletion of the 3'-phosphate group from octenoyl-CoA results in a decrease in the binding free energy by 5.23 kJ/mol, lead to the suggestion that the 3'-phosphate group serves as a functional moiety of acyl-CoA substrates in stabilizing both ground and transition states of the enzyme during catalysis (see below).

The question arises as to why the 3'-phosphate group of CoA exhibits different influences on the octanoyl-CoA-dependent and butyryl-CoA-dependent reactions of the enzyme, and in par-

Scheme 3 Butyryl-CoA-dependent reaction of the enzyme

Abbreviations: But-CoA, butyryl-CoA; Crot-CoA, crotonoyl-CoA.

Scheme 4 Octanoyl-CoA-dependent reaction of the enzyme

Abbreviations: Oca-CoA, octanoyl-CoA; Oce-CoA, octenoyl-CoA.

ticular why the turnover rate of the enzyme increases by 4-fold on deletion of the 3'-phosphate group from octanoyl-CoA but decreases to one-fifth on deletion of the 3'-phosphate group from butyryl-CoA substrate. Before attempting to answer these questions, it is essential to review some of the subtle features of the octanoyl-CoA- and butyryl-CoA-dependent reactions of the enzyme.

Schopfer et al. [12] first demonstrated that the butyryl-CoA-dependent reductive half-reaction of the enzyme involves a biphasic decrease in absorption in the oxidized flavin region (450 nm) and a rapid single-exponential increase followed by a slow single-exponential decrease in absorption in the CT-complex region (560 nm). Of these reaction phases, the fast and slow phases increased and decreased (hyperbolically) as a function of butyryl-CoA concentration respectively. These, together with other experimental data, led to the proposal [12] that the fast and slow phases during the butyryl-CoA-dependent reactions originated from the conversion of E-FAD-butyl-CoA into E-FADH₂-crotonoyl-CoA and E-FADH₂-crotonoyl-CoA into E-FADH₂-butyl-CoA (owing to a competitive displacement of crotonoyl-CoA by a high concentration of butyryl-CoA from the reduced enzyme site) respectively (Scheme 3).

A similar kinetic mechanism was deduced in our laboratory for the IPCoA-dependent reductive half-reaction of the enzyme [4]. We further demonstrated that the E-FADH₂-enoyl-CoA CT complex, formed by either butyryl-CoA or IPCoA substrate, serves as an exclusive electron donor (to FcPF₆) during the oxidative half-reaction of the enzyme [2]. The enzyme species formed on the decay of the CT complex, i.e. E-FADH₂-butyryl-CoA/IPCoA (owing to competitive displacement of enoyl-CoA by acyl-CoA from the reduced enzyme site; see Scheme 3) is responsible for the 'oxidase' activity of the enzyme [3,6,8]. In addition, with IPPP as substrate, it seems evident that the conversion of E-FADH₂-product into E-FADH₂-substrate is somehow blocked by the presence of FcPF₆ in the reaction mixture (resulting in the suppression of the oxidase activity of the enzyme) [8]. It is therefore clear that the observed slow phase of the butyryl-CoA-dependent reductive half-reaction is not involved during the catalytic turnover of the dehydrogenase reaction of the enzyme.

In contrast with the butyryl-CoA- and IPCoA-dependent reactions, Kumar and Srivastava [5,6] demonstrated that the octanoyl-CoA-dependent reductive half-reaction involves a biphasic increase and decrease in absorption in the CT-complex region (545 nm) and the oxidized flavin region (450 nm) respectively. Hence the slow phase observed during the octanoyl-CoA-dependent reaction (i.e. the increase in absorption in the CT-complex region) is not equivalent to the slow phase of the butyryl-CoA-dependent reaction (i.e. the decrease in absorption in the CT-complex region, originating from a competitive displacement of crotonoyl-CoA by butyryl-CoA from the reduced enzyme site). With octanoyl-CoA as substrate, an

additional phase, characterized by a decrease in absorption in the CT-complex region (equivalent to the slow phase of the butyryl-CoA-dependent reaction) occurs on a longer time scale ($t_{1/2} > 5$ min) [6]. Further kinetic studies revealed that the octanoyl-CoA-dependent reductive half-reaction of the enzyme involves formation of two kinetically distinct forms of the CT complexes (denoted as CT₁ and CT₂), and both these complexes serve as electron donors (to FcPF₆) during the oxidative half-reaction of the enzyme (Scheme 4) [5,6,13].

Besides the above difference in the microscopic pathways of the butyryl-CoA- and octanoyl-CoA-dependent reactions, we realized that the rate-limiting step of the butyryl-CoA-dependent reaction is the forward fast rate constant (i.e. the conversion of E-FAD-butyryl-CoA into E-FADH₂-crotonoyl-CoA, as shown in Scheme 3) of the reductive half-reaction, whereas that of the octanoyl-CoA-dependent reaction is the dissociation 'off-rate' of octenoyl-CoA (the reaction product of octanoyl-CoA) from the oxidized enzyme site [13]. The latter facts can be readily envisaged by considering the transient kinetic data of Figures 2 and 5. For example, the fast relaxation rate constant (which leads to the formation of the E-FADH₂-crotonoyl-CoA CT complex) on reduction of E-FAD by a saturating concentration of butyryl-CoA is 13.9 ± 0.4 s⁻¹ (Figure 2B). Given that under the above condition approx. 50% of E-FAD is reduced, the forward rate constant for the reductive half-reaction can be approximated as 6.9 s⁻¹ (on the basis that the interaction of E-FAD and butyryl-CoA satisfies a rapid equilibrium condition). The latter value is similar to the turnover rate (8.0 s⁻¹; Table 1) of the butyryl-CoA-dependent reaction of the enzyme. The small difference in the above rate constants is presumably due to errors in determining the steady-state and transient kinetic parameters, although we cannot rule out the possibility of FcPF₆-dependent enhancement of the fast relaxation step of the reductive half-reaction. A qualitatively similar conclusion can be drawn (after correcting the rate constants for saturating concentration of the substrate) for the dephosphobutyryl-CoA-dependent reaction of the enzyme.

However, for octanoyl-CoA the relaxation rate constant of the fast phase (at a saturating concentration of octanoyl-CoA) is 494 ± 16 s⁻¹ at 10°C (Figure 2A) and 916 s⁻¹ at 25°C (see the Results section); under this condition approx. 85% of the enzyme-bound FAD is reduced to FADH₂. Hence, even by assuming that the fast relaxation rate constant represents an equal summation of the forward and reverse rate constants, the turnover rate of the enzyme (at 25°C) should not be less than 458 s⁻¹, a value approx. 30-fold higher than the experimentally measured turnover rate of 15.6 ± 0.5 s⁻¹ (Table 1). However, the experimentally determined dissociation 'off-rate' constant of the reaction product, octenoyl-CoA, from the oxidized enzyme site (12.6 s⁻¹; Figure 5A) is similar to the turnover rate of the enzyme (Table 1). We believe that the above difference is due to the experimental errors in determining the steady-state and 'off-rate' parameters. Hence, unlike the butyryl-CoA-dependent reaction, the octanoyl-CoA-dependent reaction is limited by the dissociability of the reaction product (octenoyl-CoA) from the oxidized enzyme site. Because the dissociation off-rate constant of dephospho-octenoyl-CoA (172 s⁻¹) from the oxidized enzyme site is about 3-fold faster than the turnover rate of the enzyme involving dephospho-octanoyl-CoA as substrate, it is likely that the overall rate limitation of the enzyme is conferred, at least in part, by some other step of the reaction pathway.

In light of the above discussion, it follows that any factor that would influence the dissociability of the reaction product would affect the octanoyl-CoA-dependent turnover rate of the enzyme, whereas any factor that would influence the reductive half-

reaction would influence the butyryl-CoA-dependent turnover rate of the enzyme. It should be emphasized that deletion of the 3'-phosphate group from both octanoyl-CoA and butyryl-CoA substrates decelerates the reductive half-reaction and accelerates the dissociation 'off-rates' of the corresponding enoyl-CoA products from the oxidized enzyme site. However, because the octanoyl-CoA-dependent reaction is limited by the dissociation 'off-rate' of the reaction product, deletion of the 3'-phosphate group leads to an increased turnover rate of the enzyme. In this case, the diminution of the reductive half-reaction (on deletion of the 3'-phosphate from octanoyl-CoA) is not realized towards the overall turnover of the enzyme. On the contrary, because the butyryl-CoA-dependent reaction is limited by the reductive half-reaction, the deletion of the 3'-phosphate group leads to a decrease in the turnover rate of the enzyme. In the latter case the increased dissociability of the reaction product, crotonoyl-CoA (on deletion of the 3'-phosphate group), remains irrelevant towards the turnover of the enzyme. In summary, the origin of the differential effects of the 3'-phosphate deletion (on the steady-state turnover rate of the enzyme) lies in the different rate-limiting steps of the enzyme involving octanoyl-CoA and butyryl-CoA as substrates.

Aside from these mechanistic deductions, some energetic consequences of the influence of the 3'-phosphate deletion on the enzyme catalysis are noteworthy. For example, it is apparent that the magnitude of deceleration of the reductive half-reaction (on deletion of the 3'-phosphate group) is less pronounced with octanoyl-CoA (to approx. 1/2.4) than with butyryl-CoA (to 1/10) as substrate (Figure 2). Clearly, the presence of four additional methylene groups in the case of octanoyl-CoA (compared with butyryl-CoA) partly offsets the loss of binding energy due to deletion of the 3'-phosphate group. Whether such a compensatory effect is due solely to the stabilization of the transition state or is a trade-off between ground and transition state must await further studies.

Considering that the deletion of the 3'-phosphate group from octanoyl-CoA enhances the turnover rate of the enzyme, it might seem intuitively that the 3'-phosphate of CoA (particularly with octanoyl-CoA as physiological substrate) has been preserved merely owing to a mistake of the natural selection process. The absence of the 3'-phosphate group from octanoyl-CoA would have lowered the energy barrier between ES and the rate-determining transition state by 3.26 kJ/mol. However, this would have occurred at an expense of 5.60 kJ/mol loss of the ground-state energy within the ES complex. Cumulatively, the presence of 3'-phosphate in octanoyl-CoA has an advantage of 2.34 kJ/mol of energy barrier between the free enzyme and the rate-determining transition state. Hence, even with octanoyl-CoA as substrate, the 3'-phosphate group has an energetic advantage during the enzyme catalysis.

On the basis of the mechanistic precedents of enzyme catalysis, as well as from theoretical considerations, it has been argued that both enzymes and their cognate substrates have been under continuous evolutionary pressure towards optimization of efficiency and selectivity of biochemical reactions [26,27]. In many instances, the structures of (physiological) substrates and/or intermediary metabolites seem to be much larger than required for the relevant chemical reactions. For example, most of the glycolytic intermediates contain phosphate groups whose functions are not justifiable, particularly because the reactive centres of such intermediates are far removed from the phosphate groups. Alberly and Knowles [28] argue that such phosphate groups serve as 'handles' that help to facilitate 'uniform binding' of substrates during catalysis. Further examples of the existence of 'seemingly redundant' structural fragments are found among

most of the coenzyme structures. In cases where the experimental data are available, the deletions of such fragments (or via site-directed modification of enzymes that obviate interactions with those fragments) often lead to impaired catalytic activities of the relevant enzymes [11,29–31]. Obviously, the binding energies of 'non-reactive' fragments of the substrate structures are directed towards alleviating the transition-state barriers during enzyme catalysis [32]. It is therefore not surprising that the 3'-phosphate group of acyl-CoA substrates have functional roles during the MCAD-catalysed reaction. Such roles of the distal fragments of the substrate structures might not be correctly predictable on the basis of the structural data alone.

We thank Mr. Wallace W. Muhonen for generating Figure 1 on a Silicon Graphics workstation. This work was supported by grants from the National Science Foundation (MCB-9507292) and the American Heart Association, National Center.

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