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Studies with general acyl-CoA dehydrogenase from pig kidney

Inactivation by a novel type of 'suicide' inhibitor, 3,4-pentadienoyl-CoA

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3,4-Pentadienoyl-CoA, an allenic substrate analog, is a potent inhibitor of the flavoprotein pig-kidney general acyl-CoA dehydrogenase. The analog reacts very rapidly ($k = 2.4 \times 10^3\ min^{-1}$) with the native oxidized enzyme to form a covalent flavin adduct probably involving the isoalloxazine position N-5. This species is inactive, but activity may be regained by two pathways. The allenic thioester can be displaced ($k = 0.3\ min^{-1}$) by a large excess of octanoyl-CoA substrate upon reversal of covalent adduct formation. Alternatively, the enzyme inactivator adduct slowly decomposes ($t_{1/2} = 75$ min) to form the strongly thermodynamically favoured 2,4-diene and catalytically active, oxidized enzyme. During this latter process 15–20% of the activity is irreversibly lost probably due to covalent modification of the protein. These data suggest that 3,4-pentadienoyl-CoA should be considered a suicide substrate of the acyl-CoA dehydrogenase. The mechanism of the reactions, and in particular the 3,4→2,4 tautomerization, are consistent with a catalytic sequence initiated by abstraction of an α-hydrogen as a proton.

Of the several mammalian acyl-CoA dehydrogenases discovered in the late 1950's [1], the general acyl-CoA dehydrogenase has received most attention. This flavoenzyme catalyzes the rapid α-β unsaturation of fatty acyl-moieties of medium chain length during mitochondrial β-oxidation:

$$EFl_{ox} + R-CH_2-CH_2-CO-SCoA \rightleftharpoons EFl_{red} + R-CH=CH-CO-SCoA.$$

The reducing equivalents are subsequently transferred to electron-transferring flavoprotein and transmitted to the electron transport chain [1]. The dehydrogenation reaction shown above involves removal of both *pro*-2*R* and *pro*-3*R* hydrogens [2–5] yielding the corresponding *trans*-2-enoyl-CoA product. Several approaches suggest that the *pro*-2*R* hydrogen is removed as a proton [6–10]. In contrast, the *pro*-3*R* hydrogen appears to be transferred directly to the N-5 position of the isoalloxazine ring system as a hydride [5].

Two types of mechanism based or suicide substrates of the acyl-CoA dehydrogenases have been already described. One of considerable pharmacological interest is methylenecyclopropylacetyl-CoA. This unusual thioester is derived *in vivo* from hypoglycin, a toxic amino acid found in the unripe arillus of ackee fruit which causes severe hypoglycaemia and often death in man [11]. General acyl-CoA dehydrogenase is irreversibly inactivated by this thioester via covalent modification of the flavin prosthetic group [7]. In contrast, 3-alkynoyl-CoA derivatives or their pantetheine analogues irreversibly modify the protein [8–10], possibly by alkylation of an active center glutamate residue [8]. In both these examples, inactivation appears to be initiated by abstraction of an α-proton. In this paper we describe a third covalent inactivator of the general acyl-CoA dehydrogenase, 3,4-pentadienoyl-CoA. This allene is thermodynamically unstable as compared to its conjugated tautomer, 2,4-pentadienoyl-CoA. The allene is unlikely to serve as a substrate since α-β unsaturation would generate the corresponding cumulene, an even more thermodynamically unfavorable molecule. The interaction of 3,4-pentadienoyl-CoA with general acyl-CoA dehydrogenase might furnish further evidence for the α-carbanion mechanism. In particular the finding of a 3,4→2,4 isomerization would be directly consistent with this concept. 3,4-Pentadienoyl-CoA also provides an unusual example of a reversible form of mechanism based inactivation.

Abbreviation. EFl_{ox} (EFl_{red}), oxidized (reduced) form of general acyl-CoA dehydrogenase.

Enzymes. General acyl-CoA dehydrogenase (EC 1.3.99.3).

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EXPERIMENTAL PROCEDURES

Materials. Pig-kidney general acyl-CoA dehydrogenase was purified and assayed as described previously [12, 13]. The FAD analogs were obtained as described in the references given on Table 1 or in [14]. Apoprotein was prepared and reconstituted with FAD analogues as described earlier [14]. CoA (Li-salt) and octanoyl-CoA were from P-L Biochemicals. 3,4-Pentadienoic acid was prepared by photoisomerization of 2,4-pentadienoic acid [16] and was pure by ^{1}H-NMR, consisting of multiplets at 3.1, 4.75, and 5.25 ppm (2:2:1). 3,4-Pentadienoic acid containing 50% ^{2}H (random distribution) at position 2 was obtained by carrying out the photoisomerization in 99.8% ^2H_2O, the deuterium content was determined by ^{1}H-NMR. Thioesterification with CoA was accomplished by a modified procedure of Goldman and Vagelos [15] and the thioesters further purified on a C_{18}-reverse-phase column (Waters) using a linear gradient from 5–40% methanol and 20 mM phosphate buffer, pH 6.0. 3,4-Pentadienoyl-CoA

elutes at a methanol concentration of ≈30%. Its ^{1}H-NMR spectrum was the summation of the spectra of 3,4-pentadienoic acid and CoA. Thioester concentrations were determined by titration with 2,2′-dithiopyridine [17] after base hydrolysis (0.1 M NaOH, 3 min, 100°C).

Methods. Concentration of acyl-CoA dehydrogenase are expressed in terms of bound flavin using the experimentally determined absorption coefficient at 446 nm of 15.4 mM^{-1} cm^{-1} [12]. Ultraviolet and visible absorption spectra were recorded on a Uvikon 820 spectrophotometer. Unless otherwise noted all buffers contained 0.3 mM EDTA. Release of flavin was obtained by the trichloroacetic acid extraction method described by Mayhew and Massey [18], or by heating (100°C, 3 min), or by treatment with 4.5 M guanidine hydrochloride at pH 7.6. Rapid kinetic studies were performed with a stopped-flow instrument built by Dr Th. Raichle [19].

RESULTS

Inactivation of general acyl-CoA dehydrogenase by 3,4-pentadienoyl-CoA

General acyl-CoA dehydrogenase preincubated with three equivalents of 3,4-pentadienoyl-CoA and then assayed 1.2–220 min after mixing shows the progression of behaviour shown in Fig. 1 curves C–F. Initially (curve C), samples diluted into the standard assay system (see Experimental Procedures) show a very pronounced acceleration in rate, with activities extrapolated to zero time of close to zero increasing to a maximal value of about 75% of the control rate (curve B) after 3 min. Such lag phases are not seen with normal substrates of the enzyme (e.g. octanoyl-CoA, see curve B), nor are they observed when complexes of the dehydrogenase with the competitive inhibitors acetoacetyl-CoA or heptadecyl-SCoA are assayed in the standard system [6] (C. Thorpe, unpublished). The acceleration in rate seen in Fig. 1 is due to the progressive replacement of the inactivator by the large excess of octanoyl-CoA present in the assay. The lag phase becomes much less pronounced upon increasing time of preincubation with 3,4-pentadienoyl-CoA until after 220 min (curve F) the trace is as linear as the native control sample (curve B). This rate is 80% of the control, and no further activity is recovered on longer preincubation times or after dialysis of the treated enzyme.

In contrast to these preincubation experiments, curve G represents the addition of native dehydrogenase to an assay containing both octanoyl-CoA as substrate and 6 μM 3,4-pentadienoyl-CoA. The initial rate is 60% of control, but declines over 2 min to 11% native activity. Here, octanoyl-CoA is not in large excess and is slowly displaced from the enzyme by the very tight binding 3,4-pentadienoyl-CoA (see later). This curvature is abolished if the enzyme is preincubated for 10 min at 25°C with octanoyl-CoA and the inactivator, and the assay started by addition of phenazine methosulfate and dichloroindophenol to yield the same concentrations as before. In this case a linear rate of about 13% of the control was obtained (data not shown). In similar experiments, using higher concentrations of 3,4-pentadienoyl-CoA (e.g. 0.1 mM) practically complete suppression of activity can be achieved. As might be expected from the above, 3,4-pentadienoyl-CoA shows no activity as a substrate in the standard assay system (Experimental Procedures).

These experiments identify several aspects of the interaction of the allenic thioester derivative with pig-kidney general acyl-CoA dehydrogenase which will be elaborated later in the manuscript. 3,4-Pentadienoyl-CoA is a very potent and rapid inhibitor of the dehydrogenase. Inhibition may be reversed by two routes. The first involves replacement of the inhibitor by an excess of a tightly binding substrate such as octanoyl-CoA (curve C, Fig. 1). The second involves a slow enzyme catalyzed turnover of the inhibitor yielding 2,4-pentadienoyl-CoA.

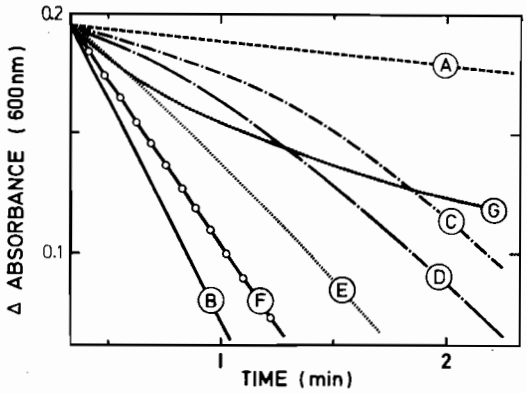


Fig. 1. *Effect of 3,4-pentadienoyl-CoA on the activity of general acyl-CoA dehydrogenase.* Native enzyme, 4 μM in 50 mM phosphate buffer, pH 7.6, was incubated with 3 equiv. of the allenic substrate analog at 25°C. Activity was determined by diluting 5 μl of the incubation mixture directly into the assay cocktail as described in Experimental Procedure, monitoring the reduction of dichloroindophenol at 600 nm. (A) Non-enzymatic blank reduction of dichloroindophenol; (B) control assay in the absence of inhibitor. (C, D, E, F) are after incubation times of 1.2, 8, 60 and 220 min respectively. (G) The assay curve when 30 μM octanoyl-CoA and 6 μM 3,4-pentadienoyl-CoA were added simultaneously to the native enzyme

3,4-Pentadienoyl-CoA reacts with general acyl-CoA dehydrogenase stoichiometrically

Fig. 2 shows a spectrophotometric titration of the enzyme with the allenic substrate analog under aerobic and anaerobic conditions. After each addition the decrease in A_{446} was complete before measurement could be made. For the reasons discussed below, it is essential that the titration be carried out rapidly otherwise a stoichiometry greater than unity will result. The titration proceeds isosbestically to yield a final spectrum characterized by a high absorbance in the near ultraviolet region, a weak maximum around 440 nm, and a very low absorbance above 520 nm (Fig. 2). This species is clearly different from that produced by reduction of the enzyme with dithionite, photochemical means or with substrate [12]. The final spectrum (curve 5) also clearly shows that conversion of oxidized flavin is essentially complete in this reaction. This contrasts with the behaviour observed with normal acyl-CoA substrates where the extent of reduction obtained on saturation varies widely and is strongly dependent on the acyl chain length used [1, 12, 20]. In the presence of oxygen, a good electron acceptor of uncomplexed reduced general acyl-CoA dehydrogenase, or of phenazine methosulfate/2,6-dichloroindophenol, an efficient electron acceptor system for product-complexed reduced enzyme, exactly the same reaction stoichiometry is observed. These data underscore the inability of the allenic thioester to serve as a normal substrate (see earlier).

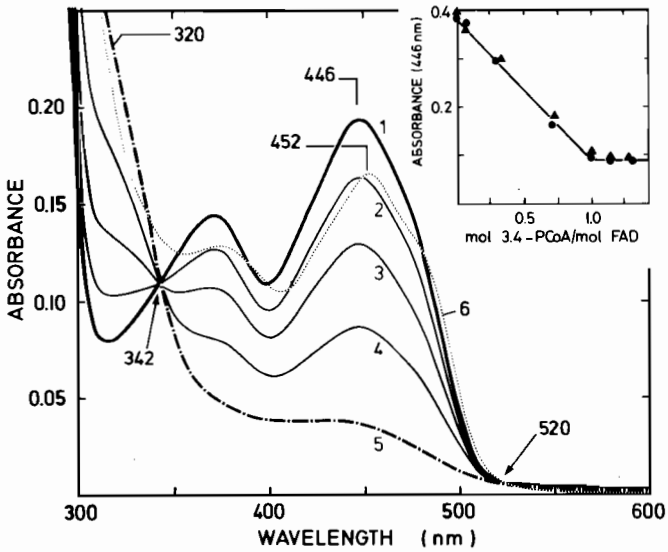


Fig. 2. *Spectral course of the reaction of general acyl-CoA dehydrogenase with 3,4-pentadienoyl-CoA*. The oxidized enzyme, 12 μM in 50 mM phosphate buffer, pH 7.6 (curve 1), was titrated at 25 °C with the concentrations of 3,4-pentadienoyl-CoA (3,4-PCoA) shown in the inset and the intermediate spectra were recorded within 2 min (curves 2–5). Arrows denote the position of isosbestic points. Curve 5 represents the spectrum obtained at maximal decrease at 446 nm, after addition of ≈ 1 equiv. of inhibitor. Curve 6 was recorded after the maximal return of the flavin absorbance (= 2.5 h). The inset shows the absorbance changes at 446 nm measured under aerobic (●——●) and anaerobic (▲——▲) conditions, and plotted as function of the allene/enzyme ratio

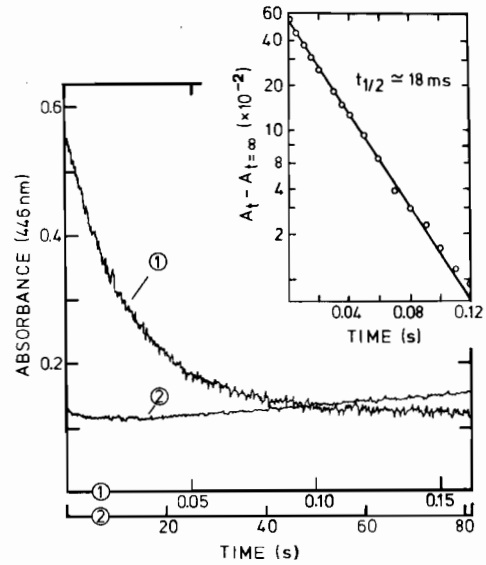


Fig. 4. *Kinetic course of the rapid absorbance changes during the reaction of general acyl-CoA dehydrogenase with 3,4-pentadienoyl-CoA*. Native enzyme, 35 μM in 50 mM phosphate buffer, pH 7.6, was mixed at 25 °C with the same volume of a 0.35 mM solution of 3,4-pentadienoyl-CoA in the same buffer. The absorbance changes at 446 nm during the first 100 ms in the 2-cm cuvette of the stopped-flow spectrophotometer followed first-order kinetics (curve 1). Curve 2 was obtained by monitoring the absorbance changes of a similar reaction over a time scale of 100 s. The inset shows a semilogarithmic plot of curve 1

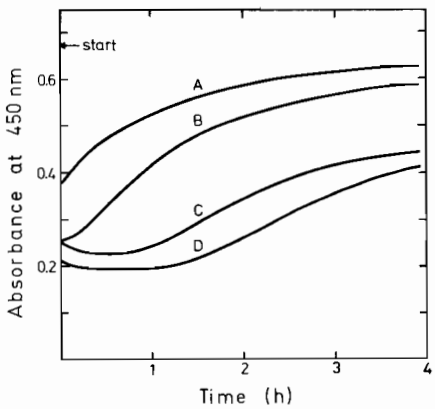


Fig. 3. *Time dependence of the slow spectral changes of general acyl-CoA dehydrogenase upon incubation with varying concentrations of 3,4-pentadienoyl-CoA*. The enzyme, 44 μM in 50 mM phosphate buffer, pH 7.6, was incubated at 25 °C with (A) 0.6, (B) 1.0, (C) 1.6 and (D) 1.9 equivalents of the analog. The reappearance of the oxidized flavin spectrum was followed at 450 nm

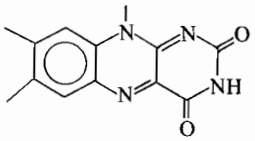
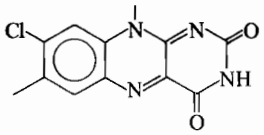
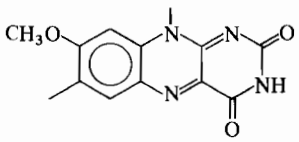
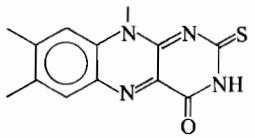
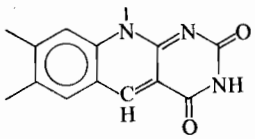
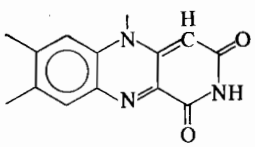
Kinetic course of the reaction of general acyl-CoA dehydrogenase with 3,4-pentadienoyl-CoA

Fig. 3 shows that after a very rapid bleaching of the flavin absorbance at 450 nm upon the addition of the inactivator, a slow return of the oxidized chromophore ensues in a reaction requiring several hours for completion (e.g. see curve A). The final spectrum obtained in the titration experiment is shown in curve 6 of Fig. 2. The main absorbance envelope is now red-shifted from 446 nm to 452 nm, and a shoulder has developed at about 480 nm indicating the continued presence of a bound thioester ligand in the reoxidized enzyme. The rate and extent of reappearance of the 450-nm absorbance is independent of the presence of the electron acceptors oxygen or phenazine methosulfate. When greater than stoichiometric levels of allene are used (Fig. 3, curves C–D), a pronounced lag is seen in the reappearance of 450-nm absorbance. Further, the duration of this lag, and the extent of reappearance of the oxidized flavin spectrum are proportional to the excess of allenic inactivator used in the incubation (Fig. 3). This behaviour does not reflect instability of the allenic thioester in free solution (not shown), but involves a slow enzyme-catalyzed turnover to a species which does not possess particular inhibitory activity. Thus the initial rates shown in Fig. 1 curves C–F increase with time, and the assays become progressively more linear (see earlier).

The rate of reappearance of the 450-nm absorption is 9×10^{-3} min^{-1} at 25 °C ($t_{1/2} \approx 75$ min) and, after the lag phase, it is independent of the concentration of the allene originally added. It is however strongly dependent on the temperature. The Arrhenius activation energy, which can be estimated from experiments such as those of Fig. 3 performed in the temperature range 12–36 °C, is = 150 kJ mol^{-1}. This high value suggests that the process involves a major molecular rearrangement.

The initial phase of the reaction of general acyl-CoA dehydrogenase with 3,4-pentadienoyl-CoA can be monitored conveniently by following the absorption decrease at 446 nm (cf. Fig. 2) in the stopped-flow spectrophotometer (Fig. 4). The course of the reaction is monophasic over at least five half-lives (see inset) yielding a rate constant of 2.4×10^3 min^{-1}. This reaction is independent of the allene concentration in the range 1.7×10^{-5} to 3.5×10^{-4} M. In

Table 1. *The effect of the redox potential of the enzyme flavin on the reaction with 3,4-pentadienoyl-CoA*
Activity is that in the standard assay (see Experimental Procedure) minus the 1% residual activity of the apoprotein. Redox potentials are for two-electron couples taken from the indicated reference. n.d., not determined. (+) Reduction is complete within 5 s

Coenzyme structure	Kinetic constants of reaction with allene		Acitvity	E_0'
	apparent reduction k_{obs}	apparent reoxidation $t_{1/2}$		
	min	min	%	mV
	2.4×10^3	75	100	−208 [26]
	+	10^3	8	−152 [27]
	+	3	n.d.	−284 [28]
	+	60	7	−126 [29]
	2.8×10^3	0	0	−311 [29] −273 [30]
	0	0	0	−280 [29]

contrast, substrate reduction of the pig-kidney general acyl-CoA dehydrogenase [21, 22] and similar acyl-CoA dehydrogenases [20, 23, 24], has been reported to proceed multiphasically. Fig. 4 (curve 2) shows the onset of the slow absorbance increases, which follows the decrease. When the same experiment as that shown in Fig. 4 was carried out using 3,4-pentadienoyl-CoA which had 50% ^2H at the α-position ($H_2C=C=CH-CH^2H-COS-CoA$, random distribution of the label), no significant isotope effect on the reaction rate was observed. The reaction of 3,4-pentadienoyl-CoA with 5-deaza-FAD enzyme, details of which will be published later [25] (A. Wenz and S. Ghisla, unpublished), proceeds at essentially the same rate (Table 1).

The effect of the redox potential of the enzyme flavin on the reaction with 3,4-pentadienoyl-CoA

One aspect of the reaction of the allenic inactivator with general acyl-CoA dehydrogenase is the monophasic, and essentially complete reduction of the enzyme flavin. This contrasts markedly with the behavior shown by normal substrates which give multiphasic absorption decreases with end points which are strongly dependent on the acyl chain length of the substrates used. In effect, the internal equilibrium K_2:

$$EFl_{ox} \sim SH_2 \overset{K_2}{\rightleftharpoons} EFl_{red} \sim S$$

is dependent on chain length [12, 22] and reflects the redox potentials of the bound flavin and bound substrate moieties. Replacement of the normal flavin by analogues of different redox potentials has been found to perturb K_2 [14]. Accordingly, such substitutions might be expected to alter the course of the reaction of the dehydrogenase with the allene and be useful in clarifying the mechanism. A series of experiments such as those shown in Figs 2 and 3 were thus carried out with general acyl-CoA dehydrogenase reconstituted with the FAD analogues shown in Table 1. The results clearly indicate a correlation between an increase of the rate of return of oxidized flavin absorption with a decrease of the flavin redox potential. This does not apply to 5-deaza-FAD general acyl-CoA dehydrogenase which is not active with normal substrate [14] but reacts rapidly with 3,4-pentadienoyl-CoA (Table 1).

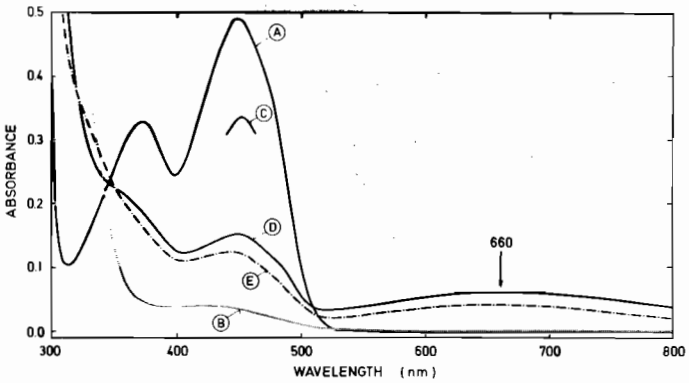


Fig. 5. *Identification of 2,4-pentadienoyl-CoA as product of the slow reoxidation of the reduced enzyme species.* The native enzyme, 32 μM in 50 mM phosphate buffer, pH 7.6 (curve A) was incubated with 53 μM 3,4-pentadienoyl-CoA. The decay of the resulting complex (curve B) was followed by monitoring the increase in 450-nm absorbance. After a 70% recovery of the original 450-nm absorbance (curve C) a few grains of solid dithionite were added to yield curve D. Curve E represents the spectrum of the complex obtained by treating dithionite-reduced enzyme with 0.1 mM of authentic 2,4-pentadienoyl-CoA. The stoichiometric reaction of the native enzyme with 4-pentenoyl-CoA yielded a similar spectrum (not shown)

The degree of irreversible inactivation corresponding to the difference between original and 'reformed' Fl_{ox} absorbance, however, does not change with the alteration of the redox potential of the coenzyme.

Identification of 2,4-pentadienoyl-CoA as the product of the reaction of 3,4-pentadienoyl-CoA with general acyl-CoA dehydrogenase

Pig-kidney general acyl-CoA dehydrogenase in its oxidized or reduced form, has been shown to yield charge transfer absorptions upon complexation with a variety of substrate analogues or products [12, 31]. The spectra obtained, in particular the λ_{max} and absorption coefficients of the charge transfer bands are unique for each CoA-derivative used [31, 32]. A chemically reasonable putative product from the reaction of the allene would be the 2,4-pentadienoyl-CoA isomer. This species has been shown by Engel and Massey to yield a bluish-green complex with reduced butyryl-CoA dehydrogenase from *Megasphaera elsdenii* [33]. The ultraviolet-visible spectrum of the analogous complex with the mammalian enzyme is shown in Fig. 5. It can be formed either on the addition of 2,4-pentadienoyl-CoA to a sample of enzyme reduced with dithionite (curve E), or upon the addition of one equivalent of 4-pentenoyl-CoA to the oxidized flavoprotein (data not shown). In the latter case the diene is produced directly at the active site of general acyl-CoA dehydrogenase by the reaction

$$\leftrightharpoons EFl_{ox} \sim H_2C{=}CH{-}CH_2{-}CH_2{-}COS\text{-}CoA \leftrightharpoons EFl_{red} \sim H_2C{=}CH{-}HC{=}CH{-}COS{-}CoA \leftrightharpoons .$$

The enzyme was incubated with a 1.7-fold excess of the allene, and the slow 'turnover' was allowed to reach completion. Upon reduction of the oxidized flavin with dithionite, an absorption band developed which was closely similar to that obtained with reduced enzyme and authentic 2,4-pentadienoyl-CoA. No other medium-chain CoA derivatives tested gave similar spectral changes. From the sensitivity and specifity of the effect we conclude, that 3,4-pentadienoyl-CoA is indeed converted to 2,4-pentadienoyl-CoA by general acyl-CoA dehydrogenase.

Release of the flavin chromophore upon reaction with 3,4-pentadienoyl-CoA

Although the interaction of 3,4-pentadienoyl-CoA with general acyl-CoA dehydrogenase leads to a spectrum clearly reflecting a reduced flavin species (Fig. 2), this form cannot transfer reducing equivalents to efficient electron acceptors of the native enzyme (see earlier). Thus frank transfer of reducing equivalents to yield the corresponding acyl product 2,3,4-pentatrienyl-CoA, a cumulene, would appear very unlikely. Rather, as has been observed with other flavoenzymes [34, 35], this behavior suggests the formation of a covalent adduct between flavin and the substrate analog. Such adducts may have sufficient stability to survive release from the protein. Accordingly after maximal bleaching of the enzyme flavin by the allenic inhibitor (Fig. 2, curve 5), the prosthetic group was released by treatment with 2% sodium dodecylsulfate, 4.5 M guanidine hydrochloride, 5% trichloroacetic acid, or by heating at 100°C for 3 min. In all cases oxidized FAD was recovered quantitatively. The same results were obtained when denaturation was performed under rigorously anaerobic conditions. These data show that whatever the nature of the reduced flavin species, it is very labile upon denaturation of the protein.

The fact that denaturation of a reduced flavin species under anaerobic conditions yields oxidized flavin quantitatively requires comment. Control experiments using enzyme reduced with octanoyl-CoA and denatured anaerobically show the same behaviour. These data are readily explained by the differing stabilities of reduced and oxidized enzyme forms towards denaturation. Since these species exist in rapid equilibrium:

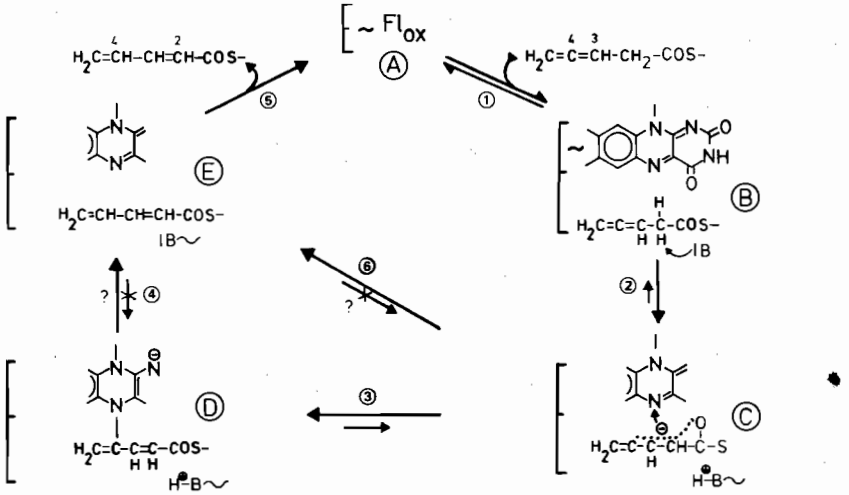
$$EFl_{ox} \sim SH_2 \leftrightharpoons EFl_{red} \sim S$$

a more rapid denaturation of the oxidized enzyme will effect release of the oxidized prosthetic group. Indeed, reduction of the native dehydrogenase either chemically, or with substrate, profoundly stabilizes the enzyme against thermal and guanidine hydrochloride denaturation (Madden, M., Lau, S.-M. and Thorpe, C. unpublished).

DISCUSSION

Scheme 1 shows the steps which are proposed to occur during the reaction of 3,4-pentadienoyl-CoA with general acyl-CoA dehydrogenase.

Initially, the allenic analog binds rapidly and reversibly to the free enzyme (A) to form the Michaelis complex (B) (step 1). An active center base residue, possibly a carboxylate, as shown by Fendrich and Abeles for the related enzyme butyryl-CoA dehydrogenase from *Megasphaera elsdenii* [8], then abstracts an α-proton from the allene-yielding species (C) (step 2). This transient anion would be expected to be stabilized through the extended conjugation system shown. The next step 3, the formal transfer of reducing equivalents to the flavin and formation of the adduct (D) must be much faster than step 6 since the adduct is formed stoichiometrically from 1 mol of inhibitor. The adduct (D), i.e. the species with



Scheme 1. *Proposed mechanism of inactivation of general acyl-CoA dehydrogenase by 3,4-pentadienoyl-CoA*

the spectrum of curve 5 in Fig. 2, is thus the primary product of reaction of the allene with the enzyme. Several points can be made concerning the structure of this species. (a) The non-reactivity with electron acceptors strongly supports the absence of normal, unsubstituted, reduced flavin in the complex. Such lack of reactivity is typical of reduced flavin derivatives blocked by covalent modification [34, 35]. (b) Since denaturation of the protein leads to the quantitative reappearance of oxidized flavin, the modification does not involve a carbon atom of the flavin such as those at positions C-4a, or C-6 [34, 35]. In such cases strong oxidants are required to reform oxidized flavin from flavin adducts linked by C–C bonds [34, 36]. Rather the rapid reoxidation observed here is typical of attachment to a heteroatom such as N-5 [35, 37]. (c) The spectrum of the product (Fig. 2, curve 5) is characterized by a very low absorbance in the visible, and a high absorbance of a bond at 300–320 nm, which is partially obscured by the protein absorbance. Flavin derived chromophores with such characteristics are 1,5-dihydroflavins carrying a conjugated substituent at the 5-position, such as 5-acyl-flavins [37–39]. Note that 1,5-dihydroflavin in general acyl-CoA dehydrogenase does not show absorption maxima at $\lambda > 300$ nm (however shoulders at ≈ 340 nm and ≈ 380 nm) [12], that N-5 alkylated flavins absorb with well resolved bands in the 325–350 nm region and that 4a,5-dihydroflavins and derivatives have maxima in the 350–400 nm part of the spectrum [35, 40]. These arguments support a structure such as compound D, Scheme 1, in which the covalent bond would be between N-5 and the 4-position of the dienoyl moiety. Species D would be expected to arise from nucleophilic attack of the delocalized anion C on the oxidized flavin electrophile. An adduct resulting from the allene C-2 attack is, however, conceivable. The structure of an adduct such as compound D, obtained by reaction of the 3,4-allene with 5-deaza-FAD-enzyme will be the subject of a forthcoming paper [25] (Wenz, A. and Ghisla, S., unpublished).

It is instructive to compare the rates of formation of the reduced flavin species (compound D) depicted in Scheme 1 with the production of the native reduced enzyme by a normal substrate such as butyryl-CoA. In the case of the allene, reduction of the flavin by steps (1+2+3) occurs with a rate constant of 2.4×10^3 min^{-1}, whereas generation of the dihydroflavin-crotonyl-CoA complex using the normal substrate, butyryl-CoA, is 10-fold slower [21, 22]. In both instances initial binding (step 1) must be much faster than the overall reduction of the flavin [21, 22].

This work shows that there are two distinct routes to regenerate active enzyme. Dilution of the adduct into a large excess of octanoyl-CoA, a very tight binding substrate of the enzyme [6], effects relatively rapid recovery of activity (Fig. 1, curve C). This is envisaged as occurring by competition of octanoyl-CoA with reformed 3,4-pentadienoyl-CoA (formed by reversal of steps 3+2+1, Scheme 1) for active enzyme (compound A). The rate constant for the reversal of steps 1+2+3 may be estimated from the regain of activity seen in curve C of Fig. 1 ($t_{1/2} \approx 2$ min, $k \approx 0.34$ min^{-1}), since octanoyl-CoA can only react with the free oxidized enzyme (compound A). This rate constant must reflect the slowest step in the reverse sequence, i.e. step 3 or a combination of steps 3 and 2 (Scheme 1). The ratio of forward and backward rate constants of about 7000 using 3,4-pentadienoyl-CoA is consistent with the essentially complete formation of the reduced flavin species shown in Fig. 2 curve 5. In contrast, the analogous reversal with butyryl-CoA-reduced enzyme occurs with a rate constant of approximately 240 min^{-1} yielding a ratio of forward to reverse rate constants of 1. The large difference in these ratios (approaching four orders of magnitude: 22 kJ) reflects, in part, the strongly exergonic isomerization of 3,4-pentadienoyl-CoA to the corresponding 2,4-diene. For example, the free energy difference between 1,3-pentadiene and 1,2-pentadiene is 63 kJ mol^{-1} [41], and this favourable value should be even higher upon conjugation with a thioester carbonyl moiety. In summary the first relatively fast mode of reformation of oxidized enzyme (compound A) can be described as a competitive displacement of the inhibitor, the limiting rate of the process being the reformation of free 3,4-pentadienoyl-CoA.

The second, much slower mode ($t_{1/2} \approx 75$ min) of reformation of active enzyme (compound A) is driven by the conversion of 3,4-pentadienoyl-CoA to its 2,4-isomer, i.e. by the removal of the allene from the equilibrium of steps 1+2+3, Scheme 1.

Two possible routes for this isomerization with reappearance of oxidized flavin can be envisaged (Scheme 1). Protonation of species D at the vinylic flavin N-5 diene-C-4 orbital to yield compound E directly via step 4 cannot be

dismissed. However, we consider the route involving reformation of compound C by reversal of step 3 more likely. Protonation of the transient carbanion (compound C) at the C-4 position to yield compound E (step 6) would compete with reactions 3 and 2. The observation that the rate of decay of compound D is indeed dependent on the redox potential of the flavin (Table 1), the rate of decay decreasing with increasing redox potential of the flavin would be in agreement with this variant. In fact the redox potential of the flavin should affect the equilibrium (and rates) of step 3, this equilibrium being shifted to the right in the case of low potential flavins, consequently increasing the rate of the (irreversible) step 6. Neither of these sequences would be expected to be effectively reversible both because of the strong thermodynamic drive toward this isomerization (see earlier), and because reversal would involve attack of oxidized flavin on the oxidized 2,4-diene species. 2→4 proton shifts have been described during the interaction of vinylacetyl-CoA with butyryl-CoA dehydrogenase from *M. elsdenii* and during the oxidation of glutaryl-CoA by glutaryl-CoA dehydrogenase from *Pseudomonas fluorescens* [8, 10].

The irreversible and very slow inactivation accompanying turnover of 3,4-pentadienoyl-CoA to the 2,4-isomer (15–20% per turnover) clearly does not result from irreversible covalent modification of the flavin since upon denaturation the latter is recovered quantitatively. This inactivation might arise from covalent modification of an essential protein residue as in the case described by Fendrich and Abeles [8]. Such a modification might also be responsible for the progressive denaturation of the enzyme observed in experiments such as those shown in Fig. 3. At which stage in the process depicted in Scheme 1 such an event occurs cannot be deduced with certainty from the present results. However, the fact that 2,4-pentadienoyl-CoA does not affect enzymatic activity suggests that this irreversible inactivation results from a side reaction prior to its release (step 5, Scheme 1), and thus probably at the level of intermediate compounds C or D.

The lack of observation of an isotope effect on the reaction with partially α-deuterated 3,4-pentadienoyl-CoA might simply be due to its intrinsically low value and to discrimination. The isotope effects observed with α-deuterated acyl-CoA derivatives vary videly depending on the nature of the substrate and on the source and specificity of the enzyme [19, 21–24]. Alternatively, the step involving breakage of the αC–H bond might not be rate limiting in the present case, as opposed to the reaction with butyryl-CoA [21, 22]. Unfortunately, the difficulties in the chemical synthesis of fully labelled 3,4-pentadienoic acid, and its chemical instability have precluded further studies of this aspect.

The finding of isomerization of 3,4-pentadienoyl-CoA to the 2,4-isomer is clearly compatible with abstraction of the α-hydrogen as a proton (step 2, Scheme 1), and its transfer to position 4 of the product. It thus would constitute evidence in favor of a carbanion-initiated mechanism, as discussed and proposed elsewhere by our groups and as also by others for the α,β-dehydrogenation reaction catalyzed by acyl-CoA dehydrogenases [6, 8, 21, 23]. In the present case radical mechanisms are very unlikely for the following reasons: In fact general acyl-CoA dehydrogenase reconstituted with 5-deaza-FAD reacts with 3,4-pentadienoyl-CoA to yield a product with spectral properties closely similar to those observed upon reaction with normal enzyme [25]. 3,4-Pentadienoyl-CoA reacts at essentially the same rate with normal and with 5-deaza-FAD enzyme (Table 1). As also discussed elsewhere [21] the finding of analogous products being formed at similar rates with the same enzyme carrying different coenzymes is a strong argument in favour of the occurrence of the same mechanistic event. On the other hand, dark reactions of 5-deaza-flavins have been shown not to proceed via radical steps, the activation energy of the latter being much too high [42].

The results in this paper show that 3,4-pentadienoyl-CoA should be considered an inhibitor of the suicide type for general acyl-CoA dehydrogenase. Like methylenecyclopropylacetyl-CoA [7], but unlike the 3-alkynoyl-inhibitors [8, 10], the flavin moiety is the primary target of this allene. However 3,4-pentadienoyl-CoA is unusual in that, although a potent covalent inhibitor of the enzyme, activity may be recovered via two distinct processes. Thus such an allenic species may be expected to be a potent, but short acting, inhibitor of fatty acid oxidation, and as such might be useful in metabolic studies.

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