Oxidative Inactivation of a Charge Transfer Complex in the Medium-Chain Acyl-CoA Dehydrogenase†

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ABSTRACT: The intense charge transfer complex between the enolate of 3-thia-octanoyl-CoA and the oxidized flavin of the medium-chain acyl-CoA dehydrogenase is discharged by the ferricenium ion with irreversible inactivation of the enzyme. Charge transfer complex formation is a necessary, but insufficient, condition for oxidative inactivation: the 3-oxa-octanoyl-CoA complex is also inactivated, whereas the comparable *trans*-3-octenoyl-CoA species is not. Complete inactivation of the dehydrogenase with 3-thia-octanoyl-CoA requires 1 molecule of thioester and apparently 3 molecules of ferricenium hexafluorophosphate. Experiments with 8-Cl-FAD substituted enzyme and the crystal structure of enzyme•ligand complexes argue that ferricenium ion−mediated oxidation proceeds through the flavin prosthetic group. Synthesis of [2-^{14}C]-3-thia-octanoyl-CoA, followed by isolation of radiolabeled peptide from the modified medium-chain dehydrogenase, showed that inactivation results in labeling the catalytic base, GLU376. Oxidative modification is accompanied by the release of CoASH. A mechanism for inactivation is proposed involving generation of a sulfonium salt which efficiently captures the carboxylate nucleophile.

The acyl-CoA dehydrogenases (Beinert, 1963; Engel, 1992) catalyze abstraction of the pro-R-α-proton with elimination of the pro-R-β-hydrogen as a hydride equivalent from normal substrates to the N-5 position of the isoalloxazine ring of the flavin moiety (Biellman & Hirth, 1970; Frerman et al., 1980; Ghisla et al., 1984). The carboxylate base, depicted in Scheme 1, is GLU376 in the medium-chain acyl-CoA dehydrogenase (Kim, 1991; Kim et al., 1993; Bross et al., 1990; Powell & Thorpe, 1988). The resulting reduced enzyme•trans-2-enoyl-CoA complex is then reoxidized in two successive 1-electron steps by two molecules of electron-transferring flavoprotein (Hall & Lambeth, 1980; Reinsch et al., 1980; Gorelick et al., 1985). The reductive half reaction depicted above shows large and approximately multiplicative deuterium isotope effects at C-2 and C-3 when butyryl-CoA is used, suggesting that dehydrogenation of this short-chain substrate is concerted (Murfin, 1974; Reinsch et al., 1980; Pohl et al., 1986; Schopfer et al., 1988).

We have previously synthesized acyl-CoA analogues in which dehydrogenation is blocked by replacement of the methylene group at C-3 by a heteroatom (Lau et al., 1988). For example, 3-thia-octanoyl-CoA (Chart 1) binds to the medium-chain dehydrogenase and rapidly (about 330/s at 2 °C, pH 7.6; Zhou and Thorpe, unpublished) forms an intense, long wavelength band absorbing at 800 nm (Lau et al., 1988). This new spectral feature is believed to represent a charge transfer band between a tightly bound acyl-CoA enolate as the donor and oxidized flavin as the acceptor (Massey & Ghisla, 1974; Lau et al., 1988; see Scheme 2): Accordingly, the enzyme catalyzes exchange of one of the α-protons of 3-thia-octanoyl-CoA in D_2O (Lau et al., 1988). Since very similar absorbance bands are generated on the addition of either 3-oxa-octanoyl-CoA or *trans*-3-octenoyl-CoA (Powell et al., 1987; Lau et al., 1988), the heteroatom in 3-thia-octanoyl-CoA is not the charge transfer donor (Lau et al., 1988). α-Proton abstraction with these substrate analogues may proceed to completion because their enolates cannot efficiently discharge reducing equivalents into the flavin cofactor. The crystal structure of the medium-chain dehy-

Scheme 1

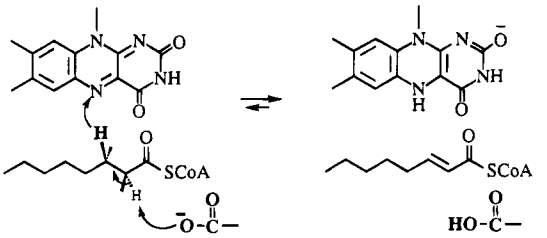
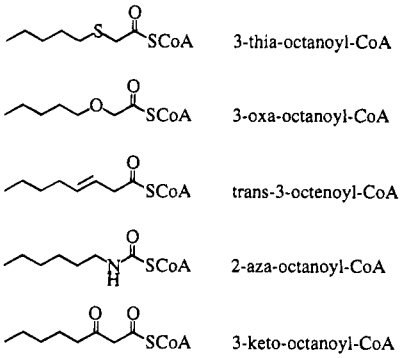
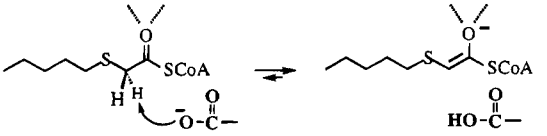


Chart 1: Some Acyl-CoA Analogues Used in This Work



Scheme 2



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drogenase shows that the substrate carbonyl oxygen atom makes H-bonding interactions with the amide N−H of GLU376 and the 2′-OH of the ribityl side chain of the flavin prosthetic group (Kim et al., 1993). Loss of this latter H-bond by replacement of the bound FAD with 2′-deoxy-FAD results in an essentially inactive enzyme (Ghisla et al., 1994). It is therefore reasonable to invoke H-bonding in the stabilization of the bound enolates of these blocked substrate analogues (Thorpe & Kim, 1995).

As part of a continuing study of these interesting and comparatively stable enzyme•enolate complexes, we have found that they can be oxidized by the 1-electron oxidant ferricenium hexafluorophosphate. In the case of the 3-thia-analogues, this leads to a new method of labeling of the active site of the dehydrogenase. This phenomenon may prove useful in the inactivation of other flavoenzymes that form charge transfer complexes with suitable nonsubstrate analogues.

MATERIALS AND METHODS

Materials. Medium-chain acyl-CoA dehydrogenase was isolated from pig kidneys as described previously (Gorelick et al., 1982; Lau et al., 1986). CoASH (lithium salt), 2,6 dichlorophenolindophenol (DCIP),[1] phenazine methosulfate (PMS), octanoic acid, DPCC-treated trypsin, *N*-ethylmaleimide (NEM), and pepsin were purchased from Sigma. Ferricenium hexafluorophosphate and 4,4′-dithiodipyridine were from Aldrich; *trans*-3-octenoic acid was from Lancaster Synthesis, *N*-butyl-maleimide was from ICN; trichloroacetic acid (TCA) was from Fisher; *n*-hexyl-isocyanate was from Kodak; and sodium dithionite was from the Virginia Smelting Co., Portsmouth, VA. Iodo-[2-^{14}C]-acetic acid was obtained from Amersham. 8-Cl-riboflavin was a generous gift of Dr. John Lambooy, University of Maryland, and was converted to 8-Cl-FAD as in Spencer et al. (1976). 8-Cl-FAD reconstituted enzyme was prepared as described in Thorpe and Massey (1983).

General Methods. Unless stated otherwise, all buffers were 50 mM potassium phosphate, pH 7.6, containing 0.3 mM EDTA. Static absorbance measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer. Concentrations of native, 8-Cl-FAD reconstituted, and oxidatively modified dehydrogenase were determined using the following extinction coefficients: 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979); 14.0 mM^{-1} cm^{-1} at 440 nm (Thorpe & Massey, 1983); and 15.4 mM^{-1} cm^{-1} at 458 nm (this paper; determined by the guanidine hydrochloride method in Thorpe et al., 1979). Radioactivity was determined with a Beckman LS-100C scintillation counter with Liquiscint (National Diagnostics) liquid scintillation cocktail. Concentration and ultrafiltration of small enzyme samples was achieved using microconcentrators (Amicon Corp., M_r 10,000) according to the manufacturer's instructions. Binding and kinetic parameters were determined from spectrophotometric titrations using Enzfitter, Elsevier Biosoft. Medium-chain acyl CoA dehydrogenase activity was determined using either ferricenium hexafluorophosphate (Lehman & Thorpe, 1990) or the PMS/DCIP system (Thorpe et al., 1979). Solutions were deoxygenated by repeated flushings with nitrogen as described earlier (Gorelick et al., 1985). Photochemical and dithionite reductions were performed as in Gorelick et al. (1985).

Synthesis and Characterization of CoA Thioesters. The 3-thia and 3-oxa acids and thioesters were synthesized and characterized as described earlier (Lau et al., 1988). *trans*-3-Octenoyl CoA and octanoyl CoA thioesters were prepared by the mixed anhydride method of Bernert and Sprecher (1977). 3-Keto-octanoyl CoA was synthesized enzymatically by the procedure of Thorpe (1986) and 2-aza-octanoyl CoA as in Trievel et al. (1995). All thioester were purified by HPLC with a Perkin-Elmer Series 400 liquid chromatograph on a semipreparative octadecylsilica column (Vydac) using a gradient of methanol and 25 mM phosphate, pH 5.3 (Cummings & Thorpe, 1994). All purified CoA thioesters were desalted on a Bio-Rad P-2 column, lyophilized, and stored frozen. All the thioesters used in this work were quantitated using an extinction coefficient of 16 mM^{-1} cm^{-1} at 260 nm (Stadtman, 1957).

Preparation of Modified Enzyme. The dehydrogenase (9 μM) was incubated with 30 μM 3-thia-octanoyl-CoA and 200 μM ferricenium hexafluorophosphate in 800 μL of 50 mM potassium phosphate buffer, pH 7.6, for 2 h. The enzyme was ultrafiltered and washed 5 times with 50 mM potassium phosphate buffer pH 7.6 to remove excess reagents. The modified enzyme showed 0.2% of the activity of the native enzyme, as judged by the ferricenium assay using 50 μM octanoyl CoA as substrate.

Effect of Visible Light on the Stability of the Charge Transfer Complex. Enzyme at a concentration of 6 μM was incubated at 2 °C in a cuvette with 15 μM 3-thia-octanoyl-CoA and 90 μM ferricenium in 50 mM phosphate buffer, pH 7.6, either 17 cm from a 150-watt floodlamp or in dim light. The decline in the charge transfer band was monitored spectrophotometrically for both irradiated and control samples.

Ferricenium Stoichiometry. The dehydrogenase (9.2 μM in 50 mM phosphate buffer, pH 7.6) was mixed with 123 μM ferricenium hexafluorophosphate and the spectrum was recorded. 3-Thia-octanoyl-CoA (13 μM) was then added and the spectrum recorded after complete discharge of the charge transfer complex (60 min). The remaining ferricenium ion was calculated from absorbance measurements at 618 nm (Lehman & Thorpe, 1990). Control experiments were performed in the absence of 3-thia-octanoyl-CoA and enzyme.

Synthesis of [2-^{14}C]-3-Thia-octanoyl-CoA. 3-Thia-octanoic acid was synthesized by mixing 20 μL of 1.08 M iodoacetic acid with 27 μCi of [2-^{14}C]-iodoacetic acid followed by 12 μL of 5 M sodium hydroxide and 5 μL pentanethiol. After stirring in dim light for 2 h, 30 μL of 2 M HCl was added followed by 120 μL of 100 mM MOPS at pH 7.4; 10 μL of 0.5 M DTT; 100 μL of 1 M ATP in MOPS buffer adjusted to pH 7; 20 mg coenzyme A in 200 μL of MOPS buffer adjusted to pH 7; 1 unit acyl CoA synthetase in 500 μL of MOPS; and 50 μL of 0.5 M magnesium chloride. The mixture was stirred for 2 h at 37 °C in dim light, ultrafiltered, and washed with MOPS buffer to remove protein. The thioester was purified from the combined filtrates on a semipreparative C18 column as before (Cummings & Thorpe, 1994), lyophilized, and stored frozen.

Structural Characterization of Radiolabeled Modified Enzyme. Modified enzyme was prepared by incubating the enzyme (23 nmoles) with a 1.5-fold excess of [2-^{14}C]-3-thia-octanoyl CoA (at a specific activity of 2860 cpm/nmole) and a 7-fold excess of ferricenium hexafluorophosphate at pH

[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; $Fc^+PF_6^-$, ferricenium hexafluorophosphate; HEPES, 4-(2-hydroethyl)piperazine-1-ethanesulfonic acid: MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate; TFA, trifluoroacetic acid.

7.6. After 2 h the enzyme was ultrafiltered and washed several times with 50 mM potassium phosphate buffer, pH 7.6, to remove excess reagents. The modified enzyme was denatured by boiling for 5 min in the dark in the presence of an 18-fold excess of NEM. The enzyme was centrifuged and the pellet washed 3 times with phosphate buffer to remove unbound FAD. Most (80%) of the radioactivity was present in the pellet (55 610 cpm) with the remainder released into the supernatant. The precipitate was resuspended in 0.1 M ammonium bicarbonate, pH 7.0, and digested at 37 °C with 2% w/w DPCC-treated trypsin. After 2 h the mixture was ultrafiltered to remove trypsin and undigested core material. This short digestion period minimized release of the label from the peptide. Peptides were isolated by reverse-phase HPLC on a Vydac octadecylsilica column (0.46 × 15 cm). Chromatograms were monitored at 220 nm and developed at 1 mL/min using the following gradient: 5 min, 100% 0.1% TFA in water; 5−125 min, linear gradient to 60% acetonitrile containing 0.1% TFA; 125−130 min, linear gradient to 100% 0.1% TFA in water. Fractions were collected every minute and sampled for radioactivity. Once the region containing the radioactive peptide was identified, peaks were collected manually in acid-washed test tubes. The recovery of radioactivity was >90% of the digest applied. The radioactive peptide was speed-vacuumed to dryness, redissolved in 50% acetonitrile containing 0.1% TFA, and sequenced using an Applied Biosystems gas-phase protein sequencer (Model 470A/120A/900A) according to manufacturer's instructions. Electrospray ionization mass spectrometry used an Autospec Q instrument. The pure tryptic peptide was dissolved in 50% acetonitrile/0.1% TFA and delivered at a flow rate of 5 μL/min (observed mass 1742.8; calculated for the derivatized peptide 1743.0).

For pepsin digests the modified enzyme was adjusted to 5% formic acid and digested with a 2% (w/w) solution of pepsin at 37 °C. After 1.5 h, the pH of the digest was adjusted to 4 with 2M KOH, and the solution was ultrafiltered and washed once with buffer. Peptides were isolated by reverse-phase HPLC as above and gave three radioactive peaks. The relative ratios of these peaks varied with each digestion, and the isolation of tryptic peptides was found to be more convenient (see above).

Isolation of FAD from Native and Modified Enzyme. Enzyme (10 μM) in 50 mM potassium phosphate buffer, pH 7.6, was denatured by incubation in a boiling water bath for 5 min in the dark. The denatured enzyme was centrifuged at 12 800 rpm and washed three times with phosphate buffer to remove free FAD. The combined supernatants were analyzed by HPLC on a semipreparative reverse-phase C18 column. The chromatograms were monitored at 260 nm and developed at 2 mL/min with the following program: 5 min, 100% water; 5−25 min, linear gradient to 65% methanol; 25−30 min, linear gradient to 100% methanol; 30−35 min, at 100% methanol; 35−40 min, linear gradient to 5 min 100% water. Denaturation of the photoreduced modified enzyme was achieved by boiling (see above), or upon the addition of 5% TCA in dim light. After 5 min in TCA, the suspension was centrifuged and the supernatant extracted five times with an equal volume of diethylether. Residual ether was removed with a stream of nitrogen and the volume and spectrum of the aqueous solution were recorded.

Trapping Released CoASH as the N-Butyl-maleimide Adduct. The medium chain dehydrogenase (8 μM) in 50 mM phosphate buffer pH 7.6 was incubated with 8 μM 3-thia-octanoyl-CoA, 95 μM N-butylmaleimide, and 103 μM ferricenium ion. After 2.5 h at 25 °C the mixture was ultrafiltered and washed twice with 375 μL phosphate buffer. The combined supernatants were analyzed by HPLC on an analytical C18 reverse-phase column. Chromatograms were developed at 0.5 mL/min using a gradient formed from methanol and 100 mM KP_i, pH 4.0, modified from DeBuysere and Olson (1983): 4 min, at 6% methanol; 8 min, linear gradient to 8%; 6 min, linear gradient to 13%; 6 min, linear gradient to 20%; 12 min, linear gradient to 45%; 5 min, at 45%; 5 min, linear gradient back to 6% methanol; and 10 min, holding at 6% methanol. Elution times were: ferricenium ion, 9.0 min; CoASH, 20.8 min; CoAS-SCoA, 33.4 min; CoAS-*N*-butyl maleimide adduct, 44.3 min. Ferrocene failed to elute with this program, and was removed with a methanol wash step.

Quantitating CoASH Release. The dehydrogenase (6.5 μM) was incubated for 90 min in the presence of 13.3 μM 3-thia-octanoyl-CoA, 142 μM 4,4′-dithiodipyridine, and 125 μM ferricenium ion. CoASH release was followed by monitoring the increase at 324 nm (Grassetti & Murray, 1967; Brocklehurst & Little, 1981). Control experiments showed that CoASH can be accurately quantitated using dithiodipyridine in the presence of the ferricenium ion: this oxidant reacts very slowly with thiols under these conditions. Controls were also run to account for the change in absorbance due to other processes. Oxidative inactivation of the enzyme in the absence of dithiopyridine leads to a slight decrease in 324-nm absorbance. The very sluggish reaction of the dehydrogenase with thiol group−specific reagents (Thorpe et al., 1979) was accounted for by a control in the absence of ferricenium ion. Finally no significant changes occurred with ferricenium and 4,4′-dithiopyridine alone.

Photoreduction of 3-Thia-octanoyl-CoA Modified Medium-Chain Acyl-CoA Dehydrogenase. The modified dehydrogenase (11 μM in 50 mM phosphate buffer, pH 7.6, containing 10 mM EDTA) was placed in a water bath at 0 °C and illuminated 2 cm from a 150-watt lamp for a total of 68 min. Half-reduction occurs after approximately 5 min illumination. Under comparable conditions, 3% reduction of the uncomplexed native dehydrogenase was observed after 40 min.

RESULTS

Ferricenium Ion Dependent Inactivation of Medium-Chain Acyl-CoA Dehydrogenase Complexed with 3-Thia-octanoyl-CoA. Figure 1 shows that the medium-chain acyl-CoA dehydrogenase is rapidly inactivated during incubations that combine the substrate analogue 3-thia-octanoyl-CoA (Lau et al., 1988), and the electron acceptor ferricenium hexafluorophosphate (Lehman & Thorpe, 1990; Lehman et al., 1990). Samples were withdrawn from the incubation mixtures, and their activity was measured using a standard assay system (PMS/DCIP; see Methods). The curve in Figure 1 represents a first-order decline in activity with an apparent rate constant of 0.095/min at 128 μM ferricenium and a residual activity of 0.5%. Inactivation requires the presence of both the 3-thia-octanoyl-CoA and the ferricenium ion because insignificant loss of activity occurs when the dehydrogenase is incubated with either reagent alone (Figure 1). Further, preincubation of 3-thia-octanoyl-CoA (35 μM) and ferricenium hexafluorophosphate (255 μM) for 45 min at pH 7.6 before the addition of enzyme (1.8 μM) had no effect on the kinetics of inactivation (not shown). Thus inactivation

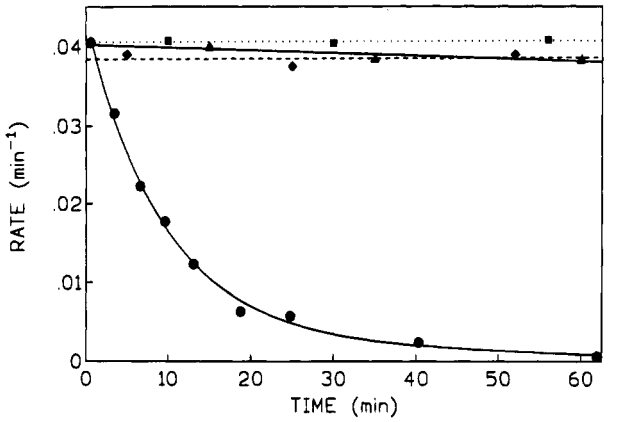


FIGURE 1: Inactivation of medium-chain acyl-CoA dehydrogenase in the presence of 3-thia-octanoyl-CoA and ferricenium hexafluorophosphate. Medium-chain acyl-CoA dehydrogenase (1 μM in 100 mM HEPES buffer, pH 7.6, containing 0.3 mM EDTA) was incubated with 28 μM 3-thia-octanoyl-CoA and 128 μM ferricenium hexafluorophosphate at 25 °C, and samples were withdrawn for assay using the PMS/DCIP procedure (●; see Methods). The curve represents a first-order decay to 0.5% activity with a rate constant of 0.095/min. Parallel incubations omitted either ferricenium hexafluorophosphate (◆), 3-thia-octanoyl-CoA (■), or both reagents (▲).

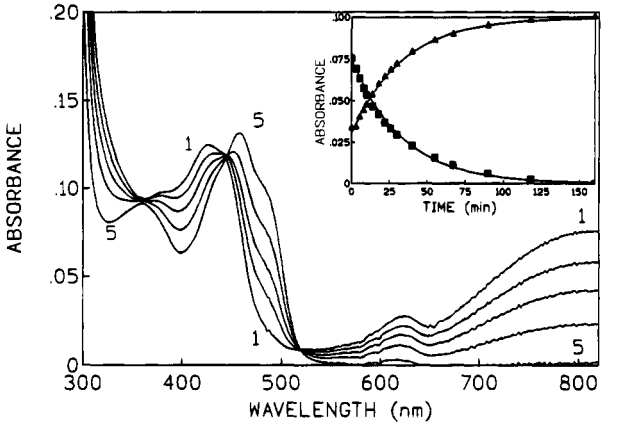


FIGURE 2: Spectral changes during the inactivation of medium-chain acyl-CoA dehydrogenase in the presence of 3-thia-octanoyl-CoA and ferricenium hexafluorophosphate. The oxidized dehydrogenase (8.8 μM) was mixed with 31 μM 3-thia-octanoyl-CoA in 100 mM HEPES buffer, pH 7.6, 25 °C. Spectra 1−5 were recorded 0.1, 8, 18, 40, and 160 min after the addition of 46 μM ferricenium hexafluorophosphate. The inset plots absorbance changes at 480 nm (▲) and 820 nm (■) with first-order rate constants of 0.03 and .031/min, respectively.

of the medium-chain acyl-CoA dehydrogenase requires the simultaneous presence of all three components: enzyme, 3-thia-octanoyl-CoA, and ferricenium ion. Inactivation with the complete system is protected by the inclusion of the redox inactive ligand 2-aza-octanoyl-CoA (Chart 1; Wang & Thorpe, 1991). Thus 72% (compared to only 8%) activity remains after 19 min when 10 μM 2-aza-octanoyl-CoA is included in incubations of 2 μM enzyme with 10 μM 3-thia-octanoyl-CoA and 150 μM ferricenium hexafluorophosphate (not shown).

Spectral Changes during Oxidative Inactivation. The changes in the visible spectrum that accompany inactivation are depicted in Figure 2. Curve 1 shows the intense charge transfer complex with a maximum around 800 nm and the characteristic blue shifted flavin peak induced on the addition of excess 3-thia-octanoyl-CoA (Lau et al., 1988). The long-wavelength band decreases on the addition of the ferricenium ion with a corresponding reappearance of a resolved oxidized

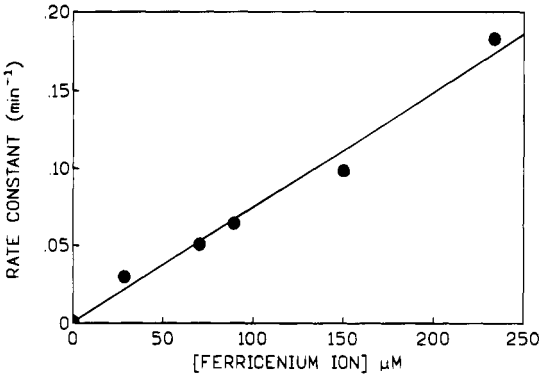


FIGURE 3: Concentration dependence of the apparent rate constant for oxidation of the charge transfer complex by the ferricenium ion. The ferricenium ion dependence (●) was established determining the rate of decrease in 820-nm absorbance using 3 μM enzyme and 30 μM 3-thia-octanoyl-CoA in 100 mM HEPES buffer, pH 7.6 and 25 °C.

flavin envelope (pseudo-first-order rate constants of 0.031 and 0.03 /min respectively; see inset). These spectral changes are accompanied by a decrease in the 618 nm absorbance due to the ferricenium ion (Figure 2). When this species is monitored at 578 nm (to avoid contributions from the absorbance band of the charge transfer complex), the oxidant declined with a rate constant of 0.03/min. The isosbestic points at 446 and 518 nm (Figure 2) and the first-order kinetics are consistent with a transformation of the charge transfer complex into an oxidized enzyme derivative without the accumulation of significant levels of an intermediate species. Repeating the experiment shown in Figure 2 under anaerobic conditions (see Methods) showed no apparent differences in behavior. Thus dissolved oxygen is not involved in the spectral changes encountered here. In addition, illumination of the charge transfer complex with visible light (see Methods) had no significant effect on the kinetics of oxidation of the long wavelength band.

Experiments comparable to that shown in Figure 2, following the decrease of the charge transfer band at a range of ferricenium concentrations, showed that the pseudo-first-order rate constant is linearly dependent on oxidant concentration up to approximately 240 μM (Figure 3). Over a comparable range of ferricenium ion concentrations, the reoxidation of the native dehydrogenase reduced with octanoyl-CoA similarly shows an essentially linear dependence (Lehman & Thorpe, 1990). The y-intercept in Figure 3 reveals insignificant decomposition of the 3-thia-octanoyl-CoA·enzyme complex in the absence of the ferricenium ion (over the time scale of these experiments; Lau et al., 1988). The rate predicted from Figure 3 for a ferricenium concentration of 128 μM (0.096/min) is in excellent agreement with the value of 0.095/min determined from the loss of activity in Figure 1.

In contrast to the strong dependence of the reaction on the ferricenium ion (Figure 3), the rate is essentially independent of the 3-thia-octanoyl-CoA concentration from 5 to 49 μM (.063 ± .001 /min using 85 μM ferricenium ion; not shown). 3-Thia-octanoyl-CoA binds rather tightly to the oxidized medium chain acyl-CoA dehydrogenase (K_d of 0.47 μM; Lau et al., 1988) and thus ligand concentrations of ≥ 5 μM would be close to saturating.

The above data suggest that inactivation of the dehydrogenase by the ferricenium ion proceeds via the enzyme·3-thia-octanoyl-CoA complex. A role of the charge transfer

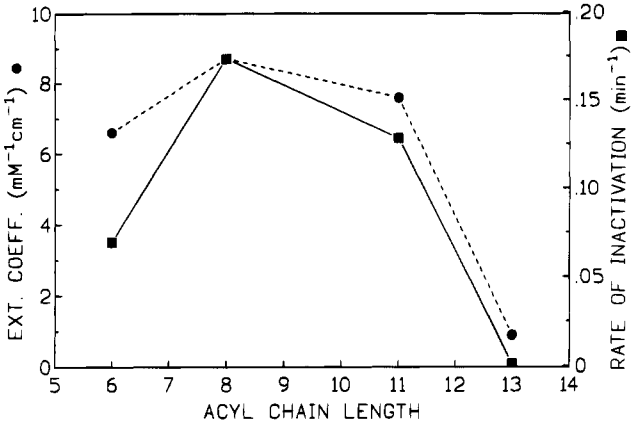


FIGURE 4: Correlation between the rate of inactivation and the intensity of charge transfer complexes observed with 3-thia-acyl-CoA analogues of varying chain length. The rate of inactivation of the medium-chain dehydrogenase was determined using 3 μM enzyme and 200 μM ferricenium hexafluorophosphate in the presence of 300 μM 3-thia-hexanoyl-CoA and 30 μM 3-thia-octanoyl-, undecanoyl-, and tridecanoyl-CoA ligands. Pseudo-first-order rates of inactivation, and the corresponding extinction coefficients for the charge transfer bands (Lau et al., 1988) are compared as a function of chain length.

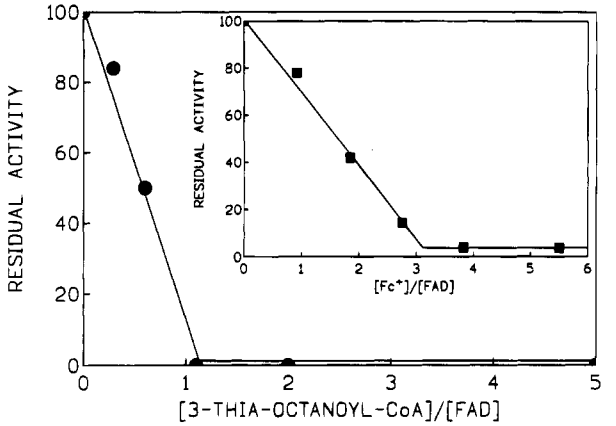


FIGURE 5: Stoichiometry of the inactivation of medium-chain acyl-CoA dehydrogenase by 3-thia-octanoyl-CoA and ferricenium hexafluorophosphate. Main figure: Aliquots of the dehydrogenase (3.5 μM) were incubated with varying concentrations of 3-thia-octanoyl-CoA in the presence of 210 μM ferricenium in 50 mM phosphate buffer, pH 7.6 at 25 °C. Residual activity was assessed after 2 h (corresponding to greater than 10 half-lives) using the PMS/DCIP assay (see Methods). Inset: Samples of 50 μM enzyme were incubated with a 3-fold excess of 3-thia-octanoyl-CoA and varying concentration of ferricenium ion. The higher concentrations used in this experiment allowed the change in activity to be complete in less than 1 h. After this time period, the mixture was diluted to give a concentration of 2 μM enzyme and assayed as usual.

complex per se in inactivation is suggested by the experiments summarized in Figure 4. Lau et al. (1988) showed that the intensity, but not the position of the charge transfer complex between 3-thia-acyl-CoA ligands and the medium-chain dehydrogenase, was markedly chain length dependent (Figure 4; circles). The pseudo-first-order rates of inactivation of the medium-chain dehydrogenase with saturating 3-thia-acyl-CoA ligands follows the same general shape. Both the charge-transfer intensity and the rate of inactivation show an optimum at about 3-thia-octanoyl-CoA (Figure 4).

Stoichiometry of Modification. The dehydrogenase was mixed with 0−5 equivalents of 3-thia-octanoyl-CoA, and inactivations were initiated with 210 μM ferricenium ion. Samples were withdrawn after 2 h and the residual activity was determined (Figure 5; main panel). Complete inactivation of the medium-chain acyl-CoA dehydrogenase requires 1.1 ± 0.1 molecules of 3-thia-octanoyl-CoA per flavin. In contrast, the apparent stoichiometry with respect to the ferricenium ion is 3.1 per flavin (two determinations; Figure 5, inset). Control experiments showed that insignificant amounts of the ferricenium ion were consumed on incubation with the free enzyme or 3-thia-octanoyl-CoA ligand. The stoichiometry was confirmed by using absorbance data to calculate the amount of ferricenium ion consumed upon inactivation of the dehydrogenase (see Methods). Two determinations gave values of 3.2 ± .02 ferricenium ions per flavin.

8-Cl-FAD Substitution Affects the Rate of Inactivation of 3-Thia-octanoyl-CoA Complexes. The medium-chain acyl-CoA dehydrogenase was reconstituted with 8-Cl-FAD (Thorpe & Massey, 1983). The substituted enzyme showed 8% activity in the PMS/DCIP, assay in good agreement with earlier work (Thorpe & Massey, 1983), and 60% of native activity using the ferricenium method (Lehman & Thorpe, 1990). Addition of 3-thia-octanoyl-CoA to the substituted enzyme gave an intense charge transfer band (10 mM^{-1} cm^{-1} at 820 nm; not shown). Inactivation of this complex (formed upon the addition of 30 μM 3-thia-octanoyl-CoA to 3 μM substituted dehydrogenase) with 196 μM ferricenium ion showed a rate constant of .017/min compared to 0.13/min observed with the native enzyme.

Table 1: Effectiveness of Electron Acceptors in the Oxidative Inactivation of the Medium-Chain Acyl-CoA Dehydrogenase•3-Thia-octanoyl-CoA Complex[a]

oxidant	$E^{o'}$ (V)	$t_{1/2}$ (s) for reoxidation of $E{\cdot}Fl_{2e}{\cdot}P$[b]	% activity[c]
$Fc^+PF_6^-$	0.38	0.02	1.2
PMS	0.08	3.0	103
$K_3Fe(CN)_6$	0.42	0.8	107
DCIP	0.22	700	99

[a] Enzyme (2 μM) was incubated with 50 μM 3-thia-octanoyl-CoA and 200 μM oxidant at 25 °C in 100 mM Hepes buffer at pH 7.6. After 2 h, the enzyme was assayed as described in Methods. [b] Values are taken from Lehman and Thorpe (1990). [c] Activity remaining compared to the control with no oxidant present.

The Effect of Other Oxidants. A number of other artificial electron acceptors were tried as alternate oxidants for the inactivation of the 3-thia-octanoyl-CoA•enzyme complex (Table 1). The half-times for the reoxidation of octanoyl-CoA-reduced dehydrogenase by these oxidants is shown for comparison (Lehman & Thorpe, 1990). The ferricenium ion is a kinetically superior oxidant by some 40-fold, and is the only reagent to inactivate the 3-thia-octanoyl-CoA complexes of the medium chain dehydrogenase at a significant rate (Table 1).

Inactivation of the Acyl-CoA Dehydrogenase by 3-Oxa-octanoyl-CoA. 3-Oxa-octanoyl-CoA generates a somewhat less intense charge transfer band than that produced with the 3-thia-analogue when added to the medium chain acyl-CoA dehydrogenase (Lau et al., 1988). This charge transfer complex was also inactivated by the ferricenium ion with a 30% smaller rate constant than that observed with the 3-thia-analogue (0.066/min using 1 μM enzyme, 128 μM ferricenium and 150 μM 3-oxa-octanoyl-CoA in 50 mM phosphate buffer, pH 7.6, 25 °C; not shown). However, unlike 3-thia-octanoyl-CoA, the 3-oxa-octanoyl-CoA complexes are relatively unstable, even in the absence of oxidant (Lau et al., 1988). For this reason, and because 3-thia-octanoyl-CoA could be conveniently prepared in a radioactive form (see

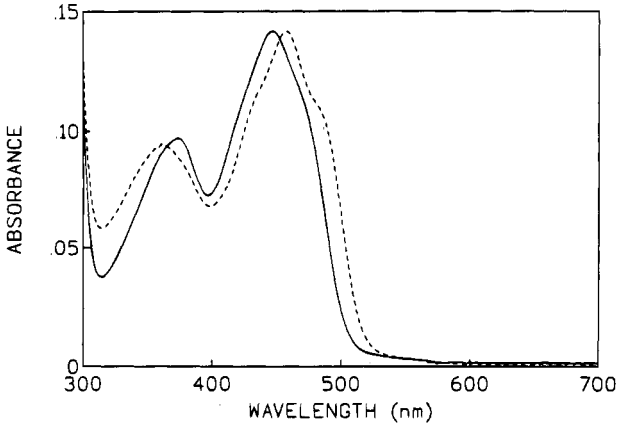


FIGURE 6: Comparison of the visible spectra of native and modified enzyme. Spectra of native and oxidatively modified medium-chain acyl-CoA dehydrogenase (both 9 μM; solid and dashed lines, respectively) are shown in 50 mM phosphate buffer, pH 7.6 and 25 °C. The extinction coefficient of the flavin bound to the modified enzyme (15.4 mM^{-1} cm^{-1}) was determined by the guanidine hydrochloride method (see Methods).

later) further characterization was restricted to the 3-thia-derivatives.

Properties of the Oxidatively Modified Dehydrogenase. The medium-chain enzyme was inactivated on a larger scale (see Methods) and repeatedly washed by ultrafiltration to remove excess 3-thia-octanoyl-CoA, ferricenium ion, and other products of inactivation (see later). No FAD was detected in these filtrates (not shown), showing that the prosthetic group remains firmly bound to the modified dehydrogenase. Figure 6 shows that the spectrum of the treated enzyme is clearly different from the native dehydrogenase with a striking 12-nm red shift of the 446-nm peak but a 10-nm blue shift of the 374-nm peak. In addition, the flavin absorption envelope of the modified enzyme shows distinctly higher resolution in its main absorbance peak. Such red shifts are typical of the acyl-CoA dehydrogenase when complexed with a variety of ligands that do not participate in charge transfer complex formation (Powell et al., 1987). In contrast, the UV spectra of the native and modified enzyme are not significantly different. In particular, no significant increase in absorbance at 260 nm is observed with the modified enzyme, suggesting that release of coenzyme A from the modified enzyme has occurred (not shown; see later).

The flavin released upon boiling the modified enzyme coeluted with an authentic sample of FAD on HPLC (not shown) suggesting that inactivation does not involve covalent modification of the flavin chromophore. Release of FAD also occurred on treatment of the modified enzyme with guanidine HCl, but at a rate much slower than that observed with the native enzyme ($t_{1/2}$ = 24 h, vs. 9 min in 3.8 M guanidine HCl, pH 7.6, 25 °C; not shown).

The modified enzyme (Figure 6) shows approximately 0.2% activity of the native enzyme (see Methods) and no activity is regained following prolonged ultrafiltration or storage at −20 °C (not shown). Unlike the native enzyme, which is rapidly reduced upon the addition of 1 equivalent of octanoyl-CoA (Hall et al., 1979; Thorpe et al., 1979), no significant spectral changes are observed over 90 min after the addition of 18 equivalents of this tightly binding substrate to the modified enzyme. As would be expected for an active site−directed modification reaction, labeling the enzyme with some fragment of 3-thia-octanoyl-CoA prevents the binding of other acyl-CoA derivatives.

Since the modification reaction clearly inhibits the reductive half-reaction of the medium-chain dehydrogenase, it was of interest to examine the competence of the second half of the catalytic cycle, in which electrons are passed from the reduced dehydrogenase to suitable oxidants (Beinert, 1963; Thorpe, 1991). Since the treated enzyme cannot be reduced with acyl-CoA substrate, nonenzymatic methods were explored. The native enzyme is efficiently photoreduced under anaerobic conditions in the presence of EDTA and catalytic levels of 5-deazaflavin (Massey & Hemmerich, 1978; Thorpe et al., 1979). However photoreduction is very slow in the absence of photocatalyst (3% reduction is observed after 40 min of illumination using 10 mM EDTA alone; see Methods). In marked contrast, the modified enzyme (11 μM) undergoes ready photoreduction in the absence of deazaflavin with the formation of a reduced flavin species (apparent extinction coefficient of 5.4 mM^{-1} cm^{-1} at 432 nm), a conspicuous shoulder at 320 nm, and an isosbestic point at 336 nm (not shown). This difference is not simply due to the presence of a bound 3-thia-octanoyl-CoA ligand. Thus an extremely slow rate of photoreduction of the native enzyme was observed in the presence of 10 μM enzyme and 19 μM 3-thia-octanoyl-CoA and 10 mM EDTA (not shown).

When the reduced modified enzyme is exposed to an oxygen atmosphere approximately 17% increase in absorbance at 432 nm occurs with a slight shift in the peak to 440 nm. This increase is consistent with the appearance of a small proportion of oxidized flavin. The remaining reduced material, however, shows essentially no further absorbance increase after overnight storage at −20 °C followed by ultrafiltration and washing with aerobic phosphate buffer. In fact, the spectral changes seen could also be generated by illumination in air-saturated buffer (not shown). In addition to its refractility toward molecular oxygen, the reduced modified enzyme was resistant to oxidation by the ferricenium ion (5 μM enzyme and 22 μM oxidant gave no significant reoxidation of the enzyme over 15 min at 25 °C). Reoxidation of the native reduced enzyme under these conditions is facile (Lehman & Thorpe, 1990).

Attempts to generate reduced modified dehydrogenase by dithionite titration were prevented by extreme kinetic sluggishness. Thus incubation of the modified enzyme (8 μM in 50 mM phosphate buffer, pH 7.6) by an 11-fold excess of dithionite produced only a 9% decrease in oxidized flavin absorbance at 450 nm over 24 h at 25 °C. The experiment was repeated including 1 μM methylviologen as a mediator and gave the same spectral changes as photoreduction (not shown). Thus both chemical and photochemical reduction of the modified enzyme yields an unusual reduced flavin species that resists reoxidation. As would be expected from the above, the reduced modified enzyme remains catalytically inactive in the ferricenium assay system (not shown).

Denaturation of the photoreduced enzyme on boiling liberated 98% of expected amount of free oxidized FAD. In contrast, treatment with 5% TCA released only about 18% of the expected oxidized flavin absorbance. The corresponding TCA precipitate was redissolved in 6 M GuHCl / 50 mM phosphate buffer and showed a new peak at 370 nm with an intensity of 85% of the original 432 nm absorbance of the reduced enzyme prior to denaturation. A variable disposition of flavin products between supernatant and protein-bound fractions depending on conditions is also encountered during the denaturation of a reduced flavin-2-octynoyl-CoA adduct of the medium-chain dehy-

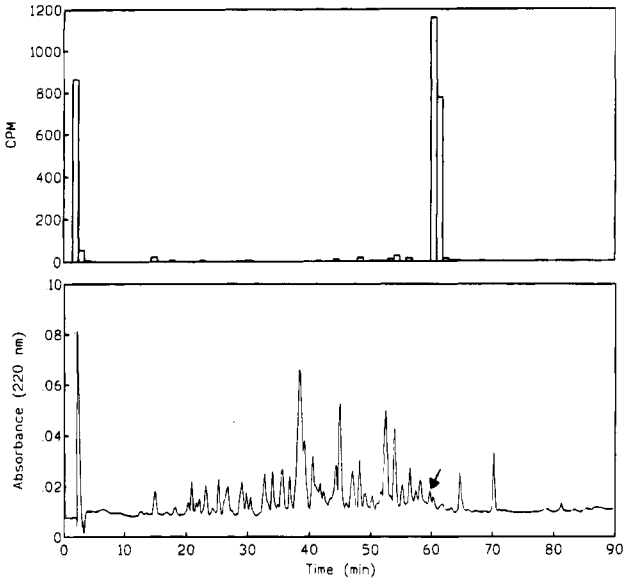


FIGURE 7: Peptide mapping of tryptic digest of modified medium-chain acyl-CoA dehydrogenase. The radiolabeled dehydrogenase was denatured by boiling and digested with DPCC-treated trypsin (see Methods). A sample (containing 1.09 nmol label; 3280 cpm) was applied to a octadecylsilica reverse-phase column, the elution was monitored at 220 nm, and 1-mL fractions were collected for radioactive counting. The total radioactivity recovered from the column was 91%.

drogenase (Zhou & Thorpe, 1989).

Coenzyme A Is Released on Oxidative Inactivation. The dehydrogenase was inactivated to less than 1% residual activity as in Figure 2 but including *N*-butyl maleimide to trap any CoASH released. The solution was ultrafiltered and the supernatant analyzed by HPLC (see Methods). A new peak was obtained which coeluted with the *N*-butyl-maleimide-SCoA adduct (see Methods; not shown). The kinetics of CoASH release were determined spectrophotometrically. 4,4′-Dithiodipyridine (142 μM) was included in the inactivation mixture (6.5 μM enzyme, 13 μM thioester, and 125 μM ferricenium ion in 50 mM phosphate buffer, pH 7.6), and the release of thiol was followed at 324 nm (Grassetti & Murray, 1967). Control experiments recorded the absorbance changes in the absence of the thiol-trapping reagent and in the absence of the ferricenium ion (see Methods). After correction for these absorbance changes, 0.95 equivalents of thiol were released in a process that was half complete in 13 min.

Isolation of Oxidatively Labeled Peptide. Since CoA was released during inactivation, a radiolabel was needed on the acyl-moiety of 3-thia-octanoyl-CoA. [2-^{14}C]-3-Thia-octanoyl-CoA was synthesized and used to show that 1.08 radiolabels were incorporated upon oxidative inactivation of the enzyme (see Methods), in agreement with the stoichiometry determined in Figure 6. Samples of the treated enzyme were denatured by boiling and redissolved in solutions of guanidine hydrochloride adjusted to pH values from 2 to 10 (see Methods). After 22 h of incubation at 25 °C, the percentage of radioactivity released from the protein was determined by ultrafiltration to be 60, 14, 28, 91, and 95% at pH values of 2, 4, 6, 8, and 10, respectively. Thus release of radiolabel was most pronounced at both low and high pH values. Trial pepsin digests of the modified enzyme generated multiple radioactive peaks (see Methods), and thus peptide maps were run using the modified enzyme subjected to a 2-h digestion with trypsin at pH 7.0, 37 °C. Figure 7 shows that 29% of the radiolabel was released in the void volume of the HPLC column with 65% of the total radioactivity recovered in a prominent peak at 61 min. The separation was repeated on a larger scale, and the 61-min peak collected manually for gas-phase sequencing (see Methods). Table 2 shows the sequence obtained in comparison with the corresponding human and pig liver medium chain acyl-CoA dehydrogenase peptide. The radioactivity emerging on each cycle of sequencing is shown in Figure 8. In view of the instability of the label established at pH 2 and 10 (see earlier), the progressive loss of radioactivity from the peptide undergoing sequencing is not unexpected. Thus at each sequencing cycle the peptide is exposed to trimethylamine and trifluoroacetic acid vapors and would be expected to release radioactivity with each cycle. However, there is a clear increase in radioactivity at cycle 6. These data strongly suggest that GLU376 is the target of oxidative inactivation of the medium-chain acyl-CoA dehydrogenase.

Table 2: Sequence Comparison of Human and Pig Liver Medium-Chain Acyl CoA Dehydrogenase with the Isolated Peptide

PLMCAD[a]	Ile-Tyr-Gln-Ile-Tyr-Glu-Gly-Thr-Ala-Gln-Ile-Gln-Arg
HLMCAD[b]	Ile-Tyr-Gln-Ile-Tyr-Glu-Gly-Thr-Ser-Gln-Ile-Gln-Arg
peptide	Ile-Tyr-Gln-Ile-Tyr-**Glu**-Gly-Thr-Ala-Gln-Ile-Gln-Arg

[a] Unpublished sequence available in the file 3mdd from the Brookhaven Protein Database. [b] Kelly et al. (1987).

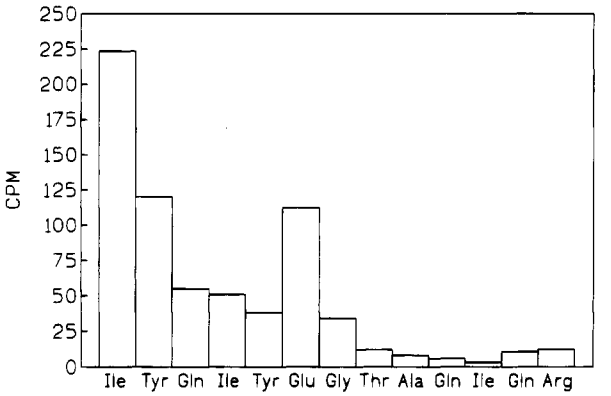


FIGURE 8: Radioactivity released during Edman sequencing. A sample of the radiolabeled peptide (996 cpm) was sequenced as described in Methods, and a fraction of the PTH-amino acids retained for radioactive counting. A total of 650 cmp were released in 13 sequencing cycles and 250 cpm were retained on the filter.

The derivatized peptide isolated as in Figure 7 was analyzed by electrospray ionization mass spectrometry (see Methods). The mass obtained (1742.8) is consistent with that for the sequence shown in Table 2 with an attached 3-thia-octanoic acid moiety (expected mass of the conjugate, 1743.0; see Discussion).

DISCUSSION

Charge Transfer Is a Necessary But Insufficient Condition for Inactivation by the Ferricenium Ion. Figure 4 shows a strong correlation between the rate of inactivation and the intensity of the charge transfer band for a series of 3-thia-acyl-CoA·dehydrogenase complexes. Inactivation is most rapid with the C-8 analogue which forms an intense, long-wavelength band with the medium-chain enzyme. Consistent with a role of a charge transfer complex in enzyme inactivation, the 2-aza-octanoyl-CoA·dehydrogenase complex, which yields no long-wavelength band, is not significantly inactivated by the ferricenium ion (Table 3). How-

Table 3: Incubation of Dehydrogenase·CoA Thioester Ligand Complexes with the Ferricenium Ion

ligand[a]	CT complex[b]	% activity[c]
2-aza-octanoyl-CoA	no	103
3-keto-octanoyl-CoA	yes (560)	114
trans-3-octenoyl-CoA	yes (820)	83[d]
3-oxa-octanoyl-CoA	yes (780)	12.2
3-thia-octanoyl-CoA	yes (800)	2.5

[a] Enzyme (1 μM) was incubated with 30 μM ligand and 200 μM $Fc^+PF_6^-$ at 25 °C. After 70 min, the enzyme was assayed as described in Methods. [b] Charge transfer complex. [c] Compared to control with ligand but no ferricenium added. [d] No further change in activity was observed over 7 h.

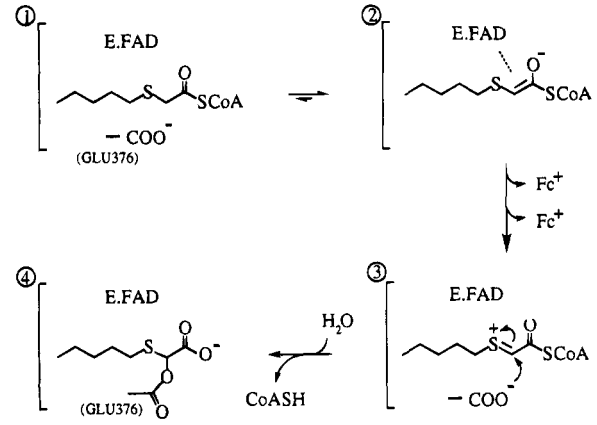
ever, charge transfer complexation is not a sufficient condition for oxidative inactivation. Thus *trans*-3-octenoyl-CoA forms very similar charge transfer bands to 3-thia-octanoyl-CoA (7100 mM^{-1} cm^{-1} at 820 nm vs. 8700 mM^{-1} cm^{-1} at 804 nm, respectively; Powell et al., 1987; Lau et al., 1988), and yet this complex is not significantly inactivated by the ferricenium ion (Table 3). In fact, conversion of *trans*-3-octenoyl-CoA to 2,4-octadienoyl-CoA apparently occurs (Schaller and Thorpe, unpublished observations). The apparent 17% decline in activity is probably associated with the transfer of this 2,4-diene into the assay cuvette along with the enzyme. No further decrease in activity occurs over a total of 7 h (Table 3).

The enolate to flavin charge transfer complex observed with 3-keto-octanoyl-CoA (λ_{max} 560 nm; Powell et al., 1987) is also not subject to oxidative inactivation (Table 3). Thus inhibition is not just a general consequence of ligand binding but appears to be an attribute of the interaction between certain charge transfer complexes and the ferricenium ion.

Oxidation Involves the Flavin Prosthetic Group. The inactivation of the medium-chain acyl-CoA dehydrogenase described above shows similarities to the oxidative interception of catalytic intermediates of nonredox enzymes termed "paracatalytic" by Christen and co-workers (Christen, 1977; Lubini & Christen, 1979). Rabbit muscle aldolase, for example, normally catalyzes the conversion of fructose 1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. In the presence of 0.5 mM ferricyanide, approximately 1 turnover in 25 generates hydroxypyruvaldehyde phosphate instead of dihydroxyacetone phosphate. This oxidation consumes 2 ferricyanide ions per hydroxypyruvaldehyde formed. Under these conditions, aldolase is also slowly inactivated with the incorporation of a triose phosphate unit and the formation of an intrachain crosslink between two active site LYS residues (Lubini & Christen, 1979; Gupta et al., 1993). Analogous paracatalytic modification of transaldolase, transketolase, and pyruvate decarboxylase have been described (Christen et al., 1976; Cogoli-Greuter et al., 1976). In addition, ribulose bisphosphate 1,5-carboxylase catalyzes the substrate-dependent reduction of tetranitromethane (Bhagwat & McFadden, 1982). Finally, glyoxylase can transfer reducing equivalents to an exogenous flavin analogue (Ueda et al., 1984).

Consideration of the crystal structure of the medium-chain acyl-CoA dehydrogenase (Kim et al., 1993) suggests that direct contact between the ferricenium ion and the bound enolate is highly improbable. The active site is extensively desolvated on binding acyl-CoA ligands (Kim et al., 1993; Kim & Thorpe, 1995) and there is no room within these complexes for a bulky (ca. 5 Å diameter) exogenous reagent.

Scheme 3



The drastic slowing of the rate of dithionite reduction on modification of GLU376 in this work and with 2-octynoyl-CoA inactivated enzyme (Freund et al., 1985) is also consistent with decreased accessibility to external reagents when the active site is occupied. Although access to the flavin via the thioester substrate site appears effectively blocked by derivatization of GLU376, the ferricenium ion could approach the si-face of the cofactor via a depression on the surface of the dehydrogenase in the vicinity of TRP166 (Kim et al., 1993). This general locus has been proposed as a possible site for interflavin electron transfer between acyl-CoA dehydrogenase and the electron-transfer flavoprotein (Kim et al., 1993; Thorpe & Kim, 1995). Direct involvement of flavin in the oxidative inactivation of the dehydrogenase by the ferricenium ion is suggested by the 7-fold slowing of the rate of inactivation of the 3-thia-acyl-CoA complex when 8-Cl-FAD substituted enzyme was used (see above). 8-Cl-FAD substitution generates a thermodynamically weaker reductant for exogenous reagents, and this analogue has proved very useful for modulation of the internal equilibrium in the medium-chain acyl-CoA dehydrogenase (Thorpe & Massey, 1983). Further evidence consistent with a role for the flavin in oxidative inactivation is that only the most facile electron acceptor for the octanoyl-CoA reduced native enzyme is effective as an oxidant in the inactivation reaction described here (Table 1).

Possible Mechanism for Oxidative Inactivation. Any working hypothesis to account for the inactivation described in this paper must account for the following observations. A heteroatom (either S or O, see above) is required at the 3-position of acyl-CoA ligands; *trans*-3-octenoyl-CoA is ineffective. Inactivation occurs via an enolate to flavin charge transfer complex, and the ferricenium oxidant appears to communicate directly with the flavin as it does during conventional turnover (Lehman & Thorpe, 1990). CoA is released as the thiol during formation of the modified enzyme; CoA disulfide is not a primary product of inactivation. The inactivation is light and oxygen independent.

Our working model for inactivation is shown in Scheme 3. While it satisfactorily accounts for these observations the finding that 3, not 2 equivalents, of ferricenium are consumed during inactivation is not yet understood. Proton abstraction by GLU376 generates the enolate to flavin charge transfer complex (2, Scheme 3). The crystal structure of the medium-chain acyl-CoA dehydrogenase indicates that such enolates would be coplanar and within Van der Waals contact of the flavin, facilitating the development of a hybrid orbital between them. This supramolecular orbital will increase

electron density on the electron-deficient flavin acceptor even in the ground state and allow oxidation to the sulfonium species (3, Scheme 3). The first-order dependence in ferricenium (Figure 3), and the failure to accumulate radical intermediates (Figure 2) suggest that the first electron transfer step is rate limiting.

Studies on the mechanism of inactivation of soybean lipoxygenase by 7-thiaarachidonic acid provide a precedent for Scheme 3 (Corey et al., 1985). Ferric ion−catalyzed oxidation of the vinyl sulfide moiety in this inhibitor is believed to generate a sulfonium ion, which is subsequently attacked by a protein nucleophile. Our proposal is clearly similar. Two-electron oxidation of an enolate generates a sulfonium species that must be efficiently intercepted by the adjacent GLU376 since only one molecule of 3-thia-octanoyl-CoA is required for inactivation (Figure 5). The proposed structure for the resulting adduct (4, Scheme 3) is consistent both with the mass spectrum of the derivatized peptide and with the acid and base lability of the linkage (see Results). Release of CoASH may simply reflect hydrolytic cleavage of the thioester in the inactivated enzyme. CoASH release is also observed on treatment of the acyl-CoA dehydrogenase with the 2-octynoyl-CoA (Freund et al., 1985).

In addition to this work, GLU376 has been identified as a target of the mechanism-based inhibitor 2-octynoyl-CoA and the affinity label and alternate substrate 2-bromohexanoyl-CoA (Powell et al., 1988; Haeffner-Gormley et al., 1995). Proximity and orientation presumably preclude labeling of other potential amino acid side chains by these site-directed inactivators of the medium-chain dehydrogenase.

In conclusion, we have identified a new mode of inactivation of the medium-chain acyl-CoA dehydrogenase. It will be interesting to see whether this sort of oxidative interception of charge transfer complexes is found in other flavoproteins.

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REFERENCES

Beinert, H. (1963) *The Enzymes, 2nd Ed. 7*, 447−466.
Bernert, J. T., & Sprecher, H. (1977) *J. Biol. Chem. 252*, 6736−6744.
Biellman, J. F., & Hirth, C. G. (1970) *FEBS Lett. 9*, 335−336.
Bhagwat, A. S., & McFadden, B. A. (1982) *FEBS Lett. 145*, 313−316.
Brocklehurst, K., & Little G. (1973) *Biochem. J. 133*, 67−80.
Bross, P., Engst, S., Strauss, A. W., Kelly, D. P., Rasched, I., & Ghisla, S. (1990) *J. Biol. Chem. 265*, 7116−7119.
Christen, P. (1977) *Methods Enzymol. 46*, 48−54.
Christen, P., Cogoli-Greuter, M., Healy, M. J., & Lubini, D. (1976) *Eur. J. Biochem. 63*, 223−231.
Cogoli-Greuter, M., Hausner, U., & Christen, P. (1976) *Experientia 32*, 766.
Corey, E. J., Cashman, J. R., Eckrich, T. M., & Corey, D. R. (1985) *J. Am. Chem. Soc. 107*, 713−715.
Cummings, J. G., & Thorpe, C. (1994) *Biochemistry 33*, 788−797.
DeBuysere, M. S., & Olson, M. S. (1983) *Anal. Biochem. 133*, 373−379.
Engel, P. C. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. III, pp 597−655, CRC Press, Boca Raton.
Frerman, F. E., Miziorko, H. M., & Beckmann, J. D. (1980) *J. Biol. Chem. 255*, 11192−11198.
Freund, K., Mizzer, J. P., Dick, W., & Thorpe, C. (1985) *Biochemistry 24*, 5996−6002.
Ghisla, S., Thorpe, C., & Massey, V. (1984) *Biochemistry 23*, 3154−3160.
Ghisla, S., Engst, S., Vock, P., Kieweg, V., Bross, P., Nandy, A., Rasched, I., & Strauss, A. W. (1994) in *Flavins and Flavoproteins* (Yagi, K., Ed) pp 283−292, Walter de Gruyter, New York.
Gorelick, R. J., & Thorpe, C. (1986) *Biochemistry 25*, 7092−7098.
Gorelick, R. J., Mizzer, J. P., & Thorpe, C. (1982) *Biochemistry 21*, 6936−6942.
Gorelick, R. J., Schopfer, L., Ballou, D. P., Massey, V., & Thorpe, C. (1985) *Biochemistry 24*, 6830−6839.
Grassetti, D. R., & Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 41−49.
Gupta, S., Hollenstein, R., Kochhar, S., & Christen, P. (1993) *Eur. J. Biochem. 214*, 515−519.
Haeffner-Gormley, L., Cummings, J. G., & Thorpe, C. (1995) *Arch. Biochem. Biophys. 317*, 479−486.
Hall, C. L., & Lambeth, J. D. (1980) *J. Biol. Chem. 255*, 3591−3595.
Hall, C. L., Lambeth, J. D., & Kamin, H. (1979) *J. Biol. Chem. 254*, 2023−2031.
Kelly, D. P., Kim, J.−J. P., Billadello, J. J., Hainline, B. H., Chu, T. W., & Strauss, A. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4068−4072.
Kim, J. J. P. (1991) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 291−298, Walter de Gruyter, New York.
Kim, J. J. P., Wang, M., & Paschke, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7523−7527.
Lau, S.-M., & Thorpe, C. (1988) *Arch. Biochem. Biophys. 262*, 293−297.
Lau, S.-M., Powell, P. J., Buettner, H., Ghisla, S., & Thorpe, C. (1986) *Biochemistry 25*, 4184−4189.
Lau, S.-M., Brantley, R. K., & Thorpe, C. (1988) *Biochemistry 27*, 5089−5095.
Lehman, T. C., & Thorpe, C. (1990) *Biochemistry 29*, 10594−10602.
Lehman, T. C., Hale, D. E., Bhala, A., & Thorpe, C. (1990) *Anal. Biochem. 186*, 280−284.
Lubini, D. G. E., & Christen, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2527−2531.
Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446−465.
Massey, V., & Hemmerich, P. (1978) *Biochemistry 17*, 9−17.
Murfin, W. W. (1974) Ph.D. Thesis, Washington University, St. Louis, MO.
Pohl, B., Raichle, T., & Ghisla, S. (1986) *Eur. J. Biochem. 160*, 109−115.
Powell, P. J., & Thorpe, C. (1988) *Biochemistry 27*, 8022−8028.
Powell, P. J., Lau, S.-M., Killian, D., & Thorpe, C. (1987) *Biochemistry 26*, 3704−3710.
Reinsch, J., Katz, A., Wean, J., Aprahamian, G., & McFarland, J. T. (1980) *J. Biol. Chem. 255*, 9093−9097.
Schopfer, L., Massey, V., Ghisla, S., & Thorpe, C. (1988) *Biochemistry 27*, 6599−6611.
Spencer, R., Fisher, J., & Walsh, C. (1976) *Biochemistry 15*, 1043−1053.
Stadtman, E. R. (1957) *Methods Enzymol 3*, 931−941.
Thorpe, C. (1986) *Anal. Biochem. 155*, 391−394.
Thorpe, C. (1991) in *Chemistry & Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. II, pp 471−486, CRC Press, Boca Raton, FL.
Thorpe, C., & Kim, J. J. P. (1995) *FASEB J. 724*, 718−725.
Thorpe, C., & Massey, V. (1983) *Biochemistry 22*, 2972−2978.
Thorpe, C., Matthews, R. G., & Williams, C. H. (1979) *Biochemistry 18*, 331−337.
Trievel, R. C., Wang, R., Anderson, V. E., & Thorpe, C. (1995) *Biochemistry 34*, 8597−8605.
Ueda, K., Shinkai, S., & Douglas, K. T. (1984) *J. Chem. Soc., Chem. Commun.* 371−372.
Wang, R., & Thorpe, C. (1991) *Biochemistry 30*, 7895−7901
Zhou, J.-Z., & Thorpe, C. (1989) *Arch. Biochem. Biophys. 217*, 261−269.

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