Reductive Half-Reaction in Medium-Chain Acyl-CoA Dehydrogenase: Modulation of Internal Equilibrium by Carboxymethylation of a Specific Methionine Residue†

John G. Cummings, Sze-Mei Lau, Patricia J. Powell, and Colin Thorpe*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Received November 25, 1991; Revised Manuscript Received June 19, 1992

ABSTRACT: Pig kidney medium-chain acyl-CoA dehydrogenase is specifically alkylated at a methionine residue by treatment with iodoacetate at pH 6.6. This residue corresponds to Met249 in the human medium-chain acyl-CoA dehydrogenase sequence [Kelly, D. P., Kim, J. J., Billadello, J. J., Hainline, B. E., Chu, T. W., & Strauss, A. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4068–4072]. The S-carboxymethylated dehydrogenase shows a drastically lowered affinity for octanoyl-CoA (from submicromolar to 65 μM), but retains about 23% of the maximal activity of the native enzyme. In addition, alkylation perturbs the internal redox equilibrium:

$$E{\cdot}FAD_{ox}{\cdot}\text{octanoyl-CoA} \overset{K_2}{\rightleftharpoons} E{\cdot}FAD_{2e}{\cdot}\text{octenoyl-CoA}$$

K_2 ranges from about 9 for the native enzyme to about 0.2 for the homogeneously modified protein. This effect is not due to a significant change in the redox potential of the free enzyme upon alkylation. Rather, carboxymethylation weakens the preferential binding of enoyl-CoA product to the reduced enzyme (K_3) compared to octanoyl-CoA binding to the oxidized dehydrogenase (K_1) that is required to pull the substrate thermodynamically uphill. Thus, the ratio of dissociation constants, K_1/K_3, decreases from about 15 000 for the native enzyme to only 330 upon carboxymethylation of Met249. Binding studies with a variety of acyl-CoA analogues and manipulation of enzyme redox potentials by substitution of the natural prosthetic group by 8-Cl-FAD confirm the thermodynamic effects of alkylation.

The reductive half-reaction in the medium-chain acyl-CoA dehydrogenases is initiated by formation of a Michaelis complex between the enzyme and an acyl-CoA thioester such as octanoyl-CoA (K_1 in Scheme I; Beinert & Page, 1957; Thorpe et al., 1979). Reduction of the enzyme-bound flavin prosthetic group in step K_2 entails transfer of a hydride equivalent from the *pro-R* C-3 position of octanoyl-CoA to the N-5 position of the isoalloxazine ring (Ghisla et al., 1984; Pohl et al., 1986). The resulting reduced enzyme species, $EFAD_{2e}{\cdot}P$, owes its purple color to a charge-transfer interaction between reduced flavin and tightly bound *trans*-2-octenoyl-CoA (Massey & Ghisla, 1974; Murfin, 1974; Reinsch et al., 1980; Thorpe & Massey, 1983; Lau & Thorpe, 1988).

Scheme I

$$EFAD_{ox} + SH_2 \overset{K_1}{\rightleftharpoons} EFAD_{ox}{\cdot}SH_2 \overset{K_2}{\rightleftharpoons} EFAD_{2e}{\cdot}P$$

The internal redox equilibrium, K_2, is chain length dependent. Good substrates, such as octanoyl-CoA, effect almost complete reduction of the enzyme flavin, yielding predominantly $EFAD_{2e}{\cdot}P$ (Scheme I; Beinert, 1963; Hall et al., 1979; Thorpe et al., 1979; Ikeda et al., 1985; Lau & Thorpe, 1988). In contrast, K_2 with poor substrates (such as butyryl-CoA and palmitoyl-CoA) lies toward the left, favoring the oxidized flavin component ($EFAD_{ox}{\cdot}SH_2$). Thus, this internal equilibrium step appears to reflect the chain length specificity shown by the enzyme in turnover (Hall et al., 1979; Ikeda et al., 1985). One way to artificially modulate K_2 is by substitution of the normal FAD prosthetic group in the medium-chain enzyme by analogues of differing redox potentials (Thorpe & Massey, 1983). For example, replacement with the more oxidizing 8-Cl-FAD provides sufficient thermodynamic leverage (see later) to allow even poor acyl-CoA substrates to reduce the enzyme essentially completely (Thorpe & Massey, 1983). This paper shows that S-carboxymethylation of a single methionine residue provides a second way to perturb the internal equilibrium between bound substrate and product.

EXPERIMENTAL PROCEDURES

Materials. Coenzyme A (lithium salt) and butyryl-, octanoyl-, and palmitoyl-CoA were from Pharmacia. The following acyl-CoA analogues were synthesized as indicated: octyl-SCoA, *trans*-2-octenoyl-CoA, and coenzyme A sulfonate (Powell et al., 1987); 3-thiaoctyl-SCoA and 3-thiaoctanoyl-CoA (Lau et al., 1988); and 2-azaoctanoyl-CoA (Wang & Thorpe, 1991). 8-Chlororiboflavin was a generous gift of Dr. John Lambooy, and was converted to the FAD level as described earlier (Spencer et al., 1976). Iodoacetic acid was obtained from Sigma, and the 2-^{14}C-labeled material was from Amersham. Pig kidney medium-chain acyl-CoA dehydrogenase was purified as described earlier (Lau et al., 1986).

General Methods. Unless otherwise stated, all buffers contained 0.3 mM EDTA. Concentrations of native, carboxymethylated, and 8-Cl-FAD-substituted acyl-CoA dehydrogenase were determined spectrophotometrically using the following molar extinction coefficients: 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979); 15.3 mM^{-1} cm^{-1} at 445 nm (Mizzer & Thorpe, 1980); and 14.0 mM^{-1} cm^{-1} at 440 nm (Thorpe & Massey, 1983), respectively. Anaerobic titrations were performed as in Gorelick et al. (1982). Binding and kinetic parameters were determined from spectrophoto-

† This work was supported by a grant from the U.S. Public Health Service (GM 26643).

* Correspondence should be addressed to this author. Telephone: 302-831-2689. Fax: 302-831-6335.

0006-2960/92/0431-8523$03.00/0

metric titrations using a nonlinear regression analysis program (F-Curve III) written by Dr. J. Noggle of this department or via Enzfitter, Elsevier Biosoft. Values of association–dissociation equilibria are given throughout as dissociation constants. In the case of linked equilibria involving an initial binding step (K_1) followed by K_2 (see Discussion), the apparent dissociation constant is given by

$$K_{app} = K_1/(1 + K_2)$$

Rapid reactions were followed with a Kinetic Instruments stopped-flow spectrophotometer with peripherals and software from On Line Instruments Systems Inc. Thioesters were prepared from the corresponding free acids by the mixed anhydride procedure (Bernert & Sprecher, 1977), and purified and characterized as in Powell et al. (1987).

Preparation of Carboxymethylated Acyl-CoA Dehydrogenase. Carboxymethylation with 30 mM iodoacetic acid was performed as described previously (Mizzer & Thorpe, 1980), except excess reagents were removed by centrifuge ultrafiltration (Centricon PM10 microconcentrators), washing with 100 mM phosphate buffers, pH 6.6. The modified acyl-CoA dehydrogenase exhibited 5–6% of the activity shown by the native enzyme in the phenazine methosulfate/2,6-dichlorophenolindophenol assay (Thorpe, 1981) and in the ferricenium assay (Lehman et al., 1990) using 30 μM octanoyl-CoA. K_m determination for octanoyl-CoA using the alkylated enzyme employed the ferricenium assay method.

Two methods were used to prepare carboxymethylated 8-Cl-FAD-substituted enzyme, and both gave comparable behavior toward acyl-CoA substrates. In the first, FAD was removed from the iodoacetate-treated enzyme (60 nmol) using a modification of the acid/ammonium sulfate procedure (Mayer & Thorpe, 1981) and reconstituted at 4 °C with a 1.2-fold excess of 8-Cl-FAD over 2 h as described previously (Mayer & Thorpe, 1981; Thorpe & Massey, 1983). In the second method, native enzyme was reconstituted with 8-Cl-FAD (Thorpe & Massey, 1983) and alkylated with 30 mM iodoacetate as described above.

Structural Characterization of Carboxymethylated Enzyme. Medium-chain acyl-CoA dehydrogenase (195 nmol) was alkylated for 10 h with 30 mM iodo[2-^{14}C]acetic acid (at a specific activity of about 2500 dpm/nmol) in 0.5 mL of 100 mM phosphate buffer adjusted to pH 6.6. Excess reagent was removed by repeated washings with pH 6.6 buffer using centrifuge ultrafiltration. The modified enzyme (6.9×10^5 dpm) was treated with 6 M guanidine hydrochloride (1.0 mL, pH 6, 25 °C) containing a 3-fold molar excess of *N*-ethylmaleimide over total thiols (Thorpe et al., 1979). After 1 h, the mixture was dialyzed for 12 h versus 4 L of water and then for 4 h versus 0.1 M NH_4HCO_3, pH 7.9.

The denatured alkylated protein was digested by the addition of 2% w/w DPCC-treated trypsin at 0 and 2 h, and the mixture (containing a total of 5.6×10^5 dpm) was ultrafiltered after 4 h to remove trypsin and undigested or core protein. The filtrate contained most of the radioactivity (4.4×10^5 dpm). The peptides were concentrated by lyophilization and applied to a Vydac semipreparative octadecylsilica 0.94 × 25 cm column.

Analytical chromatograms were monitored at 206 nm and developed at 2 mL/min with the following solvent gradient: 5 min, 0.1% trifluoroacetic acid (TFA)[1] in water; 5–125 min, linear gradient to 60% acetonitrile containing 0.1% TFA; 125–135 min, linear gradient to 0.1% TFA in water. Fractions were collected every minute and aliquots counted to locate radioactive peaks (see Figure 1). The radioactive peptide was collected manually in acid-washed tubes in subsequent runs using a shortened gradient: 5-min wash; 5–45 min to 20% acetonitrile; 45–55 min to 60% acetonitrile; 55–65 min to 0.1% TFA in water. The recovery of radioactivity for each preparative run was consistently >90% of the amount applied. The pooled material eluted as a single peak on re-injection, and was isolated in an overall yield of 23% based on the radioactivity of the modified enzyme prior to denaturation. Automated sequencing was performed using an Applied Biosystems gas-phase protein sequencer (Model 470A/120A/900A) according to the manufacturer's instructions. The entire peptide isolation procedure was repeated with a second batch of enzyme with identical results.

Redox Potential Determinations. Redox potentials were measured using the spectrophotometric method introduced by Massey (1990). The medium-chain acyl-CoA dehydrogenase (5–15 μM in 50 mM phosphate buffer, pH 7.6) was deoxygenated in an anaerobic cuvette in the presence of 200 μM xanthine, 1–5 μM methyl viologen, and 5–10 μM indigodisulfonate ($E^{0'}_{pH7.6} = -0.121$ V; Lenn et al., 1990). Xanthine oxidase was added to the anaerobic mixture from a syringe to a final concentration of 10–25 nM. Spectra were recorded at regular intervals throughout the reduction on an HP8452A diode array spectrophotometer. The concentrations of oxidized and reduced enzyme were calculated from the absorbance at 468 nm (an isosbestic point for the reduction of indigodisulfonate). The concentrations of oxidized and reduced dye were determined from the absorbance at 610 nm with minimal interference from the small amount of blue semiquinone formed during these reductions. The data were plotted as described earlier (Massey, 1990; Minnaert, 1965). The midpoint potential for the carboxymethylated enzyme was determined in the same way.

RESULTS

Isolation and Characterization of S-Carboxymethylated Peptide. Treatment of medium-chain acyl-CoA dehydrogenase from pig kidney with 30 mM iodoacetate at pH 6.6 affects the carboxymethylation of 1.08 molecules of methionine with the accumulation of only traces of (carboxymethyl)cysteine and histidine (Mizzer & Thorpe, 1980). A tryptic peptide map of alkylated enzyme (see Experimental Procedures) shows one dominant radioactive peak (Figure 1). This peptide was collected (see Experimental Procedures) and sequenced (Table I). Radioactivity emerged over several cycles of gas-phase sequencing, but peaked at residue 3 with a low recovery of regenerated methionine. Such behavior is consistent with the comparatively labile *S*-(carboxymethyl)methionine residue (Goren et al., 1968; Gundlach et al., 1959; Kleanthous et al., 1990). The tryptic octapeptide was positioned within the primary sequence of the medium-chain acyl-CoA dehydrogenase by comparison with published human and rat sequences (Table II; Kelly et al., 1987; Matsubara et al., 1987). Thus, iodoacetate alkylates a single methionine residue in the pig kidney enzyme, corresponding to Met249 in the human

[1] Abbreviations: CM, carboxymethylated; SCAD, MCAD, and LCAD, short-, medium-, and long-chain acyl-CoA dehydrogenase, respectively; IVDH, isovaleryl-CoA dehydrogenase; TFA, trifluoroacetic acid.

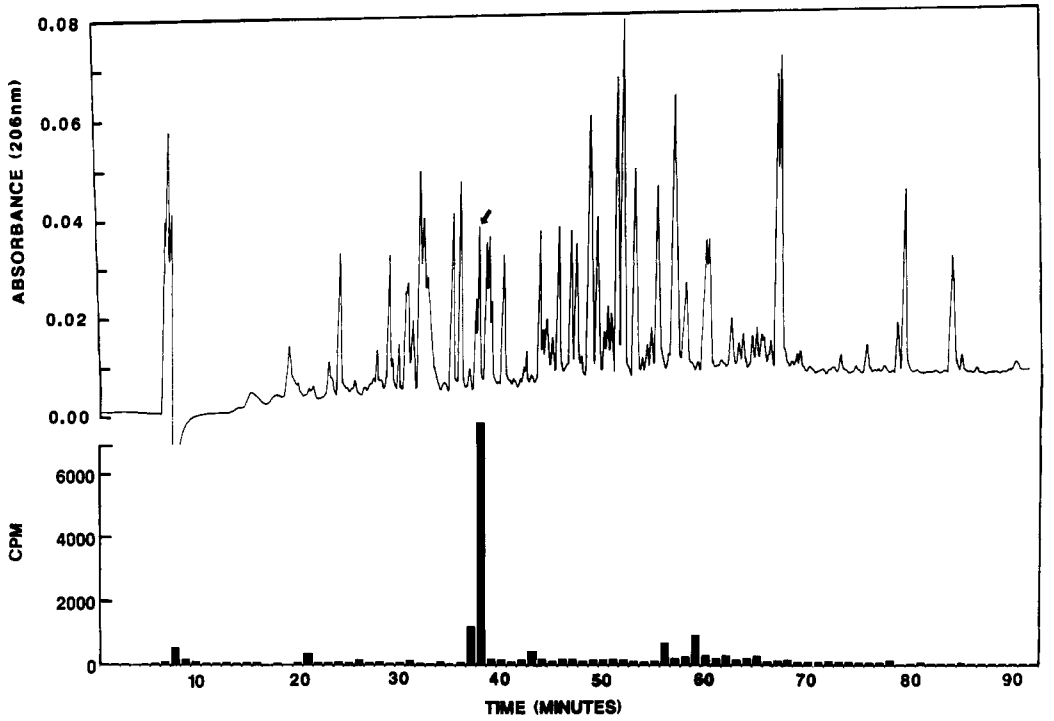


FIGURE 1: HPLC profile of a tryptic digest of medium-chain acyl-CoA dehydrogenase alkylated with iodo[2-^{14}C]acetate. The modified enzyme was digested with trypsin and applied to an octadecylsilica semipreparative column developed as described under Experimental Procedures. The peak marked by an arrow accounted for a total of 45% of the radioactivity applied to the column. It was subsequently collected manually using a shallower gradient, and the shoulder at shorter retention time was discarded.

Table I: Sequence of Modified Peptide from Carboxymethylated Medium-Chain Acyl-CoA Dehydrogenase

cycle	amino acid	yield (pmol)[a]	radioactivity (dpm)[b]
1	Ile	1748	49
2	Ala	1368	70
3	Met	149[c]	385
4	Gly	800	266
5	Thr	497	119
6	Phe	898	0
7	Asp	567	49
8	Lys	400	21

[a] About 1900 pmol was applied to the sequencer (4700 dpm). [b] Total radioactivity in fractions from the sequencer. [c] Consistent with partial regeneration of methionine from *S*-(carboxymethyl)methionine peptides (Goren et al., 1968; Gundlach et al., 1959; Kleanthous et al., 1990).

sequence. Interestingly, this residue is conserved in all of the acyl-CoA dehydrogenases sequenced to date (Table II; short-, and medium-, and long-chain and isovaleryl-CoA dehydrogenase; Kelly et al., 1987; Matsubara et al., 1987, 1989).

Modification of Met249 Perturbs the Redox Equilibrium in Enzyme–Substrate Complexes. A comparison of the effect of octanoyl-CoA on the native and carboxymethylated enzyme is shown in the inset to Figure 2. Unlike the native enzyme, which is extensively reduced by octanoyl-CoA to yield the purple charge-transfer complex (inset, dashed curve 3), the carboxymethylated dehydrogenase undergoes comparatively small spectral changes with the appearance of a weak long-wavelength absorbance at 600 nm (inset, line 2). This long-wavelength band is unstable (decaying to the dotted line after 20 min), and hence it was overlooked during previous prolonged titrations of the carboxymethylated enzyme with octanoyl-CoA (Mizzer & Thorpe, 1980). Thus, octanoyl-CoA appears to be capable of a partial reduction of the carboxymethylated enzyme, and further experiments were designed to confirm this observation.

Replacement of the natural prosthetic group by 8-Cl-FAD effects a marked change in the behavior of the carboxymethylated medium-chain dehydrogenase (Figure 2, main panel). Reduction of this more oxidizing analog (about 60 mV more positive than FAD; Moore et al., 1978) now proceeds essentially to completion, with the formation of a prominent long-wavelength band centered at 560 nm whose intensity is some 70% of that shown by unmodified 8-Cl-FAD-substituted enzyme (Thorpe & Massey, 1983). Thus, the small spectral changes seen in the inset to Figure 2 reflect a thermodynamic perturbation of the interaction between octanoyl-CoA and the carboxymethylated enzyme that can be compensated by the substitution of a more oxidizing FAD analogue.

Carboxymethylation of the 8-Cl-FAD-substituted acyl-CoA dehydrogenase seriously weakens the apparent affinity of octanoyl-CoA for the enzyme. Before carboxymethylation, titrations of the 8-Cl-FAD-substituted enzyme with octanoyl-CoA give essentially stoichiometric reduction (Thorpe & Massey, 1983). After chemical modification, a severalfold excess of octanoyl-CoA is now needed to reduce the enzyme completely (Figure 2). More detailed studies of ligand binding will be presented later in this paper.

Titrations with the long-chain substrate palmitoyl-CoA further illustrate the perturbation of the internal equilibrium, K_2, induced by carboxymethylation of Met249. When the native enzyme is considered first (see inset to Figure 3), K_2 lies to the left in the presence of palmitoyl-CoA, but can be driven to the right by 8-Cl-FAD substitution (compare dashed and dotted lines; Thorpe & Massey, 1983). Carboxymethylation essentially undoes the thermodynamic advantage provided by the flavin replacement (main panel, Figure 3). Now the alkylated 8-Cl-FAD-substituted enzyme responds to palmitoyl-CoA with slight bleaching of the flavin comparable to that seen with the native unsubstituted enzyme.

These experiments suggest that the approximately 5% activity shown by the carboxymethylated enzyme in the standard assay using 30 μM octanoyl-CoA substrate (see Experimental Procedures) is not due to the residual activity of native unmodified dehydrogenase but reflects, in part,

Table II: Comparison of Peptide Sequences Containing Met249

source	amino acids							
	1	2	3[a]	4	5	6	7	8
pig CM-MCAD[b]	Ile	Ala	Met	Gly	Thr	Phe	Asp	Lys
human MCAD[c]	Val	Ala	Met	Gly	Ala	Phe	Asp	Lys
rat MCAD[d]	Ile	Ala	Met	Gly	Ala	Phe	Asp	Arg
rat SCAD[e]	Ile	Ala	Met	Gln	Thr	Leu	Asp	Met
rat LCAD[e]	Tyr	Leu	Met	Gln	Glu	Leu	Pro	Gln
rat IVDH[e]	Val	Leu	Met	Ser	Gly	Leu	Asp	Leu

[a] Corresponds to Met249 in the MCAD sequence. [b] Determined in this work (see Experimental Procedures). [c] Kelly et al. (1987). [d] Matsubara et al. (1987). [e] Matsubara et al. (1989).

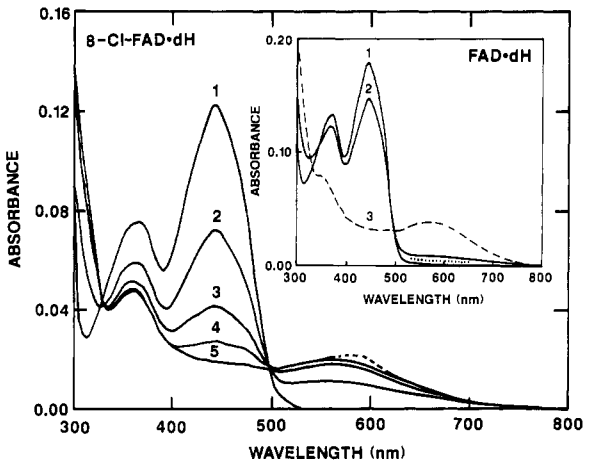


FIGURE 2: Anaerobic titration of carboxymethylated 8-Cl-FAD-substituted acyl-CoA dehydrogenase with octanoyl-CoA. Native apoprotein was reconstituted with 8-Cl-FAD and treated with iodoacetic acid as described under Experimental Procedures. The modified enzyme (curve 1, 8.6 μM in 0.82 mL of 100 mM potassium phosphate buffer, pH 7.6, 25 °C) was titrated with 12, 24, 48, and 156 μM octanoyl-CoA (curves 2–5, respectively). Over time, a further minor increase in absorbance at 590 nm is observed, possibly due to the accumulation of small amounts of the blue semiquinone (Thorpe et al., 1979). The inset shows the spectrum of the carboxymethylated enzyme containing FAD before (curve 1) and immediately after the anaerobic addition of a 26-fold excess of octanoyl-CoA (297 μM, curve 2). The dotted line indicates the position of the long-wavelength band after an additional 20 min. For comparison, the dashed line (curve 3) is the spectrum generated on the addition of excess octanoyl-CoA to an equivalent concentration of the native unmodified dehydrogenase.

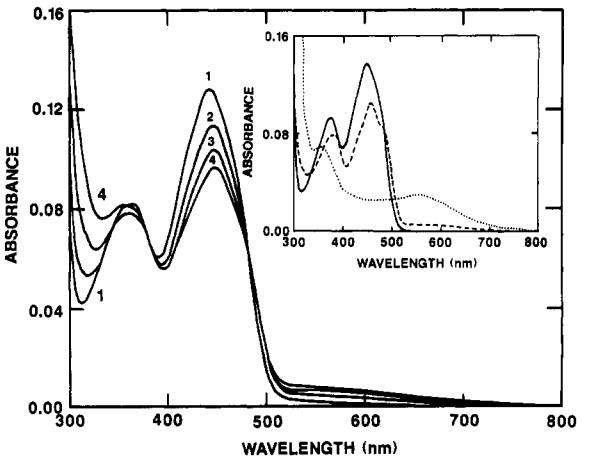


FIGURE 3: Titration of carboxymethylated 8-Cl-FAD-substituted acyl-CoA dehydrogenase with palmitoyl-CoA. The carboxymethylated 8-Cl-FAD-substituted enzyme (curve 1, 9 μM in 100 mM phosphate buffer, pH 7.6, 25 °C) was titrated with 11, 43, and 140 μM palmitoyl-CoA (curves 2–4, respectively). The inset shows the effect of saturating levels of palmitoyl-CoA on the native [(- - -) Thorpe et al., 1979] and 8-Cl-FAD-substituted unmodified acyl-CoA dehydrogenase [(···) Thorpe & Massey, 1983]. The spectrum of the native enzyme is shown by the solid line.

perturbation of an internal redox step. Further evidence that Met249 is not a catalytically essential residue comes from studies with a mechanism-based inhibitor of the acyl-CoA dehydrogenases.

Interaction of the Carboxymethylated Enzyme with 3,4-Pentadienoyl-CoA. 3,4-Pentadienoyl-CoA rapidly forms reduced flavin adducts with native medium-chain acyl-CoA dehydrogenase (Wenz et al., 1985). Figure 4 shows that addition of 1 equiv of the allene to the carboxymethylated enzyme bleaches the flavin chromophore almost completely, giving spectra very similar to those observed with the native dehydrogenase (Wenz et al., 1985). The reduced flavin adduct is, however, considerably more stable when formed with the carboxymethylated enzyme, reverting to oxidized flavin and the isomerized product 2,4-pentadienoyl-CoA at a rate about 6-fold slower than the native enzyme (data not shown; Wenz et al., 1985).

The formation of a covalent adduct at the N-5 position of the isoalloxazine ring drives reduction of both native and carboxymethylated enzymes to completion. Thus, the rates of reduction of the native and carboxymethylated enzyme can be readily compared using 3,4-pentadienoyl-CoA without the necessity of simultaneously replacing FAD by the 8-Cl derivative. Stopped-flow experiments reveal a profoundly slower reduction (some 800-fold: see legend to Figure 5) when the alkylated enzyme is reacted with a 4.4-fold excess of 3,4-pentadienoyl-CoA (43 μM) at pH 7.6, 2 °C.

Effect of Carboxymethylation on the Midpoint Potential of the Medium-Chain Dehydrogenase. Two extreme explanations could underly the behavior of the carboxymethylated enzyme toward acyl-CoA substrates. Alkylation could perturb either the redox potential of the free enzyme or the relative binding affinities of acyl-CoA substrate for the oxidized enzyme versus enoyl-CoA product for the reduced enzyme (see Discussion). Figure 6 indicates that the midpoint potentials at pH 7.6 for the two-electron reduction of carboxymethylated dehydrogenase and native dehydrogenase are essentially the same (–142 ± 6 mV, three determinations, compared to –139 ± 5 mV, four determinations, respectively). The value for the unmodified enzyme is in good agreement with a recent measurement (–136 mV at pH 7.6; Lenn et al., 1990). Thus, carboxymethylation appears to selectively modulate binding discrimination between substrate and product. Accordingly, a comparison between the binding affinity of native and alkylated enzymes was undertaken for a number of acyl-CoA analogues.

Binding of Acyl-CoA Analogues to the Carboxymethylated Enzyme. The results of a series of spectrophotometric titrations of native and carboxymethylated enzyme are shown in Table III. All the ligands except 3-thiaoctanoyl-CoA gave spectral changes extrapolated to saturation that were qual-

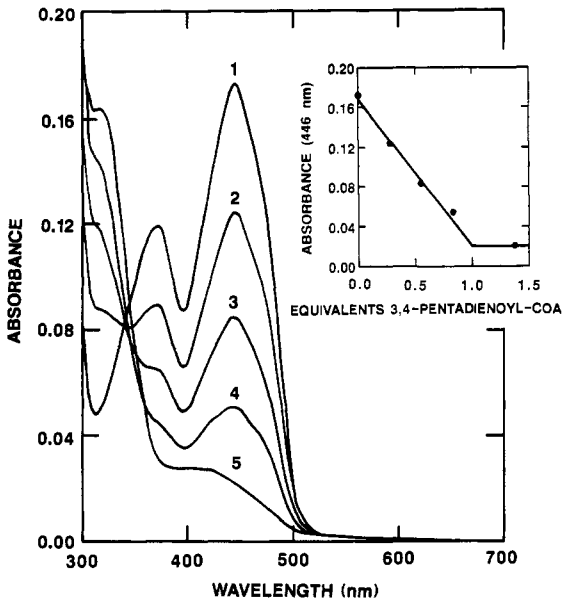


FIGURE 4: Reduction of carboxymethylated medium-chain acyl-CoA dehydrogenase by 3,4-pentadienoyl-CoA. The native dehydrogenase was alkylated with iodoacetate as described under Experimental Procedures, and the modified enzyme (12.3 μM, curve 1) was titrated with 0.28, 0.55, 0.83, and 1.38 equiv of 3,4-pentadienoyl-CoA in 100 mM phosphate buffer, pH 7.6, 25 °C (curves 2–5, respectively). Spectra were recorded a few minutes after each addition. The inset plots the absorbance at 446 nm after each addition of allene.

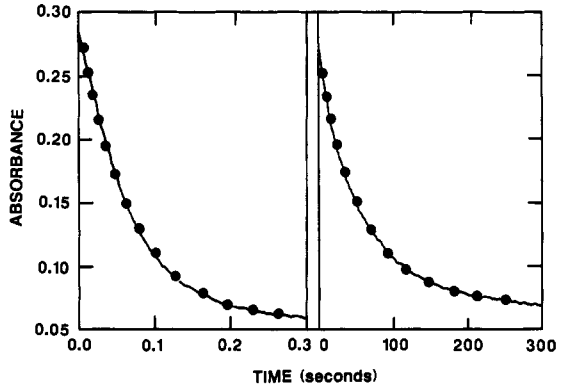


FIGURE 5: Comparison of the reduction of native and carboxymethylated acyl-CoA dehydrogenase by 3,4-pentadienoyl-CoA in the stopped-flow spectrophotometer. Native (left panel, 0.3 s) or carboxymethylated enzyme (right panel, 300 s) was mixed in the stopped-flow absorbance spectrophotometer with an equal volume of 3,4-pentadienoyl-CoA to give final concentrations of 9.7 and 43 μM enzyme and inhibitor, respectively, in 50 mM phosphate buffer, 2 °C. The solid line represents absorbance data at 446 nm (2-cm path length), and the filled circles correspond to calculated rate constants of 13/s and 1.57 × 10^{-2}/s (left and right panels, respectively).

itatively similar for both native and alkylated enzyme. Importantly, the impact of carboxymethylation on binding affinity was not uniform, but was greatly dependent on the acyl-CoA analogue used. Thus, the thioether acyl-CoA substrate analogues octyl-SCoA and 3-thiaoctyl-SCoA bind more weakly by factors of 25–30-fold (Table III). In contrast, the binding of *trans*-2-octenoyl-CoA and two redox-inactive analogues, 2-azaoctanoyl-CoA and 3-thiaoctanoyl-CoA, is weakened much more severely (by 1000–4400-fold; Table III). Such differential effects can readily explain the thermodynamic effects observed in the reductive half-reaction upon carboxymethylation of the dehydrogenase (see Discussion).

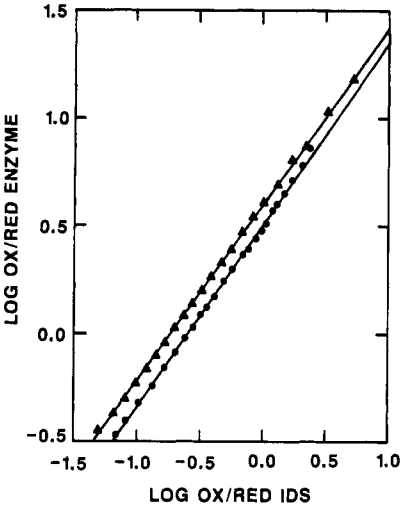
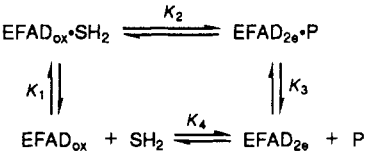


FIGURE 6: Measurement of midpoint potentials for native and carboxymethylated medium-chain acyl-CoA dehydrogenase. The redox potentials of 10 μM native and carboxymethylated acyl-CoA dehydrogenase were determined as described by Massey (1990; see Experimental Procedures). The redox potentials were calculated as described by Minnaert (1965). Native dehydrogenase (▲; −139 mV); carboxymethylated enzyme (●; −136 mV). The average value from four determinations on the native protein is −139 ± 5 mV, and is −142 ± 6 mV from three measurements on the carboxymethylated dehydrogenase.

Scheme II

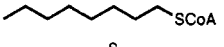
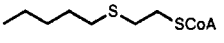
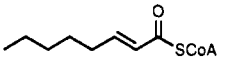
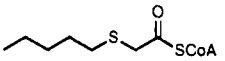
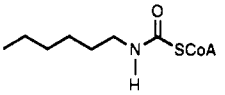


DISCUSSION

This study confirms that methionine is carboxymethylated by iodoacetic acid treatment of the medium-chain acyl-CoA dehydrogenase at pH 6.6 (Mizzer & Thorpe, 1980) and identifies the target residue within the primary structure of the enzyme. The work has demonstrated that Met249 is not catalytically essential as we previously suggested (Mizzer & Thorpe, 1980). Carboxymethylation drastically perturbs acyl-CoA ligand binding, leading to a greatly increased K_m for octanoyl-CoA (from a value of <1 μM for the native enzyme to about 65 μM; data not shown). In contrast, V_{max} is less affected, decreasing to 23% upon alkylation. Clearly, the greater than 300-fold decrease in V/K upon alkylation is dominated by binding effects. Differential binding of substrate and product is also responsible for the modulation of the internal redox equilibrium step, K_2, first recognized by Beinert and co-workers (Beinert & Page, 1957).

Consider the thermodynamic cycle shown in Scheme II, where K_1 and K_3 represent the affinities of acyl-CoA substrate (SH_2) and enoyl-CoA product (P) for oxidized and reduced enzyme forms, respectively (Thorpe et al., 1979). This cycle is completed by K_4, whose magnitude is governed by the redox potentials of free acyl-CoA dehydrogenase and free acyl-CoA substrate. A recent reinvestigation of these potentials (Lenn et al., 1990) gives values of about −136 mV for the free dehydrogenase and −41 mV for octanoyl-CoA. Thus, free substrate is thermodynamically incapable of significant reduction of the enzyme (K_4 for the octanoyl-CoA/octenoyl-CoA couple corresponds to a value of about 6.0 × 10^{-4}). However, complexes of the enzyme with octanoyl-CoA are almost completely reduced, and thus this internal equilibrium

Table III: Dissociation Constants for Binding Acyl-CoA Analogues to Native and Carboxymethylated Dehydrogenase

compound	structure	K_N[a] (μM)	K_{CM}[b] (μM)	K_{CM}/K_N
octyl-SCoA		4	120	30
3-thiaoctyl-SCoA		3.6	88	25
trans-2-octenoyl-CoA		0.09	400	4400
3-thiaoctanoyl-CoA		0.47	480	1020
2-azaoctanoyl-CoA		0.05	167	3340

[a] Dissociation constant for native enzyme. [b] Dissociation constant for carboxymethylated enzyme.

Table IV: Equilibrium Constants for the Reductive Half-Reaction for Native and Carboxymethylated Enzyme with Octanoyl-CoA

	K_1[a]	K_2	K_3[a]	K_4	K_1/K_3
native enzyme	2.0×10^{-7} [b]	9	1.3×10^{-11}	6.0×10^{-4}	15000
CM-enzyme	2.0×10^{-4} [c]	0.2[d]	6.0×10^{-7}	6.0×10^{-4}	330
CM-enzyme/ native enzyme	1000	0.022	46000	1	0.022

[a] K_1 and K_3 are expressed as dissociation constants in molar. [b] Calculated from K_{app} = 20 nM (see Experimental Procedures; Thorpe et al., 1981). [c] Calculated from K_{app} = 170 μM (see Experimental Procedures; Mizzer & Thorpe, 1980). [d] Estimated from spectral changes of CM-enzyme in the presence of octanoyl-CoA (see inset to Figure 2, curves 1 and 2).

K_2 must be pulled to the right by a strong preferential binding of octenoyl-CoA product to the reduced enzyme (via K_3) compared to octanoyl-CoA binding to the oxidize dehydrogenase (via K_1; Thorpe et al., 1979). A minimal estimate for K_2 is 9 (Lau & Thorpe, 1988), and this allows estimation of the remaining equilibrium constant, K_3, of this thermodynamic cycle (Table IV). The preferential binding of enoyl-CoA product over octanoyl-CoA substrate amounts to a factor of 15 000, and octenoyl-CoA is bound to the reduced enzyme at the picomolar level (Table IV). This extremely tight binding is compatible with the kinetic and thermodynamic stability of this reduced enzyme species (Beinert & Page, 1957; Beinert, 1963; Madden et al., 1984; Wang & Thorpe, 1991). During turnover, the dehydrogenase avoids the kinetic disadvantage usually associated with such tight binding (Fersht, 1985) by delivering electrons from the reduced enzyme·octenoyl-CoA complex directly (McKean et al., 1979; Gorelick et al., 1985; Thorpe, 1991). The resulting enoyl-CoA complex with the oxidized enzyme is weakened some 6900-fold (to about 90 nM; Powell et al., 1987), allowing displacement by substrate during the next catalytic turnover.

Table IV illustrates the impact of carboxymethylation of Met249. K_2 may be estimated from the increase in the long-wavelength absorbance at 570 nm as about 0.2 (inset of Figure 2). K_1 (Table IV) is calculated from the apparent dissociation constant for octanoyl-CoA binding to the carboxymethylated enzyme (170 μM; Mizzer & Thorpe, 1980; see Experimental Procedures). Importantly, K_4 is essentially unchanged from the native enzyme since carboxymethylation does not significantly affect the redox potential of the free dehydrogenase (Figure 6). Although the binding of both octanoyl-CoA substrate and octenoyl-CoA product are seriously weakened upon alkylation, product binding to the reduced enzyme is by far the worst affected (by a factor of 46 000 for K_3 versus 1000-fold for K_1; Table IV), accounting for the 46-fold change in the estimates of the internal redox step K_2. Interestingly, this internal equilibrium can be corrected by substitution of the normal prosthetic group by the more easily reducible analog 8-Cl-FAD. In effect, decreases in the ratio K_1/K_3 caused by alkylation are compensated by increases in K_4 associated with flavin substitution.

The side chain of Met249 is situated toward the adenine pocket of the acyl-CoA substrate binding site over 15 Å from the N-5 position of the flavin ring (Kim & Wu, 1988; Kim, 1991; J. J. P. Kim, unpublished results). Thus, the introduction of an *S*-carboxymethyl function at this locus might be expected to impair binding on steric grounds alone. However, carboxymethylation of methionine will also effect major changes in the polarity of the side chain from a relatively hydrophobic residue to a zwitterionic polar structure. Thus, it is perhaps not surprising that carboxymethylation perturbs ligand binding, although the selectivity of the interactions described here cannot be explained in molecular terms at present.

In conclusion, this work shows that Met249 is not essential for catalysis, although alkylation of this residue has profound thermodynamic and kinetic effects on the reductive half-reaction. It appears likely that chemical modification or mutagenesis of other amino acid residues within the substrate binding site might exert similar effects, because they too might weaken the selective binding of product required by the acyl-CoA dehydrogenases to pull their substrates thermodynamically uphill (Johnson & Benkovic, 1990). These observations should be kept in mind during the characterization of the naturally occurring mutants of the acyl-CoA dehydrogenases.

ACKNOWLEDGMENT

We thank Drs. Sandro Ghisla and Alexandra Wenz for drawing our attention to the reduction of the modified enzyme by 3,4-pentadienoyl-CoA, Dr. Richard Brantley for the synthesis of a number of the CoA thioester analogues used in this paper, and Dr. Huang for performing amino acid sequencing. Finally, we acknowledge the help of Dr. Jung-Ja P. Kim for advice concerning the crystal structure of the medium-chain acyl-CoA dehydrogenase and Dr. Roberta Colman for helpful discussions.

REFERENCES

Beinert, H. (1963) *The Enzymes, 2nd Ed. 7*, 447–466.

Beinert, H., & Page, E. (1957) *J. Biol. Chem. 225*, 479–497.

Bernert, J. T., & Sprecher, H. (1977) *J. Biol. Chem. 252*, 6736–6744.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman & Co., New York.

Ghisla, S., Thorpe, C., & Massey, V. (1984) *Biochemistry 23*, 3154–3160.
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.
Goren, H. J., Glick, D. M., & Barnard, E. A. (1968) *Arch. Biochem. Biophys. 126*, 607–623.
Gundlach, H. G., Moore, S., & Stein, W. H. (1959) *J. Biol. Chem. 234*, 1761–1764.
Hall, C. L., Lambeth, J. D., & Kamin, H. (1979) *J. Biol. Chem. 254*, 2023–2031.
Ikeda, Y., Okamura-Ikeda, K., & Tanaka, K. (1985) *Biochemistry 24*, 7192–7199.
Johnson, K. A., & Benkovic, S. J. (1990) *Enzymes (3rd Ed.) 19*, 159–211.
Kelly, D. P., Kim, J. J., Billadello, J. J., Hainline, B. E., 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*, pp 291–298, Walter de Gruyter, New York.
Kim, J. J. P., & Wu, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6677–6681.
Kleanthous, C., Campbell, D. G., & Coggins, J. R. (1990) *J. Biol. Chem. 265*, 10929–10934.
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., Hale, D. E., Bhala, A., & Thorpe, C. (1990) *Anal. Biochem. 186*, 280–284.
Lenn, N. D., Stankovich, M. T., & Liu, H. (1990) *Biochemistry 29*, 3709–3715.
Madden, M., Lau, S.-M., & Thorpe, C. (1984) *Biochem. J. 224*, 577–580.
Massey, V. (1991) in *Flavins and Flavoproteins*, pp 59–66, Walter de Gruyter, New York.
Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446–465.
Matsubara, Y., Kraus, J. P., Ozasa, H., Glassberg, R., Finocchiaro, G., Ikeda, Y., Mole, J., Rosenberg, L. E., & Tanaka, K. (1987) *J. Biol. Chem. 262*, 10104–10108.
Matsubara, Y., Indo, Y., Naito, E., Ozasa, H., Glassberg, R., Vockley, J., Ikeda, Y., Kraus, J., & Tanaka, K. (1989) *J. Biol. Chem. 264*, 16321–16331.
Mayer, E. J., & Thorpe, C. (1981) *Anal. Biochem. 116*, 227–229.
McKean, M. C., Frerman, F. E., & Mielke, D. M. (1979) *J. Biol. Chem. 254*, 2730–2735.
Minnaert, K. (1965) *Biochim. Biophys. Acta 110*, 42–56.
Mizzer, J. P., & Thorpe, C. (1980) *Biochemistry 19*, 5500–5504.
Moore, E. G., Cardemil, E., & Massey, V. (1978) *J. Biol. Chem. 253*, 6413–6422.
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., 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.
Spencer, R., Fisher, J., & Walsh, C. (1976) *Biochemistry 15*, 1043–1053.
Thorpe, C. (1981) *Methods Enzymol. 71*, 366–374.
Thorpe, C. (1991) in *Chemistry & Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. II, pp 471–486, CRC Press, Boca Raton, FL.
Thorpe, C., & Massey, V. (1983) *Biochemistry 22*, 2972–2978.
Thorpe, C., Matthews, R. G., & Williams, C. H. (1979) *Biochemistry 18*, 331–337.
Thorpe, C., Ciardelli, T. L., Stewart, C. J., & Wieland, T. L. (1981) *Eur. J. Biochem. 118*, 279–282.
Wang, R., & Thorpe, C. (1991) *Biochemistry 30*, 7895–7901.
Wenz, A., Ghisla, S., & Thorpe, C. (1985) *Eur. J. Biochem. 147*, 553–560.