Acyl-CoA Complexes of General Acyl-CoA Dehydrogenase and Electron Transfer Flavoprotein

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General acyl-coenzyme A dehydrogenase (G-AD) rapidly forms stable long wavelength-absorbing complexes with fatty acyl-CoA substrates of chain lengths from C₆ to C₁₆. The oxidation-reduction state of the flavin in the G-AD-substrate complex is unknown, but no EPR signal is present and the spectrum is different from the neutral semiquinone spectrum produced on oxidation of G-AD from C₉ to C₁₆. The oxidation-reduction state of the rapidly forms stable long wavelength-absorbing complex with a specific region of the enzyme and is followed by a weaker, temperature-sensitive chain length-dependent interaction of the hydrocarbon tail with an adjoining hydrocarbon binding site. The first interaction is not optically detected but the second results in both the desaturated (but not saturated) fatty acyl-CoA's with ETF, electron transfer flavoprotein, permits electron flow to acceptors (1-10); ETF is bleached by acyl-CoA in the presence of small amounts of acyl-CoA dehydrogenase (7, 14, 15).

In order for acyl-CoA dehydrogenase to turn over, it must be able to donate substrate electrons to an acceptor and must be able to dissociate the desaturated product. ETF has been shown to be able to mediate the necessary electron transfers (7-10, 14-16), but the point in the reaction at which the desaturated acyl-CoA dissociates from the dehydrogenase is not known. We have reported preliminary evidence for complex formation between ETF and Δ⁹⁻CoA (17). Evidence has also been presented that the affinity of the desaturated product for the oxidized dehydrogenase is at least as high as the affinity of the saturated substrate (11, 18). The present studies examine various interactions between acyl-CoA's, dehydrogenase, and ETF in the hope of understanding better the apparent facilitation of electron flow from fatty acyl-CoA through dehydrogenase caused by ETF. We have demonstrated that 1:1 complex formation between fatty acyl-CoA and dehydrogenase occurs rapidly; we have also observed spectrophotometrically detectable tight complexes between the desaturated (but not saturated) fatty acyl-CoA's with ETF. The role of these complexes in catalysis has not yet been established. Preliminary reports of some of these results have been presented elsewhere (19, 20).

EXPERIMENTAL PROCEDURES

Materials—Acyl-CoA derivatives were obtained from P-L Biochemicals (Milwaukee, Wis.) and as a generous gift from Prof. H. K. (19). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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β oxidation of fatty acids in mammalian mitochondria is carried out by a series of enzymes which successively and repetitively cleave off C₂-CoA fragments from fatty acyl-CoA molecules, until the fatty acyl-CoA is completely degraded to acyl-CoA. The first step of each cycle of mitochondrial β oxidation, dehydrogenation of the α,β bond (1), is carried out by a system of two different flavoproteins: a substrate-linked dehydrogenase, and another flavoprotein, called electron transfer flavoprotein which is an obligatory electron carrier from the dehydrogenase to the electron transport chain (2-10). The substrate-linked dehydrogenases, at least three different types having different chain length substrate specificities (2-6), form tightly bound air-stable intermediates with acyl-CoA derivatives appropriate to the chain length specificity of each dehydrogenase (4-6, 11-14). The presence of the acyl-CoA on the dehydrogenase appears to interfere with electron flow from acyl-CoA dehydrogenases to dichlorophenolindophenol since no reduction of acceptor occurs when acyl-CoA is added to acyl-CoA dehydrogenase alone, while acyl-CoA dehydrogenase reduced by a small amount of dithionite can rapidly reduce DCPIP⁻ or Fe(CN)₄⁻ (13). Thus, the dehydrogenase is unable to turn over in the presence of substrate alone. However, addition of ETF to acyl-CoA dehydrogenase and acyl-CoA permits electron flow to acceptors (4-10); ETF is bleached by acyl-CoA in the presence of small amounts of acyl-CoA dehydrogenase (7, 14, 15).

In order for acyl-CoA dehydrogenase to turn over, it must be able to donate substrate electrons to an acceptor and must be able to dissociate the desaturated product. ETF has been shown to be able to mediate the necessary electron transfers (7-10, 14-16), but the point in the reaction at which the desaturated acyl-CoA dissociates from the dehydrogenase is not known. We have reported preliminary evidence for complex formation between ETF and Δ⁹⁻CoA (17). Evidence has also been presented that the affinity of the desaturated product for the oxidized dehydrogenase is at least as high as the affinity of the saturated substrate (11, 18). The present studies examine various interactions between acyl-CoA's, dehydrogenase, and ETF in the hope of understanding better the apparent facilitation of electron flow from fatty acyl-CoA through dehydrogenase caused by ETF. We have demonstrated that 1:1 complex formation between fatty acyl-CoA and dehydrogenase occurs rapidly; we have also observed spectrophotometrically detectable tight complexes between the desaturated (but not saturated) fatty acyl-CoA's with ETF. The role of these complexes in catalysis has not yet been established. Preliminary reports of some of these results have been presented elsewhere (19, 20).
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When an excess of acyl-CoA substrate was used, the spectra produced were concentration-independent (see below). It can be seen that bleaching of the flavin at 448 nm was accompanied by increases in the long wavelength portion of the absorbance spectrum. C₆CoA (Fig. 2) induces the greatest decrease in 438 nm absorbance, but the long wavelength increase is maximal and about the same with C₁₄ and C₁₆CoA. C₂₀CoA and C₄₆CoA produce more highly resolved spectra with distinct shoulders at ~500 nm, which is isosbestic (Fig. 1A) for the shorter chain length derivatives. Fig. 2 shows a plot of the Δε at several wavelengths of acyl-CoA complexes of G-AD (using ε₄₄₈ = 11.4 × 10³ M⁻¹ cm⁻¹) versus chain length of the substrate. Beinert’s laboratory (4, 13, 14) has described some of these spectral changes.

Titration of G-AD with Fatty Acyl-CoA Substrates—

Aerobic titrations of G-AD with acyl-CoA substrates from C₆CoA to C₁₄CoA all showed breaks in the plots of A₄₄₈ and

Fig. 1. Air-stable acyl-CoA complexes of G-AD. A, various lots of G-AD (20 to 30 µM flavin, A₁₄₀) having A₂₇₀/A₄₄₈ ratios of 5 to 10 were pooled and diluted with KP buffer to final concentrations of 2 to 4 µM enzyme flavin. Acyl-CoA derivatives of the indicated chain lengths (C₀ to C₁₄) were added to separate samples of G-AD to final concentrations of 20 to 50 µM. B, a sample of G-AD (A₁₄₀/A₄₄₈ = 6.5) was used and diluted to 4 to 6 µM enzyme flavin (A₁₄₀) with KP buffer and acyl-CoA derivatives (C₁₂ to C₆₀) were added to a final concentration of 20 to 50 µM.

Fig. 2. Chain-length-related extinction changes in G-AD acyl CoA complexes. Data were taken from Fig. 1. Extinction coefficients for the acyl-CoA-induced absorbance changes were calculated based on A₁₄₀ = 11.4 × 10³ M⁻¹ cm⁻¹ for the oxidized G-AD and were plotted as a function of chain length of the acyl-CoA derivatives.

RESULTS

Acyl-CoA Complexes with G-AD

Effect of Addition of Fatty Acyl CoA Substrates upon the Spectrum of G-AD—Fig. 1 shows the spectra obtained on aerobic addition of excess amounts of even-numbered fatty acyl-CoA derivatives of chain lengths between C₆ and C₄₆ to G-AD. The experiments shown in Fig. 1, A and B, were done with different lots of G-AD, whose A₂₇₀/A₄₄₈ ratio varied between 0.74 and 0.79. Each acyl-CoA addition spectrum (broken lines) was obtained by adding the acyl-CoA to a different sample of the same lot of G-AD, and the spectra were normalized to the same oxidized spectrum (solid line).

C. L. Hall, unpublished observations.

when otherwise stated, A₁₄₀−C₆₀CoA was the kind gift of Dr. Robert M. Waterson of Emory University or was synthesized for chemicals, unless otherwise stated. A',₃-CxCoA was the kind gift of Dr. Mark Lively of the Georgia Institute of Technology. Glucose oxidase, type V, was obtained from Sigma; DCPIP was from Mann, Inc.

Methods—All procedures were carried out in 20 mM KP, pH 7.6, except where otherwise indicated. G-AD and ETF were purified from pig liver mitochondria as previously described (9, 21). The concentration of G-AD and ETF was measured by absorbance of the native enzymes at their visible maxima, on the basis of 4 flavins/mol of G-AD and 2 flavins/mol of ETF (9, 21) using ε₄₄₈ or ε₄₅₀ = 11,400 M⁻¹ cm⁻¹ (cf. Refs. 9, 21, and 22). Preparations of G-AD of A₂₇₀/A₄₄₈ of less than 10 were used in the experiments described herein, and ETF preparations of A₄₅₀/A₈₀₅ of 5 to 7 were chosen for most experiments.

Reduction of DCPIP was measured at 600 nm using ε = 21,000 M⁻¹ cm⁻¹ (11). Turnover in the catalytic DCPIP assay was calculated by linear regression analysis of the average of two sets of determinations of 1/υ versus 1/[ETF flavin] for each chain length at “saturating” substrate and four different ETF concentrations (see also Ref. 21). The concentration of G-AD and ETF was normalized to the same oxidized spectrum (solid line).

The experiments shown in Fig. 1, A and B, were done with different lots of G-AD, whose A₂₇₀/A₄₄₈ ratio varied between 0.74 and 0.79. Each acyl-CoA addition spectrum (broken lines) was obtained by adding the acyl-CoA to a different sample of the same lot of G-AD, and the spectra were normalized to the same oxidized spectrum (solid line).

Aerobic titrations of G-AD with acyl-CoA substrates from C₆CoA to C₁₄CoA all showed breaks in the plots of A₄₄₈ and
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MOLES C\(_\text{CoA}\)/MOLE G-AD FLAVIN

**Fig. 4.** Anaerobic titration of G-AD with C\(_6\)CoA. G-AD used was the same as in Fig. 3, A, C, D, and E. Anaerobic titration was performed as described under "Methods." For inset: \(\Delta\), 750 nm; C, 550 nm; D, 505 nm; and E, 448 nm.

**A**

\(A_{448}\) versus moles of substrate added. While any one plot, because of the scatter of points, may not permit rigorous quantitation of the stoichiometry of complex formation between acyl-CoA and enzyme flavin, examination of all of the curves in Fig. 3 and elsewhere in this paper strongly suggests formation of 1:1 complexes. It was not possible to determine spectral binding constants from these plots since binding appeared to be very tight even at micromolar enzyme concentrations. When substrate is in excess, as is shown in Fig. 1, or when air is excluded (Fig. 4), the spectra appear to show more bleaching at 490 nm than those obtained by the aerobic titrations shown in Fig. 3. This suggests that some reoxidation of the flavin occurs during aerobic titration. However, the stoichiometry and extent of bleaching appears unaltered by the presence of air. Earlier studies on the binding of acyl-CoA to the dehydrogenase also indicated some reoxidation of flavin after several manipulations, but labeled substrate was still bound (11). As a control for possible detergent or other nonspecific effects of the substrates, samples of G-AD were treated with the following substances: 30 to 50 \(\mu\)M lysophosphatidylcholine (at or above the critical micelle concentration (27)), dihydrosphingosine, sarcosine, \(C_6\) fatty acid, or \(C_6\) fatty acid plus CoA. None of these materials produced any changes in the spectrum, except for \(C_6\) fatty acid which caused the sample to become turbid. This turbidity was unaffected by subsequent addition of equimolar CoA. Thus, production of absorbance changes shown in Figs. 1 to 4 appears to require the fatty acyl-CoA ester.

**Stopped Flow Studies of Reactions between G-AD and Fatty Acyl-CoA Substrates of Varying Chain Length**—Absorbance changes seen upon addition of acyl-CoA to G-AD were followed in the stopped flow spectrophotometer. Fig. 5 shows a typical oscilloscope display and first order plot of \(A_{448}\) versus time obtained after mixing G-AD and C\(_6\)CoA. Similar biphasic first order plots were obtained from data of shown. The lines shown for each plot were drawn by eye. No change was seen in the long wavelength absorbance for each addition over 5 to 10 min, but a small increase in \(A_{448}\) was seen. Two lots of G-AD with \(A_{275}/A_{448} = 5\) and 5, respectively, were pooled and used for all titrations except for B. The sample used in B was similar (\(A_{275}/A_{448} = 6.7\)). For all experiments: \(\Delta\), 750 nm; C, 550 nm; D, 505 nm; and E, 448 nm. Final concentrations of G-AD flavin were 3 to 5 \(\mu\)M (\(A_{448}\)).
all chain lengths tested, from C₆ to C₁₀CoA. In all experiments, the more rapid first order phase encompassed about 70% of the total absorbance change. Since the critical micelle concentration is expected to increase by a factor of about 10 for every two carbons removed from the chain (28), the concentration of C₆CoA used, 20 μM, may be close to its critical micelle concentration. The remainder of the acyl-CoA derivatives used were probably well below critical micelle concentration so that the enzyme may be expected to be interacting with monomer. C₁₀CoA was not tested because the concentrations required would have been well above its critical micelle concentration of 3 to 4 μM (29).

Table I presents the first order rate constants for absorbance changes at 448 and 550 nm, observed after rapid mixing of various fatty acyl-CoA substrates with G-AD, using several ratios of reactants. Changes at 550 nm were studied for chain lengths of 6 to 10; these chain lengths produced sufficiently large extinction changes (see Fig. 1). The fastest rate constant measured (448 nm) at 1:1 acyl-CoA/G-AD flavin (Table I) was observed with C₆CoA. The few observations at 550 nm generally agreed with those at 448 nm. Experiments with different ratios of C₆CoA/G-AD flavin or C₆CoA/G-AD flavin show that the rate constant does not increase with increasing substrate concentration. The observation of first order kinetics under circumstances where enzyme and substrate are mixed in approximately equimolar quantities suggests that a prior second order event (such as substrate binding to enzyme), occurred within the dead time of the stopped flow apparatus. The data of Table I also suggest that in some cases (see data for C₆CoA and C₆CoA) addition of excess substrate may be moderately inhibitory.

Fig. 6A shows a plot of the first order rate constants for the first phase of bleaching of G-AD at 448 nm versus chain length for C₆CoA. The data of Table I also suggest that in some cases (see data for C₆CoA and C₆CoA) addition of excess substrate may be moderately inhibitory.

**Table I**

| Substrate | Fatty acyl-CoA/G-AD flavin | \( k'_{1/2} \) (s⁻¹) | \( k'_{2/1} \) (s⁻¹) |
|-----------|---------------------------|-------------------|-------------------|
| C₆CoA     | 2                         | 2.2               | 1.2               |
|           | 4                         | 2.3               | 1.1               |
|           | 5                         | 3.0               | 1.0               |
| 25        |                           | 3.0               | 1.0               |
| 55        |                           | 1.0               | 1.0               |
| C₆CoA     | 1.25                      | 63                | 10^b              |
| C₆CoA     | 0.275                     | 198               | —                 |
|           | 0.33                      | 177               | —                 |
|           | 0.5                       | 118               | —                 |
|           | 0.60                      | 98                | —                 |
|           | 1.0                       | 98                | 12                |
|           |                           |                   | 80^b              |
|           | 4.0                       | 70                | —                 |
| C₆CoA     | 1.25                      | 115               | 13                |
| C₆CoA     | 1.25                      | 40                | 19                |
| C₆CoA     | 1.25                      | 6                 | —                 |

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* a, not measured, see text.

^ Average of two different values.

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**Fig. 5.** First order plot for bleaching of G-AD by C₆CoA (Aₓ). Stopped flow spectrophotometry was performed as described under "Methods." Data were obtained and analyzed as described under "Methodes." Three experiments shown on the plot, measured at different time scales (●, 2 ms/division; □, 10 ms/division; and ○, 20 ms/division). The inset shows a trace of the photograph of the oscilloscope display of two experiments recorded at 50 mV/division (ordinate) and 2 ms/division (abscissa). A sample of G-AD prepared as described under "Methods" was used (Aₓ/32/₆CoA = 7) at G-AD flavin concentration of 15 μM in one syringe and C₆CoA at 15 μM in the other syringe.

**Fig. 6.** A, Chain length dependence of first order rate constants for G-AD reduction. First order rate constants were determined as in Fig. 5 (also shown in Table I). Multiple experiments at different time scales were employed for all combinations. Different lots of G-AD were used with Aₓ/32/₆CoA = 6 to 10. Data were analyzed as in Fig. 5 (see also "Methods" and "Results"). Data obtained at 448 nm is shown. Each point represents the rate constants seen when approximately equal amounts of substrate and G-AD [flavin] were mixed. Concentrations of G-AD flavin and acyl-CoA were as in Table I. B, Chain length dependence on rate of ETF-mediated DCPIP reduction by G-AD and of KₑETF. Catalytic turnover was determined as described under "Methods" and "Results," using the DCPIP reduction assay. Final concentrations of all reactants were: G-AD[flavin] = 8.3 × 10⁻⁸ M (Aₓ), Aₓ/32/G₆CoA = 6.5; [acyl-CoA] = 200 μM for C₆CoA, 24 μM for C₄CoA, 28 μM for C₅CoA, 21 μM for C₆CoA, 18 μM for both C₇ and C₈CoA, and 29 μM for C₉CoA. ETF concentrations were varied at constant [G-AD] to determine apparent Kₑ values, and turnovers were obtained by extrapolation of 1/₉ versus 1/ETF [flavin] to "infinite" ETF[flavin] to determine maximal turnover.
of the substrate. Comparison with Fig. 2 reveals that, with the exception of C10CoA, the first order rate constant for bleaching of G-AD at 448 nm by a given substrate correlates rather well with the extent of the absorbance change at 448 or 550 nm caused by that substrate.

Effect of Fatty Acyl Chain Length upon Turnover and $K_m$ of ETF—If turnover (Fig. 6B) in the DCPPIP assay system at saturating ($\sim 10 \times K_m$) acyl-CoA and “saturating” ETF (by extrapolation of $1/v$ versus $1/[ETF flavin]$) (9) can be regarded as reflecting the rate constants for the overall reaction, it can be seen (Fig. 6B; note differences in the scales of the ordinates of Curves A and B in Fig. 6) that the rates of bleaching of G-AD flavin by C12C16CoA are not rate-limiting and that turnover of the DCPPIP reduction system is much slower than the bleaching of G-AD flavin by the best substrates. Nevertheless, the DCPPIP turnover for each chain length tested appears to parallel the rate constant for the bleaching of the flavin. For substrates of chain length below 6 and above 12, the first order rate constant for bleaching of G-AD by substrate is very slow and appears to become rate-limiting. C12CoA (or possibly C18CoA) was previously suggested to be the best substrate, based on activity in the DCPPIP assay (30). Fig. 6B also shows that, in general, the apparent $K_m$ for ETF (determined graphically from a $1/v$ versus $1/[ETF flavin]$ plot) tends to follow the turnover numbers up to a chain length of about C12.

Dependence of Spectrum of G-AD-C16CoA Complex upon Temperature—The above results, that the chain length of the substrate appears to affect the extent of flavin bleaching and therefore presumably the efficacy of electron transfer from substrate to enzyme flavin, seems to be in conflict with the observations of tight (1:1) binding of all substrates (Ref. 11, and see Figs. 3 and 4 above). The data might be explained if one assumes that there are at least two regions in the binding site for substrate: one for the CoA head group, which is tightly bound, and at least one hydrophobic region which interacts with the hydrocarbon chain. Tight binding of the acyl-CoA head group (spectrally invisible) with varying degrees of interaction of the hydrophobic tail (dependent on chain length) might then account for the chain length dependence of the absorption changes.

The possibility that hydrophobic (or lipophilic) interactions are involved in the binding of acyl-CoA substrates was investigated by studying the effects of temperature on the spectrum of a G-AD-acyl-CoA complex. C16CoA was chosen for the experiment since the spectral changes induced by that substrate are intermediate between the “best” and the “worst” substrates.

G-AD was mixed with C16CoA at 26°C and placed in both the sample and reference beams of the spectrophotometer; the base-line spectrum was recorded. The temperature of the sample compartment was then dropped to 8.5°C and the difference spectrum between G-AD-C16CoA at 8.5°C (sample cell) and at 26°C (reference cell) was recorded (heavy trace). The temperature of the sample cell was then raised to 37°C and the difference spectrum was recorded (reference cell remained at 26°C) (lighter trace). The C16CoA used in this experiment was the kind gift of Dr. Robert Bell of Duke University (synthesized in his laboratory).

These changes, which corresponds to the long wavelength isosbestic point seen for all G-AD-acyl-CoA complexes except C18 and C20. These results are consistent with the hypothesis that hydrocarbon-hydrocarbon interactions, which are readily disrupted by heat, are contributing to the binding of acyl-CoA substrates to G-AD.

The Reduction State of the Flavin in G-AD—The reduction state of the flavin in the G-AD-acyl-CoA complex is not known with certainty. It was reported (31, 32) that G-AD could be reduced to a semiquinone form with small amounts of dithionite, and that addition of $\Delta^n$-CoA to the semiquinone resulted in a spectrum very similar to that seen when C10CoA-G-AD is added to oxidized G-AD. However, no electron paramagnetic resonance (EPR) signal was seen with C10CoA-G-AD complex (31, 32). We have also looked for an EPR signal for flavin radical in our preparation, but find none under the conditions employed.

When G-AD was photoreduced anaerobically in the presence of EDTA but without acyl-CoA substrate, a spectrum suggesting a neutral flavin semiquinone was observed (Fig. 8 and see Ref. 29). However, inspection of the spectrum suggests that only part, perhaps half, of the flavin has been converted to this state. One to three hours of irradiation under conditions...
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ETF is known to accept electrons from G-AD (14, 15). Little is known however, regarding the fate of the dehydrogenated substrate during or following electron transfer. Since special mechanisms may be required for the handling of substrates/products with large hydrophobic regions (e.g. as seen for fatty acid synthetase, Refs. 33 to 35), and because intermediate chain length acyl CoA's are not found in vivo during β oxidation (36), and also because of the reported high affinity of both saturated and unsaturated acyl-CoA for dehydrogenase (11, 18) the possibility that product transfer from G-AD to ETF might accompany electron transfer was investigated.

Photoreduction of ETF—ETF can be photoreduced anaerobically (Fig. 9) to a form having a spectrum typical (25) of anionic (or "red") flavosemiquinone before proceeding to "full" reduction (not shown). Fig. 9 shows changes in absorbance for three time points during the irradiation. Fig. 9 (inset) shows a plot of changes in absorption and fluorescence during the photoreduction of another sample of ETF. The apparent lag in the optical changes seen in Fig. 9 (inset) is probably due to residual O₂ and the fluorescence point at 10 min is probably an artifact. Reduction to the (anionic type) semiquinone usually requires 30 to 60 min under the conditions described under "Methods," and the semiquinone is stable anaerobically in the dark. This apparent semiquinone form has less than 10% of the original ETF fluorescence. Further irradiation (usually 30 to 60 min more) is required for full reduction (not shown). The fully photoreduced spectrum resembles the dithionite-reduced spectrum in the 450 nm region (7, 8) and the extinction of the 375 nm band is about 70% of the extinction of the oxidized flavin.

Titrations of ETF Semiquinone with α,β-unsaturated Acyl-CoA Derivatives—When ETF semiquinone produced as above is titrated with Δ⁵,⁵-C₆CoA, a long wavelength absorption band appears in the spectrum, as well as a concomitant increase in A₃₇₃ and E₄₉₅ (E = fluorescence emission) and decrease in A₄₉₅ (Fig. 10). This spectrum resembles (at shorter wavelengths) the characteristic double-banded ETF spectrum of the oxidized enzyme but has a strong absorption band at longer wavelengths, extending beyond 750 nm. The increase at 750 nm indicates that the final spectrum observed cannot simply be oxidized enzyme but has some of the character of a charge-transfer complex. It can be seen from the inset of Fig. 10 that maximum absorbance changes have occurred at 1 mol of substrate/mol of flavin semiquinone. In another experiment, however, the stoichiometry appeared less than 1 (0.6). Thus, it may be conjectured that the complex observed can be one which contains the elements of 2 mol of oxidized flavin and 1 to 2 mol of reduced substrate, with the distribution of electrons between substrate and flavins undefined. Essentially similar titration data were obtained in another similar experiment, using a similar sample of ETF, described for Fig. 9 (inset). Ω, A₃₇₃; □, A₄₉₅; ■, A₃₅₀ × 10; Δ, E₄₉₅; and ○, A₃₇₃.

The uncertainty in stoichiometry reflects difficulties in assessing the concentration of enzymatically active thioester in these α,β-unsaturated fatty acyl-CoA's. This problem is particularly acute in the experiments with Δ⁵,⁵-C₆CoA; in these experiments, sharp endpoints for the titrations were observed, but stoichiometry could not be assigned.
rated acyl-CoA binds to photoreduced ETF. Titration of photoreduced ETF (having a spectrum approximately equivalent to that of the photoreduced species seen in Fig. 9) with saturated C₆CoA rather than Δ²₃-C₆CoA produced some increase in 450 nm absorbance, but no long wavelength absorbance was seen. Absorbance changes were slow and there was no sharp break. Addition of Δ²₃-C₆CoA to oxidized ETF produced no detectable changes in the spectrum; preliminary attempts to study binding by equilibrium dialysis, gel filtration, or by ultrafiltration have not yet yielded usable data.

**Titration of ETF with C₆CoA in the Presence of G-AD—**
The data of Fig. 11 show the results of titration of ETF with C₆CoA (saturated) in the presence of catalytic quantities of G-AD. Since the experiment described in Fig. 10 started with ETF partially reduced by light, the total number of electrons introduced into that system was limited to those derived from the photoreduction. In the experiment described in Fig. 11, the potential extent of reduction of ETF is much greater since an "unlimited" quantity of reduced substrate, C₆CoA, is made available. Examination of the absorbance changes at 438 nm and of fluorescence emission at 490 nm (Fig. 11, inset) shows that extrapolation of the initial and final points of the titration provides an intersection indicating a stoichiometry of 1 mol of C₆CoA/mol of ETF flavin. However, intermediate points suggest that the reduction may be occurring in more than one phase.

The long wavelength absorbance (see inset) becomes maximal at 1:1 stoichiometry, suggesting that in the final form, each flavin binds 1 mol of acyl CoA, presumably (but not demonstrated), Δ²₃-C₆CoA (unsaturated). The absorbance at 375 nm shows a small, persistent, and reproducible increase after the first increments of titrant, suggesting transient formation of anionic semiquinone.

We wished to study the effects of addition of C₆CoA to a system which contained ETF and G-AD in more nearly equal amounts since this may be expected to represent better the conditions of physiological electron transfer (cf. Ref. 10). Of the many ratios of ETF to G-AD which could have been chosen, a 1:1 mixture of ETF and G-AD was used. Since G-AD contains four flavins/mol and ETF contains two, the ratio of G-AD flavin to ETF flavin is 2:1 in this experiment. Examination of the absorption spectra seen during the titration (Fig. 12A) shows an apparent isosbestic point at about 495 nm. However, close examination of the spectra at 340 nm shows that more than two flavin species must have been present. Thus, the apparent isosbestic point at 495 nm may be fortuitous. It should also be noted that the spectrum seen after addition of 11.8 μM C₆CoA to the system represents the point of the titration and that this amount of acyl-CoA is more than enough to furnish the electrons needed for full reduction of the system. Higher quantities of C₆CoA were added, but these data are not shown in Fig. 12A; the higher concentration points in Fig. 12C show the results of these additions. Examination of Fig. 12B shows that fluorescence of ETF is decreased by addition of substrate to this system. Fig. 12C shows that this change, when extrapolated from earlier titration points, indicates a stoichiometry of 1 mol of substrate/mol of ETF flavin; there is some indication of a second phase during this titration. These fluorescence data strongly suggest that the electron first added to the system appear ultimately in the ETF rather than the G-AD flavin. The absorption data obtained at 440 nm show that these spectral changes are multiphasic and, although extrapolation of the initial points gives approximately 1 mol substrate/0.5 mol of total flavin, the maximal ΔA₄₄₀ is not achieved until 1 mol of substrate/1 mol of total flavin has been added. The changes at 550 nm are gradual and maximize at a stoichiometry of 1.2 mol of C₆CoA/mol of total flavin as do those at 750 nm (not shown). Changes at 375 nm show an initial increase, followed by a gradual decrease with a stoichiometry of approximately 1 mol of C₆CoA/mol of total flavin.

**Fig. 11.** Anaerobic titration of ETF with C₆CoA in the presence of catalytic G-AD. The G-AD used was the same as in Fig. 8; final concentration, 0.15 μM [flavin]. A sample of ETF of A₄₅₀ = 7 was used; final concentration, 6.5 μM flavin. Anaerobic titration was performed as described under "Methods." Inset, data from titration. Fluorescence changes were also measured during the titration. Δ, A₄₅₀; ○, A₁₃₆; ▲, A₄₅₀ and ○, A₃₅₀ × 10.

**Fig. 12.** Anaerobic titration of 1:1 G-AD and ETF with C₆CoA. G-AD and ETF were the same samples as in Fig. 11. Anaerobic titration was performed as described under "Methods." A, absorbance changes during titration; B, fluorescence changes during titration; C, data of A and B. Three x-ordinates are used to relate optical changes to stoichiometry based on total flavin, ETF flavin, and G-AD flavin (see text). Changes at 750 nm were the same as at 550 nm.
These studies have dealt with the formation and nature of complexes between saturated and desaturated fatty acyl-CoA derivatives with general acyl dehydrogenase and with ETF. There is reason to believe that these complexes may be catalytically meaningful. Since they are formed with apparently high binding constants for both enzymes and with small whole number stoichiometry (probably 1 substrate molecule/flavin), their composition suggests specific rather than non-specific interaction. In the case of general acyl dehydrogenase, stopped flow studies have indicated that formation of complexes between G-AD and fatty acyl-CoA occurs sufficiently rapidly to permit inclusion of this step in a catalytic scheme. The demonstration of adequately fast rates has not yet been made for ETF; indeed, preliminary stopped flow studies, not reported in detail, indicate that the reaction is relatively slow, on the order of a half-time for formation of about 5 min. Despite this slow rate, we are not prepared to discard the notion that the observed complexes with ETF are not without catalytic meaning: physiologically, interaction between G-AD, ETF, and subsequent members of the electron transport chain takes place in a highly specific milieu which has not been reproduced in these experiments. The fact that ETF forms complexes which appear to be 1:1, and that there seems to be a strong preference for measurable association between an oxidized form of substrate and a reduced form of ETF tends to argue that these complexes may well merit continued consideration as parts of a catalytic scheme.

What is the nature of these complexes? It has long been recognized that the acyl-CoA dehydrogenases form distinct spectral species when mixed with their acyl-CoA substrates (2-6, 9-14, 18-21), and that the binding of acyl-CoA to dehydrogenase appears tight. The present studies of the dehydrogenase with a wide range of even-numbered chain length substrates show that the extent of absorbance changes caused by addition of substrate to G-AD appear highly dependent upon chain length. The resulting spectra, however, are qualitatively similar (Figs. 1 and 2), although in the case of C6-CoA and C8-CoA, a new transition at 505 nm appears. The magnitudes of the perturbations in the spectrum of G-AD elicited by each acyl-CoA substrate are generally correlated with the first order rate constants for the first phase of the bleaching of the flavin, with the exception of C8-CoA (Figs. 2 and 6A and Table I). Similar correlations between absorbance changes and rate of DCPIP reduction for C8, C10, C12, and C14-CoA were previously made (13, 18, 32).

The oxidation-reduction state of the long wavelength-absorbing G-AD acyl-CoA complexes is not clear from the present studies. The species has previously been suggested to be a semiquinone (32), but no EPR signal has been observed either by us or by other workers. It is possible that correct conditions for observation of signal have not been achieved. Addition of dithionite does not abolish the long wavelength absorbance of the complexes (not shown), even though it results in a small (~10%) additional decrease in 448 nm absorbance (9, 21). The 1:1 stoichiometry together with the persistence of the long wavelength absorption band after dithionite addition and the absence of an EPR signal indicate that the species seen upon addition of acyl-CoA substrate to G-AD does not represent simply a typical semiquinone. The spectra suggest some contribution from a charge-transfer interaction, but we are not prepared to define the precise oxidation-reduction state of the interacting components. Some covalent flavin-substrate complexes have been reported (97, 38) to have long wavelength absorbance. In summary, although the exact distribution of electrons cannot be assessed, it seems clear that the G-AD-acyl-CoA intermediates are tight, specific complexes.

The chain length dependence of interactions of acyl-CoA substrates of varying chain lengths with G-AD can be interpreted according to Equation 2. Despite the observation of tight 1:1 complexes with all substrates, both the magnitude of the spectral changes and their rates of appearance depend upon chain length of substrate. However, the spectra produced with all substrates tested appear to be qualitatively similar with similar ratios of $\Delta A_{448}/\Delta A_{600}$, suggesting the formation of different quantities of the same species. The latter finding suggests a chain length-dependent equilibrium for formation of the long wavelength species and supports our conclusion that the finding of a tight 1:1 binding for all substrates. This apparent paradox can be resolved by proposing the following physical model for Equation 2: ES represents a rapidly formed, tightly associated complex of the acyl-CoA head group with a specific site on the enzyme and is not accompanied by bleaching at 450 nm or production of long wavelength absorbance. Interaction of the hydrocarbon chain with another region of the active site, represented by E*-S', is less tight, depends on the chain length of the substrate, and is accompanied by the spectral changes. Such a model accounts for the observed 1:1 stoichiometry (tight binding to the acyl-CoA site), and also for the observed chain length-dependent differences in extents of spectral changes since different hydrocarbon chain lengths could alter the equilibrium between $ES \xrightarrow{k_2} \xrightarrow{k_3} E^*S', without altering the stoichiometry. The model also accounts for the chain length-dependent rate differences for production of the long wavelength species since $k_2$ might be expected to be influenced by chain length. The idea that binding of acyl-CoA to G-AD consists of a primary association of the acyl-CoA moiety, followed by a weaker, chain length-dependent equilibrium association of the hydrocarbon chain is also suggested by the failure of C16 fatty acid to produce changes similar to those of C14-CoA. The fatty acyl side chain alone is insufficient for binding since a mixture of fatty acid plus CoA, as previously mentioned, does not produce spectral perturbation.5

This model is also supported by the experiment in Fig. 7, in which complexation of a 1:1 mixture of C14-CoA with G-AD was enhanced at low temperature and diminished at higher temperature. Such a temperature dependence may be interpreted as the facile disruption of hydrocarbon—hyd
drocarbon bonds by heat. These studies have demonstrated that ETF as well as G-AD can form complexes with derivatives of fatty acyl-CoA substrates. The data shown in Figs. 10 to 12 and discussed under "Results" show that tight complexes between some reduced form of ETF and oxidized acyl-CoA do occur and suggest a specific association between their components. When G-AD is present during titration of ETF with C6-CoA (saturated), the transient appearance of increases in 375 nm absorbance suggests (Figs. 11 and 12) that ETF semiquinone is involved in the electron transfer. Under equilibrium conditions, ETF reduction appears to occur prior to full reduction of the dehydrogenase (Fig. 12) even though the electrons are presumably being transferred via the G-AD. This suggests a more positive oxidation reduction potential for the ETF. Long wavelength absorbance (Figs. 10 to 12) suggests participation of a charge-transfer complex.

These data are consistent with the following model: electrons introduced into the G-AD + ETF system are transferred via G-AD initially to ETF. Formation of semiquinone can be observed during the early phases; this then decays, and subsequent events all point to the gradual formation of the reduced complexed forms of all the flavins. Acyl-CoA transfer to reduced ETF does not occur in parallel with electron transport to ETF since appearance of 570 nm absorbance does not parallel loss of ETF fluorescence but increases gradually as does that at 550 nm (Fig. 12A). The end point of 1 mol of acyl-CoA added to 1 mol of total flavin suggests that both ETF and G-AD flavins can bind acyl-CoA. Since this process occurs continuously during the titration, we cannot specify from the data of Fig. 12 which oxidation state of acyl-CoA interacts with which oxidation state of the two enzymes. Nevertheless, the final acyl-CoA complexes involve flavins which are reduced rather than oxidized.

Under normal physiological conditions, free acyl-CoA intermediates of β-oxidation cannot be isolated from tissue (36). It is recognized that fatty acyl-CoA derivatives interact strongly with enzymes of the fatty acid oxidizing system (36, 38, 40) and with the enzymes of the fatty acid synthetase complex (32–35). Thus, the mechanism of catalysis of fatty acid oxidation must accommodate the process not only of the formation of ES complexes but also of the dissociation of enzyme-product complexes.

In the case of the complex between acyl-CoA and dehydrogenase, this dissociation could, in theory, be accomplished by the simple release of the desaturated derivative upon the removal of the reduced dehydrogenase. But our studies, as well as those of other laboratories (11, 18) indicate that, without ETF, this does not occur.

The fact that a reduced form of ETF has now been shown to form "tight" complexes with ω,ω-desaturated acyl-CoA substrates permits consideration of the possibility that ETF functions in fatty acid oxidation by taking product as well as electrons from dehydrogenase-substrate complex. If this proves to be so, the question of how the ω,ω-desaturated product is removed from ETF would then emerge. Displacement of product by succeeding molecules of substrate could provide a general mechanism (see also Ref. 41); specific interactions among other enzymes of fatty acid oxidation (enoyl hydratase, etc.) and intermediate substrates have yet to be explored.

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