Identification of 2-Tetradecylglycidyl Coenzyme A As the Active Form of Methyl 2-Tetradecylglycidate (Methyl Palmoxirate) and Its Characterization As an Irreversible, Active Site-directed Inhibitor of Carnitine Palmitoyltransferase A in Isolated Rat Liver Mitochondria*

(Received for publication, December 23, 1983)

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Methyl-2-tetradecylglycidic acid (MeTDGA) has been hypothesized to inhibit fatty acid oxidation by irreversible, active site-directed inactivation of carnitine palmitoyltransferase A after being converted to TDGA-CoA. Using synthetic TDGA-CoA, this hypothesis has been confirmed. Assessing enzyme inhibition in an isolated rat liver mitochondrial system, TDGA-CoA (synthetic or enzyme prepared) was more potent than TDGA or MeTDGA and retained activity in the absence of CoA or Mg+-ATP. It inhibited palmitoyl-CoA but not palmitoyl carnitine oxidation. Enzyme inactivation was exponential, stereospecific, and fast (t(1/2) = 38.5 s with 100 nM (R)-TDGA-CoA). TDGA-CoA was identified as a complexing type irreversible inhibitor (K is ~ 0.27 μM) by the double reciprocal relationship between the pseudo-first order inactivation rate and its concentration, by the inverse dependence of the second order rate constant on its concentration, and by the independence of the first order rate from the enzyme concentration. Palmitoyl-CoA, CoA, and malonyl-CoA protected the enzyme, while L-carnitine and palmitoyl-l-carnitine were without effect. [3-14C]TDGA-CoA labeled a protein, M, = 90,000, with a time course which paralleled that of enzyme inhibition; maximum specific binding was 16 pmol/mg of mitochondrial protein.

Long chain fatty acids must first enter the mitochondria by a carnitine-dependent process (1, 2) to be available for β-oxidation. Since its operation was first hypothesized by Fritz and Yue (3), this process has been shown to be composed of three distinct steps: (a) transfer of the fatty acyl moiety from CoA to carnitine by an acyltransferase located on the outer surface of the inner mitochondrial membrane (CPT-A); (b) translocation of acylcarnitine from the cytosol into the mitochondrial matrix; and (c) transfer of the acyl group to intramitochondrial CoA by CPT-B. Work in this area has been frustrated by the difficulties characteristic of membrane-associated enzyme systems (1) but motivated, at least in part, by the suggestion that the carnitine palmitoyl transferase system was rate-limiting for long chain fatty acid oxidation (4, 5). Recently, McGarry and Foster (6) have championed the outer enzyme as a key regulatory site for fatty acid oxidation with control being attributed to changes in tissue levels of malonyl-CoA. This proposal has generated significant controversy, and, as a result, the carnitine palmitoyltransferase system continues to be the subject of much active investigation.

As with many enzymes, the discovery of inhibitors has contributed to the understanding of the carnitine palmitoyltransferase system (for reviews see Refs. 1 and 7). Inhibition by 2-bromopalmitoyl-CoA, for example, has been used to identify the "outer" enzyme after solubilization and fractionation (8) and has been offered as evidence that CPT-A and CPT-B are different enzymes (8, 9). Competitive inhibitors such as mersalyl and (+)-decanoylcarnitine have been particularly valuable in the study (10) and assay (2) of the carnitine-acylcarnitine translocase. Newer, fluorescent analogs of acyl-CoA and acylcarnitine promise to add another dimension to the work in this field (11). Not surprisingly, inhibitors have been pursued for their potential therapeutic value with (+)-acylcarnitine being discussed relative to the treatment of diabetic ketoacidosis (12, 13) and oxefencine for use in ischemic heart disease (14).

Randle and coworkers (15, 16) hypothesized that excessive oxidation of fatty acids might be a significant factor in diabetes and other diseases by virtue of its restraint of glucose utilization. This hypothesis suggested a greater potential for inhibitors of fatty acid oxidation, and hence of the carnitine palmitoyltransferase system, as the primary treatment of hyperglycemia. Consistent with this hypothesis, the palmitate analog TDGA and its methyl ester, MeTDGA, have been reported to be effective hypoglycemic and antiketotic drugs in animals (17, 18) and man (19). Extensive work has shown them to specifically block long chain fatty acid oxidation in a variety of tissues and species (reviewed in Ref. 20), and it has been proposed that TDGA and MeTDGA produce their effect with CPT-A designating the enzyme on the outer surface of the inner mitochondrial membrane and CPT-B the intramitochondrial enzyme. Other authors have employed different conventions: "outer" and "inner" transferase (Ref. 6), CPT-I and CPT-II (c.f. Ref. 33); and CPTa and CPTb (Ref. 47).
by irreversible inhibition at the active site of CPT-A after being converted to TDGA-CoA (20, 21). With the availability of the synthetic-CoA ester, we have been able to directly address this proposed mechanism of action. Our results, which are presented here, prove that this hypothesis is correct.

EXPERIMENTAL PROCEDURES

RESULTS

Requirement for CoA Ester Formation—Previous work in vitro established that TDGA was a substrate for both microsomal (41) and mitochondrial acyl-CoA synthetases with circumstantial evidence (21) indicating that the action of these enzymes might be mandatory for TDGA to serve as an inhibitor of CPT-A. This requirement was demonstrated as shown in Fig. 1; both Mg-ATP and CoA are necessary for the activity of TDGA or MeTDGA, but not for TDGA-CoA.

This requirement for CoA ester formation was also reflected in the comparative potencies of TDGA, MeTDGA, and TDGA-CoA against CPT-A. While TDGA and MeTDGA were indistinguishable under our assay conditions, TDGA-CoA prepared synthetically (Fig. 2) and enzymatically (results not shown, IC$_{50}$ = 25.8 nM) displayed superior activity. By parallel line analysis, the CoA ester was greater than 1.8 times as potent as TDGA.

Effect of TDGA-CoA on Mitochondrial Fatty Acid Oxidation—Previous work in hepatocytes (41) using MeTDGA had shown inhibition of ketone and CO$_2$ production from palmitate, but not palmitoyl carnitine or octanoate, suggesting a specific effect on CPT-A. To confirm this specificity using the CoA ester, isolated mitochondria were preincubated for 5 min with or without 1 mM TDGA-CoA after which the state 3 oxidation of palmitoyl-CoA or palmitoyl carnitine was determined. While the oxidation of palmitoyl-CoA was inhibited roughly 80% under these conditions (11 nanoatoms of O/min/mg versus 53 for control), that of palmitoyl carnitine was not affected (82 versus 77 for control).

Stereospecificity of CPT-A for Inhibition—Of the two possible configurations at the oxirane ring, only (R)-TDGA-CoA as inhibitors of CPT-A was compared to the CoA ester of the mixed isomers as described in the legend to Fig. 2. Parallel line analysis was employed to estimate the potency of (R)-TDGA-CoA relative to (R),(S)-TDGA-CoA as 1.7.

Kinetics of CPT-A Inactivation—As had been previously observed with the free acid (20, 21), TDGA-CoA inactivation of CPT-A followed an exponential time course characteristic of irreversible inhibitors. As only one of the two stereoisomers was active, further characterization of the inactivation process utilized (R)-TDGA-CoA.

In our system, 100 mM (R)-TDGA-CoA inactivated the mitochondrial CPT-A with a pseudo-first order rate of 0.018 ± 0.004 s$^{-1}$ (S.E., n = 6). Initial attempts to further study the inactivation as a function of inhibitor concentration yielded nonlinear double reciprocal plots, a problem which was attributed to acyl-CoA hydrolase breakdown of the inhibitor. The impact of this appeared greatest at low inhibitor concentra-

- Portions of this paper (including "Experimental Procedures") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-3619, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
- M. T. Ryzlak and G. F. Tutwiler, unpublished observation.
tions. Because the inactivation process proved too fast to be studied at higher inhibitor concentrations ($t_{1/2} = 38.5 \, \text{s} \, \text{at} \, 100 \, \text{nM} \, (R)-\text{TDGA-CoA}$), our solution was to include palmitoyl-CoA during the inactivation phase to protect the inhibitor. Under these conditions (10 $\mu$M palmitoyl-CoA), a typical double reciprocal relationship was reproducibly observed between the pseudo-first order rate and the inhibitor concentration (Fig. 4). That the inactivation process involved an initial, reversible association between the inhibitor and the enzyme (i.e. complexing type irreversible inhibition (42)), as opposed to following a direct second order mechanism, was indicated by the inverse dependence of the second order rate ($k/|I|$) on the inhibitor concentration (Fig. 4, inset). This mechanism was further supported by finding that the rate of inactivation was independent of the enzyme concentration (results not shown). Having established that it was a meaningful parameter, an association constant ($K_i$) of 0.27 $\mu$M was calculated for (R)-TDGA-CoA (see the legend to Fig. 4).

Protection of CPT-A from (R)-TDGA-CoA Inactivation—As a long chain fatty acyl-CoA analog, it seemed likely that (R)-TDGA-CoA was acting at the active site of CPT-A. Evidence to support this possibility was obtained by looking at the ability of various natural compounds to protect against the inactivation produced by 100 nM (R)-TDGA-CoA. As can be seen in Table I, palmitoyl-CoA, CoA, and malonyl-CoA all slowed the inactivation rate at 10 $\mu$M, while 400 $\mu$M L-carnitine and 10 $\mu$M palmitoyl carnitine failed to influence the inactivation process.

The protective effect of the natural substrate, palmitoyl-CoA, against the inactivation by 100 nM (R)-TDGA-CoA was

![Fig. 4. Kinetic characterization of (R)-TDGA-CoA as a complexing type irreversible inhibitor of CPT-A. The pseudo-first order inactivation by 20, 50, 100, 200, and 500 nM (R)-TDGA-CoA was determined in the presence of 10 $\mu$M palmitoyl-CoA which was added to reactions at the same time as the inhibitor. The double reciprocal plot was generated by appropriate linear regression of the inactivation rate against the inhibitor concentration. The x intercept ($1/(K_i + [S]/K_i)$) was used to calculate the $K_i$ for (R)-TDGA-CoA (0.27 $\mu$M) by substituting $[S] = 10 \, \mu$M and $K_i = K_i = 27 \, \mu$M (determined under our conditions). The reciprocal of the y intercept (0.036 $\text{s}^{-1}$) provided an estimate of the inactivation rate at saturating concentrations of (R)-TDGA-CoA. Inset, the second order rate was calculated, and the reciprocals (($1/K$) were plotted against the inhibitor concentration with the line determined by appropriate linear regression. The correlation was significant ($p < 0.01$, $r = 0.96$).](file)

![Fig. 5. Palmitoyl-CoA protection of CPT-A against inactivation by (R)-TDGA-CoA. The pseudo-first order inactivation rate produced by 100 nM (R)-TDGA-CoA was determined as described under “Experimental Procedures” except that coenzyme A was omitted from the reaction mixture. The indicated compounds in distilled water were added concurrently with the inhibitor to give the desired final concentration. Results are expressed as the percentage of the control (no competing compound) rate and are presented as mean ± SE with the number of determinations in parenthesis where appropriate.](file)

| Compound          | Concentration | Percentage of control rate |
|-------------------|---------------|----------------------------|
| L-Carnitine       | 400 $\mu$M   | 102.6                      |
| Palmitoyl-carnitine | 10 $\mu$M | 95.5 ± 14.9 (2)            |
| Palmitoyl-CoA     | 10 $\mu$M    | 53.7 ± 6.0 (2)             |
| Coenzyme A        | 10 $\mu$M    | 53.3                       |
| Malonyl-CoA       | 10 $\mu$M    | 29.1 ± 5.8 (2)             |

The amount of TDGA-CoA associated with the $M_r = 90,000$ protein was estimated from the radioactivity in the solubilized mitochondrial samples over that in the zero time controls. As
ular weight scale was established by linear regression of the migration of the markers against the log of their molecular weight. The marker proteins were ovalbumin (M_r = 45,000), bovine serum albumin (M_r = 92,500), and carbonic anhydrase (M_r = 31,000). The displayed molecular weight scale was established by linear regression of the migration of the markers against the log of their molecular weight.

Analysis of incubated mitochondrial samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The marker proteins were counted, and the increase in radioactivity over zero time determined. Tracks on the fluorograph described in the legend to Fig. 6 were scanned using a densitometer interfaced with an HP 3390A integrator, and the integrated density of the band at M_r = 90,000 plotted against time. For comparison, the time course of enzyme inactivation by 200 nM unlabeled TDGA-CoA is presented, with the plotted values representing the average of two determinations at each time point. B, increase in band intensity with time as determined by sodium dodecyl sulfate sample counting. The 5-μl aliquots taken prior to electrophoresis were counted, and the increase in radioactivity over zero time determined. Results were converted to picomoles of [3-14C]TDGA-CoA using the specific activity, and the average of duplicate determinations plotted against time. (Table I) as would be expected if the enzyme had to first act on TDGA-CoA as a substrate. The failure of palmitoyl carnitine to protect the enzyme from inactivation (Table I) also argues against such a suicide-type mechanism.

DISCUSSION

Confirming the hypothesis of Tutwiler et al. (20), we conclude that TDGA-CoA is the active inhibitor derived from either TDGA or MeTDGA and that TDGA-CoA specifically inactivates CPT-A by irreversibly binding at the active site through a stable, covalent bond.

It should be clear that the formation of a CoA ester is necessary for inhibitory activity. Building upon previous observations on the utilization of TDGA and MeTDGA as substrates by the microsomal acyl-CoA synthetase (41), on the required presence of ATP and CoA (21), and on the ability of microsomes to restore TDGA-sensitivity to stored mitochondria (20), the present results prove this point by demonstrating activity for TDGA-CoA in the absence of cofactors (Fig. 1), by documenting its superior potency relative to TDGA and MeTDGA (Fig. 2), and by establishing comparable activity for an enzymatically synthesized preparation of TDGA-CoA. What may not be quite as clear is whether TDGA-CoA itself is the actual inhibitor. As a close analog of palmitoyl-CoA, the possibility exists that this compound might undergo conversion to the carnitine ester, however, this is not supported by the evidence. Using intact mitochondria, the formation of TDGA-carnitine (i.e. by CPT-A) is not detectable (20). Furthermore, carnitine addition to our isolated mitochondrial system is not required for inactivation by TDGA-CoA to occur (in contrast to 2-bromopalmitoyl-CoA (7, 43)), and doing so does not increase the inactivation rate.

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**Fig. 6.** Inactivation of CPT-A by [3-14C]TDGA-CoA: analysis of incubated mitochondrial samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A, photograph of the dried 10% gel showing the protein staining with Coomassie Blue. The marker proteins (S) are phosphorylase B (M_r = 92,500), bovine serum albumin (M_r = 69,000), ovalbumin (M_r = 45,000), and carbonic anhydrase (M_r = 31,000). The displayed molecular weight scale was established by linear regression of the migration of the markers against the log of their molecular weight. B, photograph of the fluorograph obtained after exposure for 1 week to Kodak X-AR5 film at −70 °C. Positioning and size reduction are identical to that in A. The doublet at M_r = 90,000 may be an artifact of gel overloading, as this is not observed after O’Farrell two-dimensional analysis of labeled mitochondria (46). The faint appearance shown in Fig. 7B, this displayed the same time-related increase as seen for the band density in Fig. 7A. Based upon a maximum observed uptake of approximately 8 pmol for 500 μg of mitochondrial protein and assuming one binding site per M_r = 96,000 peptide, the amount of enzyme protein in mitochondria was estimated as 1.4 μg/mg.

**Fig. 7.** Inactivation of CPT-A by [3-14C]TDGA-CoA: time course of label accumulation. A, fluorograph band intensity versus enzyme inactivation. Tracks on the fluorograph described in the legend to Fig. 6 were scanned using a densitometer (E-C Apparatus Corp.) interfaced with an HP 3390A integrator, and the integrated density of the band at M_r = 90,000 plotted against time. For comparison, the time course of enzyme inactivation by 200 nM unlabeled TDGA-CoA is presented, with the plotted values representing the average of two determinations at each time point. B, increase in band intensity with time as determined by sodium dodecyl sulfate sample counting. The 5-μl aliquots taken prior to electrophoresis were counted, and the increase in radioactivity over zero time determined. Results were converted to picomoles of [3-14C]TDGA-CoA using the specific activity, and the average of duplicate determinations plotted against time.

With respect to the identification of TDGA-CoA as an irreversible inhibitor, its effect on CPT-A is consistently found to be progressive with time and to follow a typical exponential course (c.f. Figs. 5 and 7A). Furthermore, previous work using TDGA showed the inhibition could not be reversed by dialysis for up to 9 days (21). Such a stable inactivation argues against TDGA-CoA being a tight binding reversible inhibitor, a conclusion which is graphically illustrated by the persistence of the labeled inhibitor through sodium dodecyl sulfate solubilization and electrophoresis (Fig. 6). Together with these results, the kinetic results can only be interpreted as reflecting a complexing type, irreversible inactivation process.

The kinetics establish that TDGA-CoA is interacting at a specific site on the enzyme. That this site is the fatty acyl-CoA specific portion of the active site is demonstrated by the ability of the appropriate substrates to protect against the inhibitor (Table I). Both palmitoyl-CoA and CoA exhibit this ability as would be predicted, while neither carnitine nor palmitoyl carnitine provide any protection at the concentrations tested. As expected from its reported activity (6), malonyl-CoA, a reversible inhibitor of CPT-A which is competitive with palmitoyl-CoA (44, 45), displays the greatest potency against the inactivation process. Further examination of the protection afforded by palmitoyl-CoA (Fig. 5) supports our interpretation of these effects as representing competition for the active site.
We have identified TDGA-CoA as a specific inhibitor of CPT-A based upon its effect on palmitate (41) and palmitoyl-CoA oxidation but not the oxidation of palmitoyl carnitine. Studies in which the inhibitor (MeTDGA) is given orally to rats confirm this conclusion (46): only the enzyme activity released by digitonin treatment of the isolated liver mitochondria (CPT-A (9)) exhibits decreased activity in comparison to that from vehicle-treated rats. Based upon this specificity and upon the inhibitor’s irreversibility and site of action, it is not surprising that we are able to follow the inactivation of CPT-A by using the radioactive compound (Figs. 6 and 7). The protein labeled in these experiments displays \( M_r = 90,000 \). To our knowledge, the behavior of the purified rat liver enzyme on sodium dodecyl sulfate polyacrylamide has not been reported, although analysis of a partially purified complex containing CPT-A and exhibiting \( M_r = 450,000 \) (Sephadex G-200) did reveal 10 to 12 bands of unreported size (47). In comparison to enzymes from other species, the rat liver CPT-A appears to be heavier than either the calf (\( M_r = 76,000 \) (48)) or the ox (\( M_r = 59,000 \) (49)) liver enzymes.

Acknowledgment—We thank Maria Castagno for her assistance in preparing this manuscript.

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2-Tetradecylglycidyl-CoA is an Irreversible Inhibitor

Identification of 2-tetradecylglycidyl-2-cysteine A as the active form of methyl 2-tetradecylglycidyl-2-cysteine A (methyl palmitate) and its characterization as an irreversible inhibitor of 4-lipase activity in isolated rat liver mitochondria.

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Materials and Methods

2-Tetradecylglycidyl-CoA is an irreversible inhibitor of 4-lipase activity in isolated rat liver mitochondria. The assay in which 2-Tetradecylglycidyl-CoA was synthesized as previously described (29) and used in the same procedure described (29). 2-Tetradecylglycidyl-CoA was synthesized by the procedure.
Identification of 2-tetradecylglycidyl coenzyme A as the active form of methyl 2-tetradecylglycidate (methyl palmoxirate) and its characterization as an irreversible, active site-directed inhibitor of carnitine palmitoyltransferase A in isolated rat liver mitochondria.

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*J. Biol. Chem.* 1984, 259:9750-9755.