Yonetani-Theorell Analysis of Hepatic Carnitine Palmitoyltransferase-I Inhibition Indicates Two Distinct Inhibitory Binding Sites*

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Analysis of inhibitor studies indicates that carnitine palmitoyltransferase-I has two separate sites for inhibitor binding. One site is located on the cytoplasmic side of the mitochondrial outer membrane. Malonyl-CoA, the most important physiological inhibitor of carnitine palmitoyltransferase-I, binds primarily to this site, but it can also bind to another site. A second inhibitory site is located at the active site of carnitine palmitoyltransferase-I. Coenzyme A, a product/inhibitor of carnitine palmitoyltransferase-I, binds primarily to this site and can inhibit carnitine palmitoyltransferase-I at physiological concentrations, but can also attenuate malonyl-CoA inhibition. Analogs of malonyl-CoA and other simpler compounds containing two carbonyl groups but no coenzyme A moiety inhibit only at the cytoplasmic site, indicating that this site has an absolute requirement for two carbonyl groups but has no absolute requirement for a coenzyme A moiety. Inhibitors acting through the active site included the active-site-directed inhibitor (+)-hemipalmitoylcarnitininium. These studies support the existence of two regulatory binding sites for the control of hepatic fatty acid oxidation: (a) the active site, for regulation by the inhibitory binding of coenzyme A and acetyl-CoA, and (b) a separate regulatory malonyl-CoA-binding site that is physically separated from the active site.

Carnitine palmitoyltransferase (EC 2.3.1.21) activities are expressed in rat liver mitochondria both inside and outside the mitochondrial inner membrane barrier to the diffusion of coenzyme A derivatives of fatty acids (1). The inner carnitine palmitoyltransferase (II) resides on the inner aspect of the mitochondrial inner membrane (2, 3). The exact location of the outer mitochondrial carnitine palmitoyltransferase-I has been the subject of much debate over the past few years, but that question seems to have been resolved by Murthy and Pande (4), who have presented evidence indicating that this enzyme is located in the mitochondrial outer membrane. Carnitine palmitoyltransferase-I is regulated by malonyl-CoA, its physiological inhibitor (5), and its activity and sensitivity to inhibition by malonyl-CoA are altered by changes in physiological and pathophysiological conditions (6–11). Quantitatively, the most significant change that occurs with the onset of diabetes (10, 11) or the feeding-starvation transition (12) is a 10-fold increase in the apparent $K_i$ for malonyl-CoA.

The relationship between binding sites for acyl-CoA and malonyl-CoA has not been clear; it has not been established whether they bind at the same site, or whether they bind to the same polypeptide (13–17). Experiments using protease treatment of intact mitochondria have indicated that carnitine palmitoyltransferase-I inhibition by malonyl-CoA is diminished by proteases while activity is only minimally affected (4). We have reported the effects of protease treatment on several additional inhibitors of carnitine palmitoyltransferase, some of which have mechanisms of inhibition that are different from malonyl-CoA (18, 19). Those studies indicated that malonyl-CoA and 4-hydroxyphenylglyoxylate (HPG) lose their inhibitory potency during protease treatment, whereas inhibition by the substrate analog 2-bromopalmitoyl-CoA was found to be protected from protease action by the mitochondrial outer membrane (18, 19). Although HPG and malonyl-CoA seem quite unrelated in structure, both have their inhibitory sensitivity altered by fasting (12, 20), and it was of interest to see whether they would interact at the same inhibitory site.

Coenzyme A and acetyl-CoA inhibit carnitine palmitoyltransferase-I and are structurally similar to malonyl-CoA (13). It was of interest to ascertain whether these compounds inhibited by binding to the same site as malonyl-CoA. We have examined the relationship between binding sites by performing Yonetani-Theorell double-inhibitor analyses using different inhibitors of carnitine palmitoyltransferase-I. The data presented here suggest that malonyl-CoA inhibits by interacting with carnitine palmitoyltransferase-I in a different manner than would be expected from a simple competitive inhibitor binding at the active site.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria and Protease Treatment—Male, Sprague-Dawley rats (180–240 g) obtained from Harlan Industries (Indianapolis, IN) were fed Purina Rat Chow (Ralston Purina Co., Richmond, IN) and water ad libitum. Rats were killed by decapitation, and livers were removed rapidly for preparation of mitochondria. Pig livers for mitochondrial membranes were obtained from a local slaughterhouse immediately after the animals were killed. Intact mitochondria were isolated by the method of Johnson and Lardy (21), with the modifications previously published (19). Mitochondrial outer membranes were isolated by the method of Parsons et al. (22), and their purity was assessed as described previously (11). Protease treatment of mitochondria was

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The abbreviation used is: HPG, 4-hydroxyphenylglyoxylate.
indicate that coenzyme A and malonyl-CoA bind to two converging lines (23). However, after protease treatment, this inhibition was not altered by protease treatment. The data for the same site, but the experiment will yield two intersecting lines if the inhibitors bind at two independent sites (23). We used this procedure to study possible interactions of coenzyme A and HPG with malonyl-CoA in control and protease-treated mitochondria.

Two parallel lines are not expected here since coenzyme A is the two inhibitors before and after protease treatment, suggesting they inhibit studies. Fig. 3 shows inhibition of carnitine palmitoyltransferase by malonyl-CoA and HPG in control and protease-treated mitochondria at a fixed concentration of malonyl-CoA and increasing concentrations of HPG. These data indicate that the inhibitory effects of HPG and malonyl-CoA, especially at higher concentrations, are not additive as they were with coenzyme A and malonyl-CoA. After protease treatment, the inhibitory effects of both HPG and malonyl-CoA were almost completely eliminated in accord with previous observations (4, 18, 19). These plots of 1/v versus HPG concentration, showing parallel lines, are in agreement with two inhibitors competing for a single site (23). Curvature of the plot is due to cooperative inhibition (12). After protease treatment, the Yonetani-Theorell plots are also in accord with two inhibitors binding at the same site although inhibition is almost entirely eliminated. Fig. 4 indicates that when malonyl-CoA inhibition is measured in the absence or presence of a fixed concentration of HPG, parallel lines are again obtained in the Yonetani-Theorell plot, confirming binding of these compounds to the same site. With the concentration of acyl-CoA substrate used in these experiments, curved lines are seen for malonyl-CoA inhibition only at concentrations greater than 10 μM (12).

RESULTS

Fig. 1 shows inhibition of carnitine palmitoyltransferase-I by malonyl-CoA in control and protease-treated mitochondria in a system in which the concentration of coenzyme A either was absent or was kept fixed at 50 μM. This concentration of coenzyme A gave 42% inhibition in the absence of malonyl-CoA, and inhibition was not altered by protease treatment. The data indicate that coenzyme A and malonyl-CoA bind to two independent inhibitory sites since the Yonetani-Theorell plot shows converging lines (23). However, after protease treatment, this plot gives two parallel lines that are almost horizontal, the slight slope being caused by the slight inhibition by malonyl-CoA. This indicates that protease treatment eliminates almost all of the malonyl-CoA inhibition without affecting inhibition by coenzyme A. When the concentration of malonyl-CoA was fixed and the concentration of coenzyme A was varied (Fig. 2), similar results were obtained, supporting the idea that coenzyme A and malonyl-CoA bind at two different sites. It is clear from this figure, however, that inhibition by 10 μM malonyl-CoA is attenuated at concentrations of coenzyme A greater than 50 μM. At 500 μM coenzyme A, there is very little additional effect of malonyl-CoA, suggesting that coenzyme A at high concentrations competes with malonyl-CoA for binding to the malonyl-CoA site, but has little or no inhibitory effect itself. Fig. 2B indicates that protease treatment left the coenzyme A-binding site intact, while extensively damaging the malonyl-CoA site. Two parallel lines are not expected here since coenzyme A is the variable inhibitor. Complete destruction of the malonyl-CoA sites in Fig. 2B would result in a single line. In other experiments, acetyl-CoA and propionyl-CoA behaved exactly as coenzyme A before and after protease treatment, suggesting they also bind to the active site (results not shown).

With HPG a much different picture emerged from double-inhibitor studies. Fig. 3 shows inhibition of carnitine palmitoyltransferase by malonyl-CoA and HPG in control and protease-treated mitochondria at a fixed concentration of malonyl-CoA and increasing concentrations of HPG. These data indicate that the inhibitory effects of HPG and malonyl-CoA, especially at higher concentrations, are not additive as they were with coenzyme A and malonyl-CoA. After protease treatment, the inhibitory effects of both HPG and malonyl-CoA were almost completely eliminated in accord with previous observations (4, 18, 19). These plots of 1/v versus HPG concentration, showing parallel lines, are in agreement with two inhibitors competing for a single site (23). Curvature of the plot is due to cooperative inhibition (12). After protease treatment, the Yonetani-Theorell plots are also in accord with two inhibitors binding at the same site although inhibition is almost entirely eliminated. Fig. 4 indicates that when malonyl-CoA inhibition is measured in the absence or presence of a fixed concentration of HPG, parallel lines are again obtained in the Yonetani-Theorell plot, confirming binding of these compounds to the same site. With the concentration of acyl-CoA substrate used in these experiments, curved lines are seen for malonyl-CoA inhibition only at concentrations greater than 10 μM (12).
Two Sites for Inhibition of Carnitine Palmitoyltransferase-I

Fig. 2. Yonetani-Theorell plot for inhibition of carnitine palmitoyltransferase by increasing coenzyme A in the absence and presence of a fixed concentration of malonyl-CoA. Carnitine palmitoyltransferase activity was measured as a function of coenzyme A concentration in the absence (○, ■) and presence (□, □) of 10 µM malonyl-CoA in control (panel A) and protease-treated (panel B) mitochondria. Results are means of two separate preparations of mitochondria.

Fig. 3. Yonetani-Theorell plot for inhibition of carnitine palmitoyltransferase by increasing 4-hydroxyphenylglyoxylate in the absence and presence of a fixed concentration of malonyl-CoA. Carnitine palmitoyltransferase activity was measured as increasing concentrations of 4-hydroxyphenylglyoxylate in the absence (○, ■) and in the presence (□, □) of 10 µM malonyl-CoA in control (panel A) and protease-treated (panel B) mitochondria. Palmitoyl-CoA and carnitine concentrations were 40 µM and 0.2 mM, respectively. Results are means of two separate preparations of mitochondria.

(+)-Hemipalmitoylcarnitinum is a substrate analog for carnitine palmitolytransferase (24) and a potent inhibitor of heart and liver carnitine palmitoyltransferase-I (25). Using mitochondrial outer membranes, we confirmed that this compound is a potent inhibitor of carnitine palmitoyltransferase-I (IC50 = 20 µM, Fig. 5B). Protease treatment of mitochondrial outer membranes caused a 20% loss of activity, but there was no change in the sensitivity of carnitine palmitoyltransferase-I to inhibition by this active site-directed inhibitor. The malonyl-CoA analog Ro 25–0187 is also a potent inhibitor of carnitine palmitoyltransferase-I (IC50 = 0.2 µM, Fig. 5B), much more potent than malonyl-CoA (12). In contrast to results with the substrate analog, protease treatment resulted in a complete loss of carnitine palmitoyltransferase-I sensitivity to inhibition by this malonyl-CoA analog. These data confirm the carnitine palmitoyltransferase of isolated mitochondrial outer membranes behaves exactly as the overt carnitine palmitoyltransferase of intact mitochondria with respect to proteolysis, and they substantiate that the overt mitochondrial carnitine palmitoyltransferase being studied here is carnitine palmitoyltransferase-I located on the mitochondrial outer membrane with its malonyl-CoA-binding site facing outward (cytosolic) and its active site protected within the mitochondrial outer membrane. Effects of proteolysis on inhibition by these two very potent inhibitors also confirm that the malonyl-CoA site and the active site are entirely different. Since there is no remaining inhibition by Ro 25–0187 after proteolysis and since there is no difference in inhibition by (+)-hemipalmitoylcarnitinum before and after proteolysis, there seems to be no interaction of either of these inhibitors with an alternate site.

DISCUSSION

Because of the close structural similarity between malonyl-CoA and free coenzyme A, it might be expected that they would bind at the same site; however, our data indicate that coenzyme A binds at the active site of carnitine palmitoyltransferase-I, as does acyl-CoA, 2-bromopalmitoyl-CoA, and (+)-hemipalmitoylcarnitinum, but malonyl-CoA does not bind at that site. Inhibition of carnitine palmitoyltransferase-I at the malonyl-CoA-binding site by HPG and Ro 25–0187, which have no coenzyme A moiety, contributes to a resolution of this question. The lack of an absolute requirement for the coenzyme A moiety is also shown by inhibition of carnitine palmitoyltransferase-I by malonic acid and oxalic acid (at 1 mM) of 15 ± 2% and 10 ± 1% (means ± S.E., n = 3), respectively. At the same concentration, malonic acid mono-ethyl ester and phenylpyruvate inhibited...
FIG. 4. Yonetani-Theorell plot for inhibition of carnitine palmitoyltransferase by increasing malonyl-CoA in the absence and presence of a fixed concentration of 4-hydroxyphenylglyoxylate. Carnitine palmitoyltransferase activity was measured at increasing concentrations of malonyl-CoA in the absence (○, ○) and in the presence (□, □) of 0.5 mM 4-hydroxyphenylglyoxylate in control (panel A) and protease-treated (panel B) mitochondria. Palmitoyl-CoA and carnitine concentrations were 40 μM and 0.5 mM, respectively. Results are means of two separate preparations of mitochondria.

FIG. 5. Inhibition of carnitine palmitoyltransferase by (+)-hemipalmitoylcarnitinium (panel A) and Ro 25-0187 (panel B) in control (open symbols) and protease-treated (closed symbols) mitochondria. Inhibition of carnitine palmitoyltransferase by (+)-hemipalmitoylcarnitinium (○, ○) or Ro 25-0187 (□, □) was measured using 40 μM palmitoyl-CoA and 0.5 mM carnitine. Results are representative of two experiments with isolated pig liver mitochondrial outer membranes.

carnitine palmitoyltransferase-I by 31 ± 4 and 29 ± 3% (means ± S.E., n = 3), respectively. Proteolysis abolished the inhibitory ability of all four compounds. The latter two compounds have functional groups identical with the former two but have a more hydrophobic nature. These results suggest that the coenzyme A moiety may increase the potency of inhibitors acting at the malonyl-CoA site, but high potency at this site does not require the compound to be a true coenzyme A ester since Ro 25–0187 is a better inhibitor than malonyl-CoA, but has no coenzyme A moiety. Ro 25–0187 has a short hydrocarbon chain linked to a naphthalene ring system that may act as a lipophilic substitute for coenzyme A to increase its potency over that of its predecessor, HPG. Thus, the coenzyme A moiety of malonyl-CoA is not essential for the binding of malonyl-CoA to its regulatory site, but the dicarbonyl function (possessed by malonyl-CoA, HPG, and Ro 25–0187) is absolutely essential.

It has been suggested that malonyl-CoA binds at the active site of carnitine palmitoyltransferase-I since it is a competitive inhibitor with respect to acyl-CoA and because substrates decrease specific binding of radiolabeled malonyl-CoA to mitochondrial membranes (16). We have shown that malonyl-CoA is a competitive inhibitor of carnitine palmitoyltransferase-I but not a simple competitive inhibitor (12). Our data indicating that there are two sites for inhibition of carnitine palmitoyltransferase-I, with the malonyl-CoA regulatory site being located outside the outer membrane, are consistent with competitive inhibition since it is not required that a competitive inhibitor bind to the active site, but merely that the binding of the inhibitor and competitive substrate be mutually exclusive and that the inhibition be surmountable by increasing substrate concentration. These criteria can be met by allosteric binding as well as binding to the active site.

Whether the malonyl-CoA regulatory site is located on a separate polypeptide or on the same polypeptide as the catalytic site cannot be distinguished by current data. With a kinetic approach, one cannot distinguish between inhibitory binding directly to carnitine palmitoyltransferase-I or to an auxiliary malonyl-CoA-binding protein which has been suggested to regulate carnitine palmitoyltransferase activity (26, 27). However, conclusions from the kinetic analyses reported here should be valid in either case, i.e. a malonyl-CoA-binding site may be located on a domain of carnitine palmitoyltransferase-I that is distinct from the carnitine palmitoyltransferase-I active site, or it may be located on an entirely different, but associated, protein. As the studies reported here were being prepared for publication, the cloning and sequencing of a cDNA
for hepatic carnitine palmitoyltransferase-I was reported (28). The deduced amino acid sequence suggests a polypeptide of 773 amino acids with a single 20-amino-acid membrane spanning region (residues 103–122), whereas the deduced amino acid sequence of carnitine palmitoyltransferase-II suggests a polypeptide of 658 amino acids with no membrane spanning region (29). Interestingly, the putative membrane spanning region of carnitine palmitoyltransferase-I lies adjacent to a 102-amino-acid N-terminal region that is not found in other carnitine acyltransferases (28). This region should lie outside the mitochondrial outer membrane and may contain a malonyl-CoA-binding domain, or it may interact with putative malonyl-CoA-binding proteins. Other recent data indicate that this N-terminal region is not removed after insertion into the outer membrane but is present in the mature protein (30). In summary, current evidence indicates that malonyl-CoA does not bind to the active site of carnitine palmitoyltransferase-I, but acts at a regulatory site that is located outside the mitochondrial outer membrane, while other inhibitors can act at the active site. The data, however, do not rule out the possibility that the malonyl-CoA-binding site may be located on a protein different from carnitine palmitoyltransferase-I.

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