J. Enzyme Inhibition, 1995, Vol. 9, pp. 303–308
Reprints available directly from the publisher
Photocopying permitted by license only

KINETIC, CIRCULAR DICHROISM AND FLUORESCENCE STUDIES ON HETEROLOGOUSLY EXPRESSED CARNITINE PALMITOYLTRANSFERASE II

WILLIAM R. MANN,[1,*] BING YAN,[2] CAROL J. DRAGLAND[1] and PHILIP A. BELL[1]

[1]*Metabolic Diseases and* [2]*Central Technology Departments, Preclinical Research, Sandoz Research Institute, Sandoz Pharmaceuticals Corp., East Hanover, New Jersey 07936–1080, USA*

(Received 12 May 1995)

K_m estimates for carnitine and palmitoyl-CoA of heterologously expressed rat liver carnitine palmitoyltransferase-II (rCPT-II) were 950±27 μM and 34±6 μM, respectively. V_{max} for the enzyme was 1.8 μmol/min/mg purified protein. Consistent with an ordered reaction mechanism in which palmitoyl-CoA binds first, SDZ CPI 975, a reversible carnitine palmitoyltransferase inhibitor containing both carnitine and alkyl moieties, inhibited rCPT-II competitively with carnitine and uncompetitively with palmitoyl-CoA. Substrate-enzyme interactions were examined by circular dichroism (CD) and fluorescence. Both carnitine and palmitoyl-CoA alone induced conformational changes in the enzyme; dissociation constant estimates by CD for carnitine and palmitoyl-CoA were 41±5 μM and 7±2 μM, respectively.

KEY WORDS: Carnitine palmitoyltransferase, kinetics, SDZ CPI 975, circular dichroism, fluorescence

INTRODUCTION

The carnitine acyltransferase systems [carnitine acetyltransferase (EC 2.3.1.7), carnitine octanoyltransferase and carnitine palmitoyltransferase (EC 2.3.1.21)] of eukaryotic cells mediate the transfer of acyl-CoA esters into the mitochondrial matrix from the cytosol. The carnitine palmitoyltransferase (CPT) system consists of CPT-I, situated in the outer mitochondrial membrane, a translocase, and CPT-II, both of which are associated with the inner mitochondrial membrane.

The reaction catalyzed by CPT-I (acyl-CoA + carnitine → acylcarnitine + CoA) is considered rate-limiting for β-oxidation of long-chain fatty acids.[1] Under physiological conditions, CPT-II catalyzes the reverse, but otherwise identical, reaction (acylcarnitine → acyl-CoA + carnitine). Whereas dissociation of CPT-I from its membrane environment renders it inactive, CPT-II remains active when solubilized.[2]

*Correspondence.

Product inhibition studies have demonstrated an ordered reaction mechanism for CPT-II, with acyl-CoA binding before carnitine.[3]

CPT-II may be an appropriate surrogate for CPT-I in structural studies. We have recently purified milligram quantities of active, heterologously expressed rat liver rCPT-II (rCPT-II) to near-homogeneity from a baculovirus expression system.[4] Here we describe the kinetic characteristics of the enzyme and circular dichroism and fluorescence studies examining the interaction of rCPT-II with carnitine and palmitoyl-CoA. Also described are studies with SDZ CPI 975, a novel CPT inhibitor designed as a transition state analog and containing both carnitine and alkyl moieties.

MATERIALS AND METHODS

The chemical synthesis of (-)-3-Carboxy-*N,N,N*-trimethy-2-(tetradecyloxy)-phosphinyloxy-1-propanaminium hydroxide, inner salt (SDZ CPI 975) has been described elsewhere.[5] rCPT-II purified to >95% homogeneity[4] was used in these studies. For CD and fluorescence studies, Tween 20 was removed from the material using Extracti-Gel D columns (Pierce).

Enzyme assays were performed in triplicate in a 96-well plate format. The final incubation buffer (200 μl, pH 7.4) contained 150 mM Tris-HCl, 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), L-carnitine (50–750 μM), palmitoyl-CoA (25–400 μM), plus the additions indicated. Reactions were performed at 37°C, and were started by addition of rCPT-II (0.85 μg) to the other assay components. Reduction of DTNB by CoASH was followed for 3 min at 405 nm using a Molecular Dynamics Thermomax microplate reader. Reaction rates were corrected for rates obtained in the absence of rCPT-II. Lineweaver-Burk plots are shown with the best fit lines to the data points and without consideration to any kinetic model. Kinetic constants were estimated[6] from the best statistical fit of the data to models of competitive, non-competitive, and uncompetitive inhibition and are expressed as mean values of three experiments ± SEM.

Circular dichroism (CD) spectra were recorded on a JASCO J-720 spectropolarimeter at 23±0.5°C. The sample chamber was constantly flushed with nitrogen. The cell pathlength was 1 mm. Spectra of rCPT-II (in 150 mM Tris.HCl, pH 7.4) were analyzed using the SELCON program.[7] To estimate the dissociation constant (K_d), the substrate-induced CD change at 222 nm was taken as proportional to the binding product. A ΔCD concentration plot was fitted to the equation $\Delta CD = \Delta CD_{max} L/(L + K_d)$, where L is the ligand concentration. ΔCD_{max} was obtained by extrapolating the CD change to an infinite ligand concentration. By assuming that $\Delta CD/\Delta CD_{max}$ = fractional binding and total ligand concentration $L_0 \sim$ free ligand, K_d was estimated. For palmitoyl-CoA, the K_d value can be taken as the upper limit value since the ligand was not in great excess.

Fluorescence measurements of rCPT-II (in 150 mM Tris.HCl, pH 7.4) were performed on a Shimadzu RF5000U spectrofluorometer. Protein fluorescence was induced by excitation at 295 nm. Slit widths on both excitation and emission monochromators were set at 5 mm. The sample chamber was maintained at 23±0.5°C with a circulating water bath.

RESULTS

The V_{max} of the recombinant enzyme was estimated to be 1.8 μmol CoA/min/mg purified protein. K_m estimates for carnitine and palmitoyl-CoA were 950±27 μM and 34±6 μM, respectively. These estimates are similar to those reported for mitochondrial CPT-II from chick liver[8] (1020 μM and 32 μM for carnitine and palmitoyl-CoA, respectively) and from beef heart[3] (1500±100 μM for carnitine). SDZ CPI 975 inhibited rCPT-II competitively with respect to carnitine (K_i = 0.83±0.03 μM), and uncompetitively with respect to palmitoyl-CoA (K_i = 0.62±0.04 μM) (Figures 1 and 2).

Based on the analysis of the rCPT-II circular dichroism (CD) spectrum, the enzyme was estimated to have the following secondary structure composition: α-helix, 47±3%; β-sheet, 8±5%; turn, 25±3%; unstructured coils, 20±5%. Using either CD or fluorescence techniques, we found that carnitine or palmitoyl-CoA were able to induce saturable conformational changes in rCPT-II (Figures 3 and 4). Based on the CD data, the dissociation constants (K_d) for carnitine and palmitoyl-CoA were estimated to be 41±9 μM and 7±2 μM, respectively.

DISCUSSION

Pharmacologic inhibition of long chain fatty acid β-oxidation and resulting inhibition of hepatic gluconeogenesis, achieved via irreversible inhibition of CPT-I, has been demonstrated to lower fasting blood glucose levels.[9] CPT-I is not catalytically active when dissociated from the outer mitochondrial membrane; CPT-II, however, remains active when solubilized.[2] CPT-II may be a suitable surrogate for CPT-I in inhibitor design, since both enzymes catalyze essentially identical reactions.

In the absence of detailed structural information, SDZ CPI 975 was designed as a transition state analog inhibitor of the CPT-catalyzed reaction.[5] We have shown SDZ CPI 975 is a reversible inhibitor of rCPT-II, inhibiting the enzyme competitively with respect to carnitine and uncompetitively with respect to palmitoyl-CoA. Our findings are consistent with initial rate and product inhibition studies which suggest that CPT-II follows an ordered reaction mechanism with carnitine binding after palmitoyl-CoA,[3] since an uncompetitive inhibitor binds to the enzyme-substrate complex but not to the free enzyme (i.e. K_{EI} is infinity).

The technique of optical rotatory dispersion studies has previously been used to demonstrate that carnitine induces a conformational change in carnitine acetyltransferase.[10] In the present study, CD and fluorescence spectroscopy were used to study the secondary structure composition of rCPT-II and the interaction of rCPT-II with carnitine and palmitoyl-CoA. Our secondary structure predictions for rCPT-II represent a first step towards obtaining detailed structure information on the enzyme. Carnitine and palmitoyl-CoA did not contribute to the CD signal in the 200–260 nm range, nor did they alter the pH of the sample solution in our experiments. The results in Figure 3 demonstrate conformational changes upon enzyme-substrate complex formation, and are consistent with the "induced fit" theory

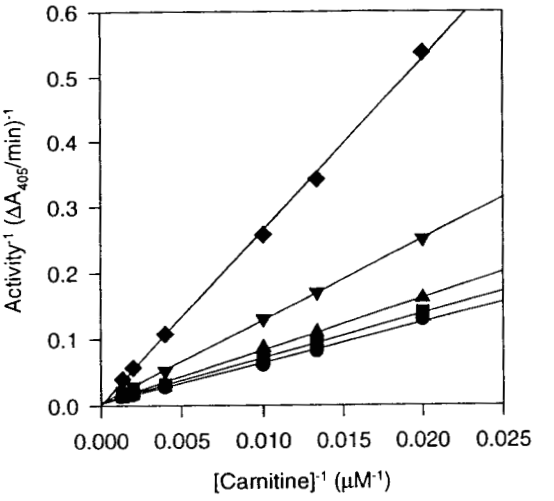

FIGURE 1 Lineweaver-Burk plot showing the effect of SDZ CPI 975 and carnitine on rCPT-II activity. rCPT-II activity was determined at the concentrations of l-carnitine indicated, in the presence of 450 μM palmitoyl-CoA. Concentrations of SDZ CPI 975 were 0 (○), 0.1 (▲), 0.3 (◆), 1.0 (▼) and 3.0 (■) μM.

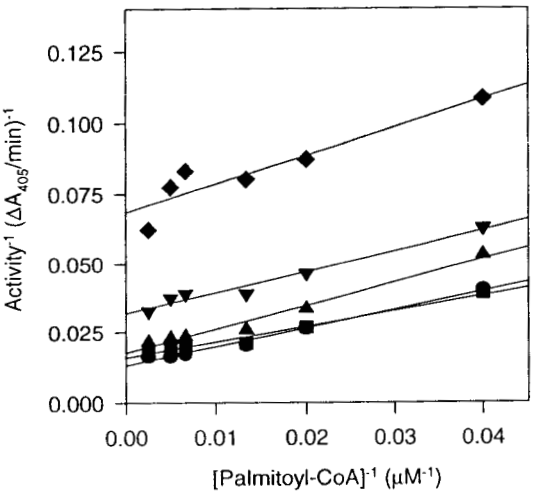

FIGURE 2 Lineweaver-Burk plot showing the effect of SDZ CPI 975 and palmitoyl-CoA on rCPT-II activity. rCPT-II activity was determined at the concentrations of palmitoyl-CoA indicated, in the presence of 500 μM l-carnitine. Concentrations of SDZ CPI 975 were 0 (○), 0.1 (▲), 0.3 (◆), 1.0 (▼) and 3.0 (■) μM.

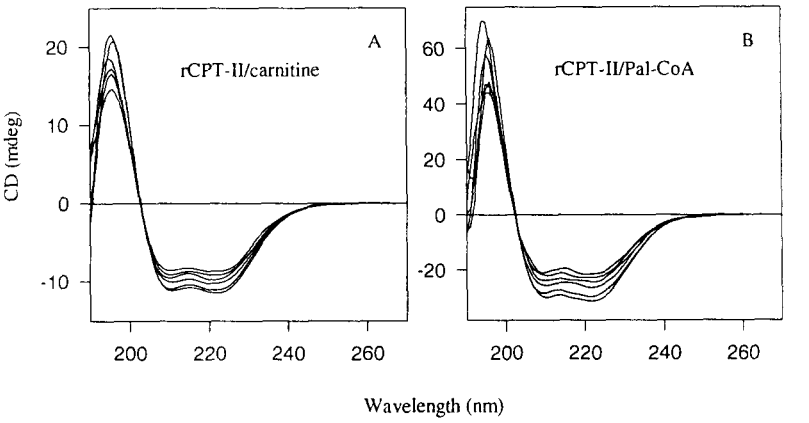

FIGURE 3 Substrate-induced changes in CD spectra. (A) Carnitine-induced changes in the CD spectrum of 1.9 μM rCPT-II. Carnitine concentrations from low to high intensities at 220 and 195 nm were 0, 10, 40, 80, 320, and 720 μM; (B) Palmitoyl-CoA-induced changes in the CD spectrum of 4.5 μM rCPT-II. The palmitoyl-CoA concentrations from low to high intensities at 220 and 195 nm were 0, 1, 2, 4, 8.4, and 17.2 μM.

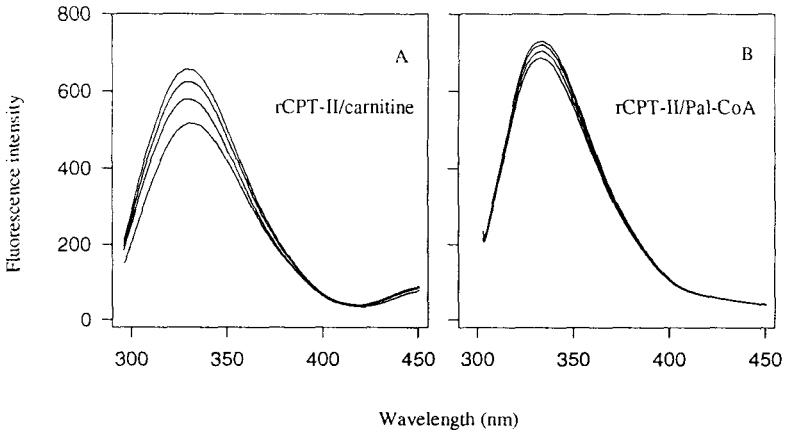

FIGURE 4 Substrate-induced changes in fluorescence spectra. (A) Carnitine-induced changes in intrinsic fluorescence emission of 3.6 μM rCPT-II. Carnitine concentrations from high to low intensities were 0, 20, 40, and 80 μM; (B) Palmitoyl-CoA-induced changes in intrinsic fluorescence emission of 4.5 μM rCPT-II. Palmitoyl-CoA concentrations from high to low intensities were 0, 4, 17.2, and 35.2 μM.

of Koshland.[11] Substrate-induced fluorescence quenching in rCPT-II (Figure 4) corroborated the conformational changes observed by CD. Our CD and fluorescence data are consistent with the notion that carnitine can specifically bind to the enzyme in the absence of palmitoyl-CoA. Our analysis of the kinetic data, however, would suggest that such binding is not catalytically productive.

References

1. McGarry, J.D. and Foster, D.W. (1979). *J. Biol. Chem.*, **254**, 8163.
2. Woeltje, K.F., Kuwajima, M., Foster, D.W. and McGarry, J.D. (1987). *J. Biol. Chem.*, **262**, 9822.
3. Nic A'Bháird, N., Kumaravel, G., Gandour, R.D., Krueger, M.J. and Ramsay, R.R. (1993). *Biochem. J.*, **294**, 645.
4. Johnson, T.M., Mann, W.R., Dragland, C.J., Anderson, R.C., Nemecek, G.M. and Bell, P.A. *Biochem. J.*, in press.
5. Anderson, R., Balestra, M., Bell, P.A., Deems, R.O., Fillers, W.S., Foley, J.E., Fraser, J.D., Mann, W.R., Rudin, M. and Villhauer, E.B. *J. Med. Chem.*, submitted.
6. Leatherbarrow, R.J. (1990). *GraFit* Version 2.0, Erithacus Software Ltd., Staines, U.K.
7. Sreerama, N. and Woody, R.W. (1993). *Anal. Biochem.*, **209**, 32.
8. Ishii, S., Ishii, H., Watanabe, T. and Suga, T. (1987). *Biochem. Biophys. Acta*, **924**, 530.
9. Wolf, H.P.O. (1992). *Horm. Metab. Res.*, **26** (Suppl.), 62.
10. Tipton, K.F. and Chase, J.F.A. (1969). *Biochem. J.*, **115**, 517.
11. Koshland, D.E. (1958). *Proc. Natl. Acad. Sci., USA*, **44**, 98.