Effects of pH on the interaction of substrates and malonyl-CoA with mitochondrial carnitine palmitoyltransferase I

Scott E. MILLS, Daniel W. FOSTER and J. Denis McGARRY*

Departments of Internal Medicine and Biochemistry, University of Texas Health Science Center at Dallas, Dallas, TX 75235, U.S.A.

(Received 4 October 1983/Accepted 10 January 1984)

The kinetics of carnitine palmitoyltransferase I (CPT I; EC 2.3.1.21) were examined in mitochondria from rat liver, heart and skeletal muscle as a function of pH over the range 6.8–7.6. In all three tissues raising the pH resulted in a fall in the K_m for carnitine, no change in the K_m for palmitoyl-CoA or octanoyl-CoA, and a marked decrease in the inhibitory potency of malonyl-CoA. Studies with skeletal-muscle mitochondria established that increasing pH was accompanied by an increase in the K_d of the malonyl-CoA binding site for this ligand, coupled with a decrease in the K_d for fatty acyl-CoA species to compete for malonyl-CoA binding. Three principal conclusions are drawn. (1) The pH-induced shift in malonyl-CoA sensitivity of CPT I is not a phenomenon restricted to liver mitochondria. (2) At any given pH within the range tested, the ability of malonyl-CoA (and closely related compounds) to inhibit enzyme activity is governed by the efficiency of their binding to the malonyl-CoA site. (3) The competitive interaction between fatty acyl-CoA substrates and malonyl-CoA as regards CPT I activity is exerted at the malonyl-CoA binding site. Finally, the possibility is strengthened that the malonyl-CoA binding site is distinct from the active site of CPT I.

The kinetics of mitochondrial carnitine palmitoyltransferase I (CPT I, EC 2.3.1.21) vary greatly, depending on the tissue studied. For example, in the rat the enzyme from heart and skeletal muscle displays a K_m for carnitine some 6- and 16-fold greater, respectively, than that from liver (McGarry *et al.*, 1983). Sensitivity of the enzyme to inhibition by malonyl-CoA spans an even wider range (about 100-fold), and appears to be positively correlated with the K_m for carnitine (Saggerson & Carpenter, 1981a; McGarry *et al.*, 1983; Mills *et al.*, 1983). Moreover, compounds structurally related to malonyl-CoA, such as succinyl-CoA and methylmalonyl-CoA, which are weak inhibitors of CPT I in liver, become increasingly more effective in heart and skeletal muscle (Mills *et al.*, 1983).

To gain further insight into the mechanism of the malonyl-CoA–CPT I interaction, we have studied the rat muscle enzyme in more detail, because of its extreme sensitivity to the inhibitor.

It was established that mitochondria from this source possess a high-affinity binding site for [^{14}C]malonyl-CoA, that this site also binds succinyl-CoA and methylmalonyl-CoA (though with successively less avidity than for malonyl-CoA), and that the activity of CPT I is governed by the degree of site occupancy, regardless of which inhibitor is employed (Mills *et al.*, 1983). Substrates for CPT I, such as palmitoyl-CoA and octanoyl-CoA, were shown to displace malonyl-CoA from its binding site (Mills *et al.*, 1983), consistent with previous reports that malonyl-CoA acts competitively with fatty acyl-CoA species towards the membrane-bound enzyme (McGarry *et al.*, 1978a; Bremer, 1981; Saggerson & Carpenter, 1981b).

During the course of our earlier work we had observed that, regardless of the tissue of origin, the sensitivity of mitochondrial CPT I to malonyl-CoA inhibition was altered by small changes in the pH of the assay mixture. The same phenomenon was reported by Stephens *et al.* (1983). This raised several questions. For example, is the effect of pH on malonyl-CoA sensitivity of CPT I the result of alterations in malonyl-CoA binding to the mito-

Abbreviation used CPT I, carnitine palmitoyltransferase I (EC 2.3.1.21).

* To whom reprint requests should be addressed.

chondria? How does pH influence other kinetic components of the system: the K_m of CPT I for its substrates, the ability of malonyl-CoA analogues to bind to the malonyl-CoA site, and the competitive interaction between substrates and malonyl-CoA at this site? Each of these issues has been addressed in the present investigation. Although the question of whether malonyl-CoA interacts with the active site of membrane-bound CPT I or with a closely associated regulatory site of the enzyme cannot be definitively answered, the results of these experiments favour the later interpretation.

Methods

Animals

Male Sprague-Dawley rats, maintained on a high-sucrose/low-fat diet (McGarry *et al.*, 1978b), and weighing about 150 g were used in the fed state.

Preparation of mitochondria

Mitochondria were isolated from liver, heart and hind-leg muscle by using methods A or C (with similar results) as described by McGarry *et al.* (1983) and were finally suspended in 0.15 M-KCl. Protein was measured by the method of Lowry *et al.* (1951).

Assay of CPT I

Enzyme activity was measured in intact mitochondria in the direction palmitoyl-CoA + [14 C]-carnitine $\rightarrow$ palmitoyl[14 C]carnitine + CoA, as described previously (McGarry *et al.*, 1983; Mills *et al.*, 1983), except that the pH of the incubation mixture was varied between 6.8 and 7.6. In the standard assay final concentrations of palmitoyl-CoA (or octanoyl-CoA), L-carnitine and fatty acid-free albumin were 50 μ M, 200 μ M and 1% respectively. With 0.2–0.4 mg of mitochondrial protein/ml of assay mixture and a temperature of 30°C, reaction rates were linear over the time periods studied (generally 4–5 min). Any deviations from this procedure are noted in the legends to Tables and Figures.

Malonyl-CoA binding to muscle mitochondria

Binding assays were performed as described previously (Mills *et al.*, 1983), except that the pH of the medium was varied from 6.8 to 7.6 and the labelled ligand used was [2- 14 C]malonyl-CoA. Experiments were conducted both at 30°C (the temperature used for CPT I assays) and at 0°C, with approx. 2 mg of mitochondrial protein. At the higher temperature, binding reached equilibrium rapidly (1–2 min) and remained constant for at least 8 min. Accordingly, incubations were routinely performed for 5 min. At 0°C the malonyl-CoA–

mitochondrial complex forms more slowly and is more stable with time (Mills *et al.*, 1983); as a consequence a 20 min incubation period was used. Assays were performed at the concentrations of [14 C]malonyl-CoA given in the Figure legends in the absence or presence of 100 μ M unlabelled malonyl-CoA. Values reported are based on specific binding of [14 C]malonyl-CoA. Although not routinely present for binding studies, ATP, carnitine, KCN and rotenone (components of the CPT I assay) were shown to have no effect on the results.

Materials

Unlabelled CoA esters were from P-L Biochemicals (Milwaukee, WI, U.S.A.). [2- 14 C]-Malonyl-CoA (58 Ci/mol) was from Amersham (Arlington Heights, IL, U.S.A.); it was found to be approx. 90% pure on the basis of high-pressure liquid chromatography analysis.

Results

Effects of pH on the kinetics of CPT I

The marked effect of pH on the inhibition of liver CPT I by malonyl-CoA, previously noted by Stephens *et al.* (1983), was confirmed. Raising the pH of the assay medium from a starting value of 6.8 resulted in a progressively rightward shift of the activity-versus-[malonyl-CoA] response curve, such that at pH 7.6 the I_{50} (the concentration of malonyl-CoA required for 50% suppression of enzyme activity) had increased almost 8-fold (results not shown).

As noted in Fig. 1, the same phenomenon was observed with mitochondria from heart and skeletal muscle, although, as expected on the basis of previous studies (McGarry *et al.*, 1983; Mills *et al.*, 1983), at any given pH the inhibitory potency of malonyl-CoA increased in the sequence liver < heart < skeletal muscle.

Table 1 documents the effect of pH on the apparent kinetic constants of the CPT I reaction in mitochondria from liver, heart and skeletal muscle. When the concentration of palmitoyl-CoA was fixed at 50 μ M, two points about the K_m for carnitine are noteworthy. First, at any given pH the apparent K_m was some 6- and 18-fold higher, respectively, in heart and skeletal muscle than in liver, in keeping with our previous report (McGarry *et al.*, 1983). Second, in all three tissues the value fell by a factor of 2 as the pH increased from 6.8 to 7.6. Calculated values for V_{max} were only minimally affected by changes in pH under conditions of limiting palmitoyl-CoA. [The concentrations of acyl-CoA referred to throughout this paper are nominal. True concentrations of the free

Fig. 1. Relationship between pH and sensitivity to malonyl-CoA of CPT I in mitochondria from different tissues. The experimental protocol described in the Methods section was followed with mitochondria from liver, heart and skeletal muscle. I_{50} refers to the concentration of malonyl-CoA required for 50% suppression of CPT I. Values are means $\pm$ S.E.M. for three experiments with each tissue.

species will have been lower, because of the presence of albumin in the reaction mixtures.]

With a fixed concentration of carnitine (200 μM) the K_m for palmitoyl-CoA did not change with pH, and in absolute terms its value, though highest in heart, was of similar magnitude (20–40 μM) in all three systems. Under these conditions V_{max} in liver was unaffected by pH, but in heart and skeletal muscle it increased 2-fold as the pH was raised from 6.8 to 7.6. Presumably this stemmed from the fact that, within the pH range tested, the concentration of carnitine used was always greatly in excess of its K_m in liver; this was not the case in heart and skeletal muscle, where 200 μM would be expected to support faster reaction rates with increasing pH (by virtue of their much higher K_m for this substrate).

Experiments using octanoyl-CoA as the varied substrate were done with skeletal-muscle mitochondria, but interpretation of the data was complicated by the fact that Lineweaver–Burk plots deviated from linearity such that accurate kinetic constants could not be assigned. Nevertheless, it was intuitively obvious that the K_m for the medium-chain substrate, though lower than that for palmitoyl-CoA, was unaffected by changes in pH, consonant with the findings obtained with the latter substrate. V_{max} with octanoyl-CoA was also lower than with palmitoyl-CoA and, as expected, increased with pH.

Effects of pH on the kinetics of malonyl-CoA binding

This series of experiments was performed with mitochondria from skeletal muscle and was designed to answer the following questions. First, could the fall in malonyl-CoA sensitivity of CPT I with increasing pH be explained by a diminution in malonyl-CoA binding to the mitochondrial membrane? Second, since fatty acyl-CoA species are competitive with respect to malonyl-CoA binding (Mills *et al.*, 1983), how does pH affect this interaction? We have previously shown (Mills *et al.*, 1983) that rat skeletal-muscle mitochondria possess a single class of high-affinity binding site for malonyl-CoA. The effect of pH on this system is shown in Table 2, line B. Raising the pH from 6.8 to 7.6 resulted in less efficient binding of malonyl-CoA (increased K_d), but was without effect on total binding capacity of the mitochondria (about 11 pmol/mg of protein). Comparison of these K_d values with the equivalent I_{50} values for CPT I inhibition (Table 2, line A) reveals two important points. First, at any given pH the K_d for malonyl-CoA binding was lower than the I_{50} for CPT I suppression. This was expected, since palmitoyl-CoA, which interacts competitively with malonyl-CoA, was present in the CPT I assay (Fig. 1, and line A of Table 2), but not in the binding experiments (line B of Table 2). Second, the change in I_{50} between pH 6.8 and 7.6 (about 7-

Table 1. Effect of pH on kinetic parameters of mitochondrial CPT I

Enzyme assays were performed as described in the Methods section, except that substrate concentrations were varied as indicated. The concentration of albumin was fixed at 1%. Kinetic constants were calculated from linear-regression analysis of Lineweaver-Burk plots. In all cases correlation coefficients between $1/v$ and $1/s$ were >0.995 . Units for $V_{\max}$ are nmol/min per mg of mitochondrial protein. Values are means $\pm$ s.e.m. for three to six experiments.

Substrate fixed	Substrate varied	Parameter measured	pH			Liver			Heart			Skeletal muscle		
			6.8	7.2	7.6	6.8	7.2	7.6	6.8	7.2	7.6	6.8	7.2	7.6
Palmitoyl-CoA (50 μ M) Carnitine (200 μ M)	Carnitine (0-1 mM) Palmitoyl-CoA (0-150 μ M)	K_m (μ M)	39 $\pm$ 2.7	28 $\pm$ 2.5	22 $\pm$ 0.33	236 $\pm$ 7.8	186 $\pm$ 18.2	127 $\pm$ 9.2	730 $\pm$ 119	526 $\pm$ 67	365 $\pm$ 49	730 $\pm$ 119	526 $\pm$ 67	365 $\pm$ 49
		$V_{\max}$ (μ M)	6.9 $\pm$ 0.90	6.7 $\pm$ 0.70	6.3 $\pm$ 0.76	10.4 $\pm$ 0.56	13.6 $\pm$ 1.3	12.5 $\pm$ 0.90	13.8 $\pm$ 0.80	19 $\pm$ 1.25	18.5 $\pm$ 1.4	13.8 $\pm$ 0.80	19 $\pm$ 1.25	18.5 $\pm$ 1.4
		K_m (μ M)	21 $\pm$ 4.4	24 $\pm$ 4.6	20 $\pm$ 4.2	35 $\pm$ 0.23	40 $\pm$ 2.9	42 $\pm$ 2.0	26 $\pm$ 1.4	29 $\pm$ 1.4	28 $\pm$ 1.2	26 $\pm$ 1.4	29 $\pm$ 1.4	28 $\pm$ 1.2
		$V_{\max}$ (μ M)	8.9 $\pm$ 1.3	9.4 $\pm$ 1.3	8.5 $\pm$ 1.2	8.4 $\pm$ 0.31	12.9 $\pm$ 0.27	15.6 $\pm$ 0.70	6.0 $\pm$ 1.1	10.8 $\pm$ 2.2	13.3 $\pm$ 2.3	6.0 $\pm$ 1.1	10.8 $\pm$ 2.2	13.3 $\pm$ 2.3

fold) was much greater than that in the K_d value (about 1.8-fold). This suggested that the presence of palmitoyl-CoA caused a disproportionate fall in the efficiency of malonyl-CoA binding at the higher pH values. Direct evidence to this effect was obtained when [14 C]malonyl-CoA binding was measured in the presence of 50 μ M-palmitoyl-CoA. As shown in line C of Table 2, the effect of palmitoyl-CoA was to increase the apparent K_d for malonyl-CoA at each pH tested, but most noticeably at the high values (cf. line B of Table 2). Thus in the presence of palmitoyl-CoA the K_d for malonyl-CoA closely approximated to the I_{50} for CPT I inhibition across the entire pH range examined (cf. lines A and C of Table 2). A similar picture emerged when the binding experiments were repeated at 0°C (lines D and E of Table 2). Although K_d values for malonyl-CoA binding were generally less at the lower temperature, they were again increased disproportionately by palmitoyl-CoA as the pH was raised.

The implication of these findings was that as pH increased palmitoyl-CoA became a more potent competitor for malonyl-CoA binding, suggesting that the affinity of the binding site for the two ligands was not being altered in the same fashion. What was not clear was whether the affinity of the malonyl-CoA site for palmitoyl-CoA was resistant to changes in pH, as was the K_m of palmitoyl-CoA in the CPT I reaction (Table 1). Theoretically, the affinity of the malonyl-CoA site for palmitoyl-CoA could be tested in two ways: either by examining directly the kinetics of labelled palmitoyl-CoA binding to mitochondria in the presence and absence of excess unlabelled malonyl-CoA, or by measuring the ability of unlabelled palmitoyl-CoA to compete with [14 C]malonyl-CoA for its receptor. We chose the latter procedure because of the large amount of non-specific binding of the long-chain acyl-CoA predictable in the former method. The results are shown in Fig. 2. Raising the pH from 6.8 to 7.6 greatly enhanced the ability of palmitoyl-CoA to displace [14 C]malonyl-CoA from its binding site. From the observed I_{50} concentrations K_d values for palmitoyl-CoA interaction with the malonyl-CoA site could be calculated by using the equation derived by Cheng & Prusoff (1973). This states that:

$$K_d \text{ (competing ligand)} = \frac{I_{50} \text{ (competing ligand)}}{1 + \frac{[^{14}\text{C}]\text{malonyl-CoA}}{K_d \text{ (malonyl-CoA)}}$$

where I_{50} (competing ligand) represents the concentration needed to decrease the binding of 0.125 μ M-[14 C]malonyl-CoA by 50%. The K_d for malonyl-CoA at each pH was taken from Table 2. The results from the experiment of Fig. 2 are

Table 2. Relationship between malonyl-CoA I_{50} for CPT I inhibition and K_d for binding to skeletal-muscle mitochondria. The data in line A were taken from Fig. 1(c). The data in lines B–E were derived from Scatchard plots of specific binding of [14 C]malonyl-CoA (5–250 nM) to muscle mitochondria where correlation coefficients >0.9 were routinely observed. Maximal binding capacities (30°C) were 11.7 ± 1.1 , 11.3 ± 0.9 and 10.9 ± 1.0 pmol/mg of mitochondrial protein at pH 6.8, 7.2 and 7.6 respectively. Values are means $\pm$ S.E.M. from three experiments.

Parameter measured	Temperature	Palmitoyl-CoA (50 μ M)	pH		
			6.8	7.2	7.6
A. I_{50} for CPT I inhibition (μ M)	30°C	Present	0.017 ± 0.0013	0.041 ± 0.0015	0.118 ± 0.0070
B. K_d for binding (μ M)	30°C	Absent	0.011 ± 0.0004	0.014 ± 0.0005	0.020 ± 0.0008
C. K_d for binding (μ M)	30°C	Present	0.029 ± 0.0020	0.049 ± 0.0060	0.122 ± 0.0120
D. K_d for binding (μ M)	0°C	Absent	0.006 ± 0.0002	0.008 ± 0.0005	0.014 ± 0.0004
E. K_d for binding (μ M)	0°C	Present	0.015 ± 0.0004	0.026 ± 0.0008	0.060 ± 0.0026

Fig. 2. Effect of unlabelled palmitoyl-CoA on the binding of [14 C]malonyl-CoA to skeletal-muscle mitochondria. The concentration of [14 C]malonyl-CoA was fixed at 0.125 μ M and that of palmitoyl-CoA was varied as indicated. Temperature was 30°C. I_{50} values (the concentration of palmitoyl-CoA needed to decrease the binding of labelled ligand by 50%) were 250 ± 14 , 105 ± 6 and 40 ± 6 μ M at pH 6.8 (○), 7.2 (△) and 7.6 (□) respectively. Values are means $\pm$ S.E.M. for three experiments.

shown in line A of Table 3. Increasing the pH from 6.8 to 7.6 caused the K_d for palmitoyl-CoA to fall from 21 to 5.5 μ M. When the experiments were repeated at 0°C, a qualitatively similar pattern was observed (Table 3, line B). In keeping with previous findings (Mills *et al.*, 1983), the affinity of the malonyl-CoA binding site was greater for octanoyl-CoA than for palmitoyl-CoA (Table 3, lines C and D), and in this case also K_d values decreased with increasing pH.

To evaluate the validity of this indirect method

for determining the affinity constants of competing substrates for the malonyl-CoA binding site, similar studies were carried out with unlabelled malonyl-CoA and its analogues as competing ligands. As shown in line E of Table 3, calculated K_d values for malonyl-CoA were similar to those obtained from the direct binding studies of Table 2 (line B). (It can be deduced from the Cheng-Prusoff equation that the I_{50} value for displacement of [14 C]malonyl-CoA by unlabelled malonyl-CoA should have ranged from 0.136 to 0.145 μ M between pH 6.8 and 7.6 respectively. Such small changes would be difficult to detect, as evidenced by the constant value of 0.15 μ M observed in line E of Table 3.) Furthermore, the K_d for succinyl-CoA and methylmalonyl-CoA changed in parallel with that for malonyl-CoA as a function of pH. Thus, in contrast with its lack of effect on the K_m of the active site of CPT I for palmitoyl-CoA and octanoyl-CoA, increasing pH markedly enhanced the affinity of the malonyl-CoA binding site for both substrates while decreasing that for malonyl-CoA and related compounds.

Discussion

It has been proposed that the enhanced suppression of liver CPT I by malonyl-CoA with a lowering of pH may be important in attenuating hepatic fatty acid oxidation and ketogenesis during ketoacidosis (Stephens *et al.*, 1983). For reasons too extensive to elaborate upon here, we are reluctant at this stage to attach such teleological significance to the phenomenon. Rather, our major purpose in pursuing the observation was to gain further insight into the properties of this complex enzyme and the biochemical mechanism through which its activity is controlled by malonyl-CoA and related molecules. We believe the present studies have been informative on both counts.

The influence of pH on the malonyl-CoA sensitivity of mitochondrial CPT I is now seen to

Table 3. Effect of pH on the competition between unlabelled CoA esters and [^{14}C]malonyl-CoA for binding to skeletal-muscle mitochondria. Experiments were carried out as described in the legend to Fig. 2, with the indicated modifications. K_d values for competing substrates were calculated as outlined in the text. Results are given as means $\pm$ s.e.m. for the numbers of experiments shown in parentheses.

Competing substrate	Temperature	pH	I_{50} (μM)				K_d (μM)			
			6.8	7.2	7.6	...	6.8	7.2	7.6	...
A. Palmitoyl-CoA (3)	30°C		250 $\pm$ 14	105 $\pm$ 6	40 $\pm$ 6		21 $\pm$ 1.2	10.3 $\pm$ 0.6	5.5 $\pm$ 1.0	
B. Palmitoyl-CoA (3)	0°C		259 $\pm$ 6	156 $\pm$ 11	61 $\pm$ 6		11.5 $\pm$ 0.3	9.6 $\pm$ 0.7	6.1 $\pm$ 0.6	
C. Octanoyl-CoA (2)	30°C		108 $\pm$ 4	35 $\pm$ 1.5	11 $\pm$ 1		9.4 $\pm$ 0.05	3.5 $\pm$ 0.00	1.6 $\pm$ 0.10	
D. Octanoyl-CoA (2)	0°C		89 $\pm$ 1	41 $\pm$ 1	12.4 $\pm$ 0.4		4.0 $\pm$ 0.05	2.6 $\pm$ 0.05	1.3 $\pm$ 0.02	
E. Malonyl-CoA (3)	30°C		0.15 $\pm$ 0.006	0.15 $\pm$ 0.006	0.15 $\pm$ 0.006		0.012 $\pm$ 0.0005	0.015 $\pm$ 0.0005	0.020 $\pm$ 0.0008	
F. Succinyl-CoA (3)	30°C		5.2 $\pm$ 0.2	6.6 $\pm$ 0.2	6.7 $\pm$ 0.15		0.427 $\pm$ 0.015	0.647 $\pm$ 0.024	0.930 $\pm$ 0.020	
G. Methylmalonyl-CoA (3)	30°C		17 $\pm$ 0.8	17.6 $\pm$ 0.3	17.4 $\pm$ 0.5		1.41 $\pm$ 0.07	1.73 $\pm$ 0.04	2.40 $\pm$ 0.10	

apply to non-hepatic tissues as well as to liver. Almost certainly, therefore, this is a widespread characteristic of mitochondria in the mammalian organism. [Note that the I_{50} values for malonyl-CoA inhibition of CPT I at pH 7.4 found in the present studies are somewhat higher than those reported by us previously (McGarry *et al.*, 1983; Mills *et al.*, 1983). The discrepancy stems from the fact that in the studies cited the final pH of the reaction mixture was inadvertently closer to 7.1 than 7.4.]

Another striking effect of pH was its ability to alter the K_m of CPT I for carnitine. Of interest is the fact that in the three tissues we examined (selected on the basis of their low, intermediate and high sensitivity to malonyl-CoA) an increase in pH over the relatively narrow range 6.8–7.6 produced a similar loss in inhibitory potency of malonyl-CoA (about 80%) coupled with an identical decrease in the K_m for carnitine (about 50%). Thus, the same inverse relationship between I_{50} for malonyl-CoA and K_m for carnitine seen previously across tissue and species lines at a fixed pH (McGarry *et al.*, 1983) also appears to hold for any given tissue when the pH is changed (see below).

Two early observations with mitochondrial membranes from rat liver (McGarry *et al.*, 1978a) had led us to suggest that, although the suppression of CPT I activity by malonyl-CoA could be overcome by increasing the concentration of palmitoyl-CoA, the interaction of substrate and inhibitor was not competitive in the classical sense. In the first place, the curve relating enzyme activity to substrate concentration conformed to a rectangular hyperbola in the absence of malonyl-CoA, but became sigmoid in the presence of the inhibitor. In addition, the detergent-solubilized enzyme lost all sensitivity to malonyl-CoA. These findings, which have since been confirmed in our and other laboratories (Bremer, 1981; Saggerson, 1982; McGarry *et al.*, 1983), pointed to a more complex interaction between CPT I and malonyl-CoA than would be expected on the basis of simple competitive inhibition. The present studies provide additional support for such an interpretation. Key observations were as follows.

First, despite the fact that the inhibitory potency of malonyl-CoA was some 50 and 100 times greater, respectively, in heart and skeletal muscle than in liver, the K_m for palmitoyl-CoA was similar in all three tissues.

Second, in any given tissue the K_m for palmitoyl-CoA in the CPT I reaction was totally resistant to changes in pH. The same was true in skeletal muscle with octanoyl-CoA as substrate (liver and heart were not tested in this case). In striking contrast, the K_d for malonyl-CoA binding to

skeletal-muscle mitochondria was profoundly pH-sensitive. Thus the pH-induced shift in malonyl-CoA sensitivity of CPT I to inhibition by malonyl-CoA was the direct result of changes in the affinity for its binding at the malonyl-CoA site. This is in accord with our earlier conclusion (Mills *et al.*, 1983) that the degree of occupancy of this site by malonyl-CoA (or structurally related compounds) governs the activity of the muscle enzyme. It is likely that the same explanation will hold for other tissues.

Third, the K_d values of the malonyl-CoA binding site for palmitoyl-CoA and octanoyl-CoA also changed markedly with pH. Interestingly, the affinity of this site for fatty acyl-CoA species and malonyl-CoA (or its analogues) responded in opposite fashion, the former increasing and the latter decreasing as the pH was raised. The net result was that, for a given increment in pH, the associated loss in malonyl-CoA sensitivity of the CPT I reaction was greater than would have been predicted simply on the basis of the altered K_d for malonyl-CoA binding seen in the absence of acyl-CoA.

What might these findings mean as regards the respective sites of interaction of substrates and inhibitors with CPT I as it exists on the mitochondrial inner membrane? Although the data do not allow a definitive answer, they could provide some clues. The fact that the affinity of the malonyl-CoA binding site for palmitoyl-CoA increased with pH, whereas the K_m for palmitoyl-CoA in the CPT I reaction was pH-insensitive, raises the possibility that the locus of malonyl-CoA binding is distinct from the active site of CPT I. We recognize that such a construct hinges on the assumption that an unchanging K_m for palmitoyl-CoA (in terms of enzyme activity with increasing pH) reflects an unchanging affinity (K_s) for this substrate at the active site. This is not necessarily true, and cannot be ascertained without more detailed knowledge of the reaction mechanism. Certainly, the fact that solubilized CPT I is insensitive to malonyl-CoA is consistent with (although it does not prove) the existence of separate active and regulatory sites.

If the above formulation is correct, the further implication would be that palmitoyl-CoA or other acyl-CoA substrates bind both to the active site (pH-insensitive) and to the regulatory component (pH-sensitive) and that the latter is the locus at which fatty acyl-CoA species exert their competitive influence against malonyl-CoA. If this is true, it follows that the binding of malonyl-CoA (or related compounds such as methylmalonyl-CoA, succinyl-CoA and glutaryl-CoA) to the regulatory site must somehow dampen the catalytic efficiency of the active site. How this is accomplished is not

known. Regardless of mechanism, if it is assumed that malonyl-CoA and acyl-CoA substrates bind to the same locus within the regulatory domain, a paradox is apparent. If mere occupancy of the regulatory site decreases enzyme activity, what prevents an acyl-CoA substrate from acting also as an inhibitor? One possibility would be that inhibitory ligands must be structurally quite similar to malonyl-CoA and possess a free carboxy group (Mills *et al.*, 1983). An alternative solution to the conundrum would be to suppose that the regulatory component contains two distinct sites, one for fatty acyl-CoA species and the other for malonyl-CoA and its analogues. According to this formulation, the binding of ligands to their respective sites would have to operate in a manner that would be indistinguishable from their binding to a common site on the basis of competitive displacement kinetics. Although direct evidence for such a model is still lacking, the fact that pH had opposite effects on the affinity of the regulatory component for fatty acyl-CoA species and malonyl-CoA would be consistent with it.

Finally, as alluded to above, another intriguing feature of this system is the reciprocal relationship noted in all of our studies between the affinity of the carnitine-binding site and that of the malonyl-CoA site for their respective ligands. The interesting possibility is raised that these two sites are closely associated, although, as shown previously (McGarry *et al.*, 1983), occupancy of one does not appear to influence the binding characteristics of the other. The fact that increasing pH enhanced the affinity of the enzyme for carnitine (which contains a positively charged nitrogen atom) while decreasing the binding of malonyl-CoA (negatively charged) could implicate the presence in the vicinity of both loci of a histidine residue which, as noted by Stephens *et al.* (1983), might be expected to titrate over the pH range tested such that its net positive charge would fall with rising pH.

In conclusion, it is evident that the malonyl-CoA/CPT I interaction is not a simple one. Clearly established is the fact that the extent of malonyl-CoA binding to mitochondria is a key determinant of enzyme activity. The data suggest that the active site of the enzyme and the regulatory locus are distinct entities. However, direct proof for this concept will require experiments with solubilized CPT I and mitochondrial membranes, before and after the enzyme has been released.

This work was supported by grants from the U.S. Public Health Service (AM 18573 and AM 07307). S. E. M. is a Recipient of National Research Service Award (AM 07139) from the National Institutes of Health. The expert technical assistance of Karen Herrmann-Thatcher and Murphy Daniels is gratefully

acknowledged. We are also indebted to Dr. Louis Hersh for helpful discussions.

References

- Bremer, J. (1981) *Biochim. Biophys. Acta* **665**, 628–631
- Cheng, Y. C. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22**, 3099–3108
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McGarry, J. D., Leatherman, G. F. & Foster, D. W. (1978a) *J. Biol. Chem.* **253**, 4128–4136
- McGarry, J. D., Stark, M. J. & Foster, D. W. (1978b) *J. Biol. Chem.* **253**, 8291–8293
- McGarry, J. D., Mills, S. E., Long, C. S. & Foster, D. W. (1983) *Biochem. J.* **214**, 21–28
- Mills, S. E., Foster, D. W. & McGarry, J. D. (1983) *Biochem. J.* **214**, 83–91
- Saggerson, E. D. (1982) *Biochem. J.* **202**, 397–405
- Saggerson, E. D. & Carpenter, C. A. (1981a) *FEBS Lett.* **129**, 229–232
- Saggerson, E. D. & Carpenter, C. A. (1981b) *FEBS Lett.* **132**, 166–168
- Stephens, T. W., Cook, G. A. & Harris, R. A. (1983) *Biochem. J.* **212**, 521–524