Importance of albumin binding in the assay for carnitine palmitoyltransferase

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Alterations in the long-chain acyl-CoA binding to albumin in the carnitine palmitoyltransferase (CPT) assay appreciably affect the reaction at commonly used substrate concentrations. Since in the CPT assay the latter are typically well below saturation or $V_{\max}$ values, the measured enzyme activity depends on both the absolute quantity of albumin in the CPT assay and any biochemical modification of its binding. The present study verifies the striking dependence of the $K_{0.5}$ for palmitoyl-CoA on albumin and the misleading 'activation' of the enzyme by compounds that also avidly bind to albumin. In assessing the intracellular physiological relevance of any modifier of CPT, the effects of protein binding in the assay assume particular importance. Indeed, any compound that alters CPT activity may do so, not directly, but as an assay artifact changing the free or unbound substrate concentrations.

Several studies have documented that malonyl-CoA is an impressive bridle of fatty acid oxidation (McGarry *et al.*, 1977, 1978; Cook *et al.*, 1978; McGarry & Foster, 1979). This compound pre-eminently inhibits CPT, a key enzyme involved in the transfer of long-chain acyl-CoA groups across the mitochondrial membrane into the enzyme-rich matrix. Nevertheless, controversy has arisen over the physiological importance of malonyl-CoA, particularly since the sensitivity of the mitochondria to this metabolite depends on the nutritional (fed or starved) or hormonal (virgin or pregnant) state (Cook *et al.*, 1980; Saggerson & Carpenter, 1981a, 1982b; Saggerson *et al.*, 1982; Robinson & Zammit, 1982). Complicating the issue as to the importance *in vivo* of malonyl-CoA in the regulation of ketogenesis have been the diverse biochemical conditions by which CPT is assayed, particularly the amount and molar ratios of albumin and substrate (Ontko & Johns, 1980; McGarry & Foster, 1981; Saggerson, 1982). For competitive inhibitors, such as malonyl-CoA, or activators of enzymes to manifest their effects, the ideal substrate concentrations for the enzyme assay should be considerably less than $V_{\max}$. Herein lies a potential problem in interpreting laboratory data generated under disparate assay conditions: the 'apparent' $K_{0.5}$ (substrate concentration at $\frac{1}{2}V_{\max}$) or K_m for the enzyme

depends to a large extent on the assay's content and type of non-enzymic binding protein. Most of the CPT assay methods published, except the original (Norum, 1964), employ albumin. We further stress the importance of the albumin concentration in the CPT assay, its influence on the 'apparent' $K_{0.5}$, and, understandably, the invariance of the $V_{\max}$ with albumin concentration. Obviously the percentage inhibition of CPT by a given concentration of malonyl-CoA will rest on the albumin-dependent $K_{0.5}$ for the enzyme, the actual substrate concentration used in the assay, and the derivative of the enzyme velocity–substrate curve at this concentration. Furthermore, the effects of long-chain fatty acids, which several authors have proposed are direct or indirect intracellular metabolic regulators (Amatruda *et al.*, 1975; McGarry & Foster, 1980; Davis-Van Thienen & Davis, 1981; Topping & Mayes, 1982), on CPT were examined in detail. Interestingly, on first inspection, they appear to be 'activators' of CPT, but this phenomenon is readily explained by their modification of substrate (palmitoyl-CoA) binding to albumin. Likewise, the effects of salicylic acid, another albumin binder, and conceivable modifier of fatty acid oxidation, on CPT activity was examined. Unless precise and standard assay conditions, with particular attention to substrate binding, are followed for CPT and numerous other enzymes, specious conclusions about 'activators' and 'inhibitors' will obscure actual intracellular events.

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

Materials and methods

Animals

Male Sprague-Dawley rats weighing 200–300 g were maintained on commercial laboratory chow and, unless otherwise stated, were starved for 18–24 h before removal of the livers.

Chemicals

CoA, its derivatives, and radiolabelled compounds were obtained from P-L Biochemicals, Milwaukee, WI, U.S.A.

Preparation and assay of liver CPT

Mitochondrial vesicles were prepared as described by McGarry *et al.* (1978). Apart from our reaction temperature (37°C) and other experimental modifications (see below), Assay II from the above reference was used to measure CPT activity. In this assay, the palmitoyl-CoA and albumin concentrations are 50 μM and 2% (w/v) respectively, and the 1 ml total volume contained 12.5 μmol of Tris/HCl (pH 7.4), 156 μmol of KCl, 3.1 μmol of KCN and 6.2 μmol of reduced glutathione. In our experiments the assay conditions were adjusted as outlined in the Figure legends. Since CPT activity was measured in mitochondrial vesicles, thereby exposing both CPT I and CPT II to the substrates, the CPT enzyme discussed throughout the text is total CPT.

Dialysis experiments

Assay mixture (1 ml) containing 100 μM palmitoyl-CoA, 0.15 μCi of [^{14}C]palmitoyl-CoA and 2% albumin (fatty acyl-CoA/albumin molar ratio approx. 0.35) was placed inside cellulose dialysis tubing of M_r cut-off 12000 (Fisher Scientific Co., Pittsburgh, PA, U.S.A.). This dialysis bag was submerged and gently stirred in 4 ml of the CPT assay mixture's buffered salts (see above; McGarry *et al.*, 1978) devoid of albumin and both labelled and unlabelled palmitoyl-CoA. Oleate was added to both solutions separated by the cellulose tubing such that their fatty acid concentrations, given in the text, were identical. The dialysis was performed at room temperature for 7 h, and the radioactivity in 400 μl of the diffusate (the solution outside the bag) was counted (aqueous scintillant ACS II; Amersham Corp., Arlington Heights, IL, U.S.A.).

Results and discussion

The relationship between CPT activity, total palmitoyl-CoA and albumin concentration is depicted in Fig. 1. At any given substrate concentration, the greater the quantity of albumin in the assay, the less palmitoylcarnitine was formed. Indeed, at the commonly used palmitoyl-CoA concentration of 50 μM , decreasing the albumin concentration from

Fig. 1. *Effects of albumin on the assay of CPT* CPT was isolated from starved male rats, and 0.22 mg of protein was used in each assay. The albumin concentrations (% w/v) were 0.01 (●), 0.5 (□) and 2 (△), and the palmitoyl-CoA concentration varied from 0 to 567 μM . The L-carnitine concentration was 175 μM . Values are means $\pm$ S.E.M. for five experiments.

Fig. 2. *Effects of oleate in the CPT assay* CPT was prepared from fed rats and 0.12 mg of protein was added to each assay (1 ml final volume). The albumin concentration (2%) in the assay was unaltered, and the oleate concentrations (mM) were 0 (●), 0.63 (○), 1.25 (□) and 2.5 (△). Values are means $\pm$ S.E.M. for four experiments.

2%, used by McGarry *et al.* (1978), to 0.01% resulted in more than a 3-fold increase in enzyme activity. These investigators and others have repeatedly suggested the importance of the fatty acid/albumin ratio in intact-mitochondrion studies (Ontko & Johns, 1980; McGarry & Foster, 1981; Saggerson, 1982); our observations confirm and further define the importance of the protein-binding concentration in the CPT assay with palmitoyl-CoA. When the assay condition contained only a minute amount of albumin (0.01%), palmitoyl-CoA concentrations above 150 μM were inhibitory, a phenomenon noted by Norum (1964) and perhaps related to the detergent effect or micelle interactions of large amounts of unbound palmitoyl-CoA. Since we dealt with high concentrations of palmitoyl-CoA and measured total CPT, not CPT I, we were not

Table 1. *Effects of oleate on the dialysis of [1-¹⁴C]-palmitoyl-CoA*

A 1 ml solution containing 100 μ M-palmitoyl-CoA, [1-¹⁴C]palmitoyl-CoA, and 2% albumin in buffered salts (see the Materials and methods section) was separated by a semi-permeable membrane from a 4 ml solution containing only the identical buffered salts. Oleate was added to each of the partitioned solutions to a final concentration as given below. After 7 h of mixing, the radioactivity (d.p.m.) in 400 μ l of the diffusate (the 4 ml solution) was measured. Values represent means $\pm$ S.E.M. for four experiments.

Concn. of oleate (mM)	Diffusate [1- ¹⁴ C]palmitoyl-CoA (d.p.m.)
0	47 $\pm$ 8
0.63	64 $\pm$ 7
1.25	86 $\pm$ 9
2.50	202 $\pm$ 15

able to discern the sigmoidal CPT I kinetics observed at relatively low substrate concentrations by Saggerson & Carpenter (1981b, 1982a) and Saggerson *et al.* (1982). A Lineweaver–Burk plot of the data revealed, at least at the two higher albumin concentrations, a common $V_{\max}$ of 10.5 nmol/min per mg of protein. Since these plots were non-linear at the low palmitoyl-CoA concentration, precise K_m values could not be determined. In their stead, $K_{0.5}$ values, i.e. palmitoyl-CoA concentrations that resulted in $\frac{1}{2} V_{\max}$ enzyme activity, were calculated. At 2 and 0.5% albumin, the respective $K_{0.5}$ values were 940 and 400 μ M. A K_m could be determined when the albumin concentration was the lowest, 0.01%. Ignoring the last point because of the above-mentioned inhibition by high concentrations of palmitoyl-CoA, the K_m was 47 μ M; this is strikingly close to the 31 μ M reported by Norum (1964), who used a radioisotopic CPT assay without albumin. Hence it is evident that the ostensible K_m or $K_{0.5}$ for palmitoyl-CoA varies directly with the assay's albumin concentration and, as a corollary, the percentage inhibition of CPT at a given concentration of malonyl-CoA could be far greater in those assays with more albumin in the mixture.

Conversely, the presence of a compound that interferes with albumin binding may 'activate' the enzyme, simply by an artifact of the assay conditions. Oleate, a long-chain fatty acid, is one such compound. If oleate is added to the assay mixture, the CPT activity, at any given concentration of total palmitoyl-CoA, is increased (Fig. 2). In the present study the albumin concentration was fixed at 2%. Oleate was without effect when albumin was omitted from the assay (results not shown). This latter observation argues against a non-specific biochemical effect of oleate on the mitochondrial

vesicles, such as altering the membrane structure, or its potential beneficial antioxidant properties. Lineweaver–Burk plots of the data revealed a similar $V_{\max}$ (7.1 nmol/min per mg of protein) but variable $K_{0.5}$ values depending on the assay's oleate content. In the absence of oleate and when the albumin concentration was 2%, the $K_{0.5}$ for palmitoyl-CoA was 450 μ M. For these particular studies CPT was prepared from fed rats. It is noteworthy that, at an identical albumin concentration in the assay, the respective $K_{0.5}$ of palmitoyl-CoA for CPT obtained from starved rats was considerably greater (see Fig. 1). Since the nutritional status of the animal appears to modify the $K_{0.5}$ for palmitoyl-CoA, this alteration in substrate–enzyme binding may partially explain the disparate sensitivity of CPT I to inhibition by malonyl-CoA which is induced by starvation. Nevertheless, as the oleate concentration in the assay was increased, the $K_{0.5}$ for palmitoyl-CoA decreased; again, this implies a disturbance of albumin–substrate binding. Suspecting substrate (palmitoyl-CoA) displacement from albumin by the oleate, dialysis experiments were performed in which albumin and [1-¹⁴C]palmitoyl-CoA were partitioned from the assay's buffered salts by a semi-permeable membrane. Oleate was added to each side of the membrane, and the amount of palmitoyl-CoA, supposedly free or unbound, that crossed was determined. The results clearly expose a displacement phenomenon; the greater the oleate concentration, the more palmitoyl-CoA was liberated from albumin and found across the membrane (Table 1). Under standard assay conditions (McGarry *et al.*, 1978) with a substrate/albumin molar ratio of 0.18, this oleate effect greatly increases the availability of substrate for the enzyme and, seemingly, appreciably 'activates' the enzyme (Fig. 2).

Salicylates avidly bind to albumin, displacing free fatty acids and, perhaps, acyl-CoA groups. If the latter does occur, a similar increase in CPT I activity, as with oleate, might be expected. Indeed, as the salicylic acid concentration in the assay was increased, the CPT I activity rose similarly until, at approx. 20 mM-salicylic acid, a peak was reached (Fig. 3). At this value, CPT I activity had doubled, but thereafter there was a progressive decline in enzyme activity with increasing salicylic acid content. This decline was attributed in part to the pH changes occurring in the assay; for instance, without salicylate in the assay, a change in the assay's pH from 7.50 to 6.85 resulted in a decrease in enzyme activity (results not shown). The increase in enzyme activity at very high salicylate concentrations may also be a direct toxic or osmotic effect of this compound on the mitochondrial vesicles.

The purpose of the present paper is to reinforce the importance of assay conditions when inter-

Fig. 3. *Salicylic acid effects on CPT activity*

CPT was obtained from starved male rats. Each assay contained 2% albumin, 50 μ M-palmitoyl-CoA and 0.12 mg of mitochondrial protein. Salicylic acid was added to the final concentrations shown. The Tris/HCl buffer, usually pH 7.4, was adjusted to pH 7.9; the pH changes in the assay mixture due to this compound are also given. Results are means $\pm$ S.E.M. for three experiments. Control (100%) CPT activity, i.e. in the absence of salicylic acid, was 0.98 ± 0.14 nmol/min per mg of protein.

preparing the percentage regulation of CPT by modifiers, especially with such disparate albumin-dependent $K_{0.5}$ values for palmitoyl-CoA. Since the $K_{0.5}$ (940 μ M) for palmitoyl-CoA with CPT from starved rats, and with 2% albumin in the assay, far exceeds the actual concentration employed, namely 50 μ M, this implies that most of the substrate is bound. Consequently, trivial changes in substrate-albumin binding, perhaps through pH, temperature, or displacement by other compounds, may dramatically influence CPT activity and the potency of a competitive inhibitor. Conversely, if the free substrate concentration is high, as seen with low-albumin low- $K_{0.5}$ conditions, the attenuation of CPT activity at any given concentration of inhibitor may not be as great, and, furthermore, alterations in the albumin binding would not assume such propor-

tions. These studies demonstrate the considerable effects of albumin and modifications of its substrate binding on the assay of CPT enzyme activity.

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