BIOCHEMICAL LETTERS

Kinetics of enzymes requiring long-chain acyl-CoA esters as substrates: effects of substrate binding to albumin

There are many problems associated with the investigation of enzymes for which long-chain acyl-CoA esters are substrates, particularly when these enzymes are bound to cell membranes. Palmitoyl-CoA forms micelles above a critical concentration, which was estimated at 3–4 μM (Zahler *et al.*, 1968), although more recent studies have suggested higher values of 30–60 μM (Powell *et al.*, 1981). Palmitoyl-CoA also has detergent properties, and attempts to circumvent these problems have usually involved the use of albumin as a binder. These problems are particularly acute with studies of the kinetics of CPT I (EC 2.3.1.21). This enzyme is associated with the outer face of the mitochondrial inner membrane and high palmitoyl-CoA concentrations may expose CPT II, which is bound to the inner face. Further, the sensitivity of CPT I to inhibition by malonyl-CoA is decreased when the enzyme is displaced from the inner membrane by detergents (McGarry *et al.*, 1978). In several recent investigations of the regulation of CPT I activity by malonyl-CoA, constant amounts of bovine serum albumin were routinely added to all assays when [palmitoyl-CoA] was varied, but the extent and effect of binding of palmitoyl-CoA to albumin was apparently not considered (see, for example, Saggerson & Carpenter, 1982). It has been remarked that the value of free [oleoyl-CoA] is not known in the presence of albumin, but all the kinetic implications of this do not appear to have been realized (McCormick & Notar-Francesco, 1983; Cook, 1984).

The purpose of this letter is to make the obvious point that more attention should be paid to the concentration of palmitoyl-CoA present in true free solution, rather than to the total palmitoyl-CoA concentration added in the presence of proteins which (non-catalytically) bind palmitoyl-CoA. We do not know of any measurements of the number of binding sites for palmitoyl-CoA on a molecule of bovine serum albumin or of their binding constants, so the free [palmitoyl-CoA] cannot be calculated. Further, it is not always

appreciated that, since binding of ligands to albumin is a bimolecular reaction, free [ligand] is not linearly related to total [ligand] plus [binder] when these are varied while maintaining their molar ratio constant.

In Fig. 1 we illustrate a model in which the presence of a constant concentration of a binding protein can generate an apparently sigmoidal relationship between the rate of reaction and [substrate] for an enzyme with classical Michaelian kinetics. With constant [binder] the fraction of substrate bound decreases with increasing total [substrate] so that free [substrate] will increase more than total [substrate]. If the values of binding constant, $K_{\text{assoc.}}$, and the concentration of the binder for the substrate are assumed, free [substrate] can be calculated and this value used to compute $v/V_{\text{max.}}$ for the enzyme in relation to total [substrate] given the K_m of the enzyme. Fig. 1 shows $v/V_{\text{max.}}$ against [substrate] plots when various values are assumed for $K_{\text{assoc.}}$ and K_m . Marked sigmoidicity is found, with values which are similar to those for many enzymes and binding proteins. Secondary Hill plots have a slope greater than unity, showing apparent positive co-operativity. The shapes of the curves (Fig. 1a) depend upon the values assigned to $K_{\text{assoc.}}$, K_m and [binder]. In the presence of a competitive inhibitor the apparent sigmoidicity is increased, because the value of $K_{m,\text{app.}}$ is decreased (Fig. 1b). If an inhibitor is introduced into this system which is also a modified long-chain acyl-CoA ester, a number of additional interactions are possible which we have not attempted to analyse, including competition for binding to albumin (Bartlett *et al.*, 1984). We have also not considered the possibilities that albumin–acyl-CoA complexes or long-chain acyl-CoA micelles may be substrates for the enzyme, or that inhibition by micelles may be relieved by binding of micelles to albumin as has been suggested for palmitoyl-CoA hydrolase (EC 3.1.2.2) (Berge, 1979). In Fig. 1(c) the effect of increasing the strength of binding while maintaining a constant [binder] is shown. In the case of extremely tight binding (curve on the extreme right), $v/V_{\text{max.}}$ only increases rapidly when the binder is saturated, that is, the binder is first titrated and, with further increase in [substrate], a typical rectangular hyperbola is seen.

Sigmoidal relationships between [palmitoyl-CoA] and the activity of CPT I have been reported in some studies in which constant [bovine serum

Abbreviations used: CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II.

Fig. 1. Plots of $v/V_{\max}$ versus [substrate]
 (a) $K_m = 5 \mu\text{M}$, binding constant ($K_{\text{assoc.}}$) = $1 \times 10^6 \text{ M}^{-1}$, no inhibitor present. Each curve represents a single concentration of binder (A) (0 – $50 \mu\text{M}$ increased in increments of $2.5 \mu\text{M}$ from left to right).
 (b) As for (a), with the addition of $5 \mu\text{M}$ -competitive inhibitor ($K_i = 5 \mu\text{M}$). (c) Effect of constant [binder] ($25 \mu\text{M}$) with increasing binding constants (0 – $8 \times 10^3 \text{ M}^{-1}$ in increments of 0.2×10^6). Initial velocities were calculated by substitution into the simple Michaelis–Menten equation of the values for unbound [S], which were calculated from the solution of $K_{\text{assoc.}} = [\text{SA}]/[\text{S}][\text{A}]$, assuming no micelle formation and instantaneous equilibrium. Each curve was constructed from 0 – $100 \mu\text{M}$ [S] in increments of $1 \mu\text{M}$. The calculations were performed on a Commart microcomputer running compiled BASIC. The results were displayed on a Watanabe xy-plotter (model WX4633). Copies of the computer program are available upon request.

albumin] were employed. Furthermore, the sigmoidicity is increased by malonyl-CoA (Saggerson & Carpenter, 1982; Cook, 1984). Although one might anticipate that regulatory enzymes such as CPT I would show allosteric behaviour, i.e. sigmoidal kinetics, any true sigmoidicity will be exaggerated by the presence of albumin.

Our approach can also be applied to a model for the effects on $v/V_{\max}$ of varying the concentration of a mixture of substrate plus binder with a constant molar ratio. It can be shown that there is no sigmoidicity, although $v/V_{\max}$ is less for any value of [substrate] compared with that in the absence of binder. The deviation from the classical hyperbolic relation between $v/V_{\max}$ and [substrate] becomes greater as [substrate] plus [binder] increases, since the percentage dissociation of the substrate–binder complex is less at higher total concentrations (result not shown). Mathematically, the relation between $v/V_{\max}$ and [substrate] cannot be linearized by using any conventional plot, although approximately straight lines may be obtained empirically with some ranges of values of [substrate]. Estimates of the apparent K_m , therefore, depend on the segment of the curve used, and these estimates shift to higher values with higher ranges of [substrate].

A solution to these problems might appear to be measurement of enzyme activity at low concentrations of long-chain acyl-CoA esters in the absence of albumin. However, when preparations of membrane-bound enzymes are used, problems may be encountered that are due to non-specific binding of acyl-CoA esters to the membrane and to the uncertain action of acyl-CoA hydrolases.

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