for a generous supply of GTP cyclohydrolase I that was used in part of this study.

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Reactivation of D-β-Hydroxybutyrate Dehydrogenase with Short-Chain Lecithins: Stoichiometry and Kinetic Mechanism†

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ABSTRACT: D-β-Hydroxybutyrate dehydrogenase (BDH), purified as soluble, lipid-free apoenzyme (inactive) from either beef heart or rat liver mitochondria, can be reactivated by short-chain lecithins in the monomeric state. The enzyme was reactivated with dihexanoyl- [PC(6:0)], diheptanoyl- [PC(7:0)], and dioctanoyllecithins [PC(8:0)]. The titration curves of enzyme activity as a function of the phospholipid concentration are consistent with a model in which the enzyme contains two identical, noninteracting lecithin binding sites. The simultaneous occupation of these sites (via an equilibrium random mechanism) is required to activate the apoenzyme. Similar results were obtained with both rat liver and beef heart apoenzymes. The maximal velocities obtained with the different lecithins were similar [110–140 μmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$]. The K_L values (the apparent dissociation constants of the lecithin-site complexes) were 1.2×10^{-4} M [PC(8:0)], 1.5×10^{-3} M [PC(7:0)], and 4.5×10^{-3} M [PC(6:0)] at 37 °C. This was confirmed by using phospholipase A_2 to compete with the dehydrogenase for the lecithin monomers. Comparison of the $\Delta G°$ values for complex formation with the different lecithins shows an average contribution of approximately 2.4 kJ/mol ($0.9RT$) per CH_2 group. The interaction of the apolar moiety of lecithin with the protein seems to be essential for effective binding of phosphatidylcholine to apoBDH. The $\Delta G°$ values, when combined with the estimated $\Delta H°$ values, suggest that the binding of lecithin to the apoenzyme is ~60% enthalpy and ~40% entropy driven.

Biological membranes are highly organized structures that consist mainly of phospholipid and proteins. D-β-Hydroxybutyrate dehydrogenase [D(−)-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30; henceforth BDH[1]] is one of the best studied phospholipid-requiring enzymes (Fleischer et al., 1974). It is tightly bound to the mitochondrial inner membrane

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[1] Abbreviations: BDH, D-β-hydroxybutyrate dehydrogenase; apoBDH, D-β-hydroxybutyrate dehydrogenase apoenzyme; PC, phosphatidylcholine; MPL, a mixture of phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol (molar ratio 1.0:0.8:0.2) isolated from rat liver mitochondria; PC(18:1), PC(8:0), PC(7:0), and PC(6:0), 1,2-dioleoyl-, 1,2-dioctanoyl-, 1,2-diheptanoyl-, and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, respectively; D-PC(7:0), 2,3-diheptanoyl-*sn*-glycero-1-phosphocholine; cmc, critical micellar concentration; DEAE, diethylaminoethyl; CM, carboxymethyl.

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(Lehninger et al., 1960; McIntyre et al., 1978a) and exhibits a specific requirement of phosphatidylcholine (henceforth PC) for activity (Sekuzu et al., 1963). The soluble, phospholipid-free protein, referred to as apodehydrogenase (or apoBDH), has been purified to homogeneity from beef heart (Bock & Fleischer, 1974, 1975) and rat liver mitochondria (Vidal et al., 1977a,b). A kinetic analysis of the reaction mechanism of BDH, with regard to substrates and coenzyme interaction, was made both for the membrane-bound enzyme in submitochondrial particles and for a purified soluble preparation reactivated with phospholipid vesicles (Nielson et al., 1973). The results were consistent with an ordered Bi-Bi mechanism (nomenclature of Cleland) where NAD^+ is the first substrate to add to the enzyme and NADH is the last product to leave. The mechanism is the same for the enzyme in the membrane or in phospholipid vesicles.

Regardless of the source of the enzyme (beef heart or rat liver), apoBDH as isolated is catalytically inactive and can be reactivated by the addition of PC or phospholipid mixtures containing PC upon formation of an active BDH–PC complex (Fleischer et al., 1974; Gazzotti et al., 1974, 1975). The binding of PC confers to the enzyme the ability to bind its coenzyme NADH (Gazzotti et al., 1974). The specificity of BDH is primarily for the polar head group of PC (Isaacson et al., 1979), whereas the hydrophobic moiety seems to be essential for efficient binding of PC to the apoenzyme (Grover et al., 1975; Vidal et al., 1977b, 1978). The interaction of apoBDH with PCs of well-defined fatty acyl composition in terms of parameters important for enzyme activity has been already reported (Gazzotti et al., 1975; Vidal et al., 1978). Although these studies gave comparative information, obtaining quantitative kinetic data is complicated when aqueous microdispersions of phospholipids are employed. In fact, the micellar nature of the lipid introduces uncertainties concerning the actual phospholipid concentration. Further complications are introduced if more than one aggregated form are present (Tinker & Pinteric, 1971), which may have different affinities and/or reactivating efficiencies for the apoenzyme. As a consequence, the experimental V_{max} and K_m are both functions of other variables that cannot be measured or calculated from the data presently available. Conversely, short-chain lecithins well below their critical micellar concentrations (i.e., as monomers) are able to bind and reactivate the apoBDH (Gazzotti et al., 1975; Vidal et al., 1977b, 1978), the BDH–PC complexes being readily dissociated. Since the phospholipid is not modified either upon complex formation or during the catalytic cycle, it is to be expected that these BDH–PC complexes will behave as an equilibrium system. Thus, classic kinetic analysis can be applied without limitations regarding the exact meaning of the experimental parameters. This paper presents the study of the kinetic mechanism of reactivation of beef heart and rat liver apoBDH with monomeric dihexanoyl-, diheptanoyl-, and dioctanoyllecithins.

Experimental Procedures

Chemicals. Sucrose, tris(hydroxymethyl)aminomethane (Tris), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), dithiothreitol (DTT), nicotinamide adenine dinucleotide (oxidized form, β-NAD), sodium DL-3-hydroxybutyrate, bovine serum albumin (BSA, type F), phenylmethanesulfonyl fluoride (PMSF), L-α-glycerophosphocholine, and oleic, hexanoic, heptanoic, and octanoic acids were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]-Octanoic acid was purchased from ICN Isotope and Nuclear Division (Cleveland, OH). Rhodamine 6G was purchased from British Drug Houses, Ltd. (England). All the other reagents were analytical grade, and the solutions were prepared in doubly distilled water, unless otherwise stated.

Fractionation Materials. Sephadex G-25 (coarse and fine grades), G-50, G-75, and G-100 (fine grade), Sepharose 4B-Cl, DEAE-Sephadex A-50, and CM-Sephadex C-50 were purchased from Pharmacia, Ltd. (Uppsala, Sweden). CPG-10-350 controlled-pore glass beads, 120–150 mesh, were purchased from Electro-Nucleonics (Fiarfield, NJ). Diaflo ultrafiltration membranes and cells were obtained from Amicon Co. (Lexington, MA). Thin-layer chromatography (TLC) was performed on glass plates covered with a layer (250 μm thick) of silica gel H (E. Merck, Darmstadt) containing 3% (by weight) magnesium acetate (Turner & Rouser, 1970). The plates were spread with Desaga equipment (Heidelberg, West Germany). Aluminum oxide (Mallinckrodt) and preactivated silicic acid (Unisil) were employed in purification of phospholipids.

Lipids. Mitochondrial phospholipids (henceforth MPL) from rat liver mitochondria were extracted and purified as described by Gazzotti et al. (1975) for beef heart MPL. Synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine [henceforth PC(18:1)] was prepared by acylation of L-α-glycerophosphocholine with oleic acid anhydride as described by Cubero Robles & Van den Bergh (1969) and was stored in hexane at −20 °C. The purified phospholipid exhibited a single species when analyzed by TLC using chloroform–methanol–water (65:25:4 by volume) as the developing system. Aqueous microdispersions were prepared from aliquots of this solution, which were dried under nitrogen, suspended in 1.0 mM EDTA–50 mM Tris-HCl buffer, pH 8.0 (3–4 μmol/mL final concentration), with a Vortex mixer, and sonicated in a MSE (Measuring and Scientific Equipment, Ltd., England) ultrasonic disintegrator (500 W, titanium probe) at 20 kHz and cavitating intensity for 30 min at 0 °C under nitrogen. These suspensions were centrifugated for 60 min at 105000*g*, and the supernatants were concentrated to about 25 μmol/mL by ultrafiltration under nitrogen using a XM-100 Diaflo membrane. The fraction of single-bilayer vesicles was separated by chromatography on Sepharose 4B-Cl as described by Huang & Thompson (1974). Synthetic 1,2-dihexanoyl-, 1,2-diheptanoyl-, and 1,2-dioctanoyl-*sn*-glycero-3-phosphocholines [henceforth PC(6:0), PC(7:0), and PC(8:0), respectively] were prepared according to Cubero Robles & Van den Bergh (1969) or purchased from Calbiochem (La Jolla, CA). Radiolabeled [^{14}C]PC(8:0) was synthesized by using [1-^{14}C]octanoic acid. The phospholipid samples were stored in anhydrous ethanol at −20 °C, and the purity was tested by TLC as indicated above. Aliquots of these solutions were dried under nitrogen and dissolved in an appropriate amount of water to obtain a final concentration of 18–20-fold the cmc of the corresponding lecithin. Clear micellar solutions were obtained with PC(6:0) and PC(7:0), while PC(8:0) separated into two phases at room temperature. The aqueous micellar solutions were kept at room temperature except PC(8:0), which was maintained at 40 °C to avoid phase separation. 2,3-Diheptanoyl-*sn*-glycero-1-phosphocholine [henceforth D-PC(7:0)] was a gift from Dr. G. H. de Haas, Utrecht State University, The Netherlands, and the aqueous solutions were prepared as those of the enantiomeric 1,2-diacyl-*sn*-glycero-3-phosphocholine. Phospholipid concentrations were determined by phosphorus analysis using the method of Chen et al. (1956) as modified by Rouser & Fleischer (1967).

Measurement of Critical Micellar Concentrations. Critical micellar concentrations (cmc) were determined by the spectral shift induced by the incorporation of Rhodamine 6G into

phospholipid micelles, according to Bonsen et al. (1972). No significant differences were found in 25 mM Tris-HCl, pH 8.1, or in 10 mM potassium phosphate buffer, pH 7.35. The values obtained were 9.8 mM with PC(6:0), 1.6 mM with PC(7:0), and 0.19 mM with PC(8:0). No difference was found between the L and D isomers of PC(7:0).

The standard assay mixture for measuring BDH activity (1.0-mL final volume) contained 10 mM potassium phosphate buffer, pH 7.35 (beef heart BDH), or 25 mM Tris-HCl buffer, pH 8.1 (rat liver BDH), 2.0 mM NAD^+, 1.0 mM EDTA, 0.3 mM DTT, and varying amounts of lipids and apoenzyme. The assay medium was preincubated for 10 min at 37 °C; an aliquot of lipid was added, followed by the apoenzyme. After an additional 2-min incubation to obtain optimal activation, the reaction was started by the addition of 20 μL of 1.0 M sodium DL-3-hydroxybutyrate. Initial reaction rates were recorded by following the reduction of NAD^+ at 340 nm in a Zeiss PM Q II spectrophotometer, and the enzyme activity was expressed as micromoles of NAD^+ reduced per minute per milligram of protein, with an extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1}. When the temperature dependence of the reaction rate with the rat liver enzyme was measured, the assay mixture had a similar composition except that 25 mM Hepes buffer was used instead of Tris, to minimize pH changes with temperature.

Phospholipase A_2. Porcine pancreas prophospholipase A_2 was prepared according to Nieuwenhuizen et al. (1974). The pure zymogen was converted into phospholipase A_2 after activation by cleavage with trypsin. The reaction was terminated by treatment with PMSF to inhibit the trypsin, and the activation peptide was removed by chromatography on Sephadex G-25. Phospholipase A_2 was then purified by chromatography on DEAE-Sephadex A-50 (Vidal et al., 1977a). The enzyme activity was measured titrimetrically according to Nieuwenhuizen et al. (1974) with a Radiometer TTT 1c autotitrator equipped with a syringe–buret SB 1a as dispenser of 0.1 N NaOH. The reaction mixture (9.0-mL final volume), containing egg yolk lipoprotein (3.2 μmol of lipid P/mL), 5 mM $CaCl_2$, and 8.3 mM sodium deoxycholate, was adjusted to pH 8.0 in a thermostated vessel at 40 °C. The alkali consumption required to maintain the pH at 8.0 for 5 min was recorded. The enzyme reaction was then started by adding 2–20 μL of the phospholipase A_2 solution, and the alkali consumption was further recorded for 2 min. One unit of phospholipase A_2 activity was defined as the consumption (release) of 1.0 μequiv of NaOH (fatty acid)/min at 40 °C. The specific activity of the purified enzyme was about 1000 units/mg of protein in the standard assay. This preparation was identical with a sample of pure phospholipase A_2 kindly supplied by Dr. G. H. de Haas, Utrech State University, The Netherlands. The extinction coefficient of the purified enzyme was $E^{1\%}_{280nm} = 13.0$.

Protein concentration was measured by the method of Lowry et al. (1951) using BSA as the standard. When the samples contained DTT, the modification described by Ross & Schatz (1973) was employed.

Dissociation Constants of Complexes between Phospholipase A_2 and Monomeric Lecithins. The affinity of phospholipase A_2 for monomeric lecithins in the absence of Ca^{2+} ions was determined by ultraviolet difference spectroscopy and equilibrium gel filtration. In the presence of monomeric lecithins, the enzyme displayed a typical difference spectrum with peaks at 282 and 289 nm (Pieterson et al., 1974a; van Dam-Mieras et al., 1975). The dissociation constants were determined from the intercepts on the abscissa axis of plots of the reciprocal A_{289} as a function of the reciprocal lecithin concentration as described by Pieterson et al. (1974a,b). The values of K_d obtained with 25 mM Tris-HCl buffer, pH 8.1, at 37 °C were 6.0×10^{-3} M with PC(6:0), 6.2×10^{-4} M with PC(7:0), and 8×10^{-5} M with PC(8:0). No significant differences in the K_d values were observed with L-PC(7:0) or D-PC(7:0), in agreement with previous studies by Bonsen et al. (1972). In 10 mM potassium phosphate buffer, pH 7.35, the K_d values obtained were 5.6×10^{-3} with PC(6:0), 5.9×10^{-4} M with PC(7:0), and 7.9×10^{-5} M with PC(8:0).

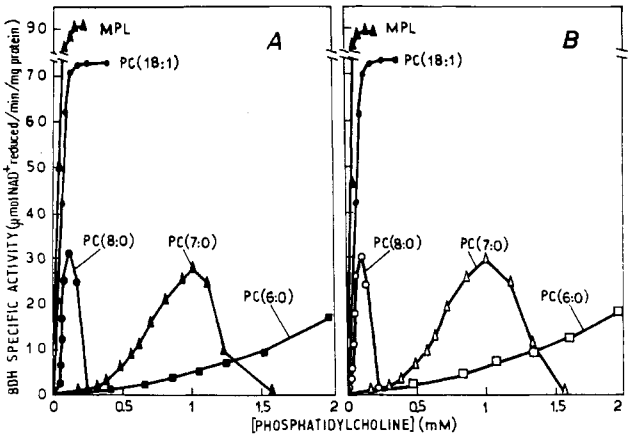


FIGURE 1: Reactivation of BDH apodehydrogenase with different lecithins. The enzyme activity was measured by using 6.0 μg of apoBDH/assay and is represented as a function of the phospholipid concentration. The lecithins employed are referred to in shorthand on each curve. (A) Beef heart BDH; (B) rat liver BDH.

The K_d values of the phospholipase A_2–PC complexes were also tested by equilibrium gel filtration on a 50 × 0.6 cm column of Sephadex G-25 preequilibrated and eluted at 37 °C with a solution of 5.6 mM PC(6:0), 0.7 mM PC(7:0), or 0.1 mM radiolabeled PC(8:0) in 10 mM EDTA–25 mM Tris-HCl buffer, pH 8.1. A sample containing 0.3 μmol of phospholipase A_2 in 250 μL of the equilibrium medium was applied, and the elution was carried out as described by Pieterson et al. (1974b). From the equal peak and trough areas in the elution pattern of the PC and the total amount of enzyme eluted in the void volume, the dissociation constants were calculated. The values obtained were 6.6×10^{-3} M with PC(6:0), 6.0×10^{-4} M with PC(7:0), and 8.12×10^{-5} M with PC(8:0).

The dissociation constants of the phospholipase A_2–PC complexes in 10 mM EDTA–10 mM Hepes buffer, pH 7.35, measured with the same techniques were 6.2×10^{-3} M with PC(6:0), 5.7×10^{-4} M with PC(7:0), and 8×10^{-5} M with PC(8:0). The value for PC(7:0) is in agreement with that previously reported by Pieterson et al. (1974a,b).

It must be noted that at an equilibrium concentration of 1.2 mM PC(7:0) or 0.185 mM PC(8:0), the ratio [PC bound]/[PC free] is higher than 1.0, which means that when the concentration of free PC approaches the cmc, the assumption that one molecule of phospholipase can bind one molecule of PC is no longer valid. This probably reflects the existence of aggregated lipid (Tausk et al., 1974) to which the phospholipase A_2 displays a higher affinity.

Results

Reactivation of ApoBDH with Long-Chain and Short-Chain Lecithins. The reactivation of beef heart and rat liver apodehydrogenases with several PCs was studied by titrating each phospholipid to a constant amount of apoenzyme to obtain the respective reactivation curve as a function of the phospholipid concentration (Figure 1A,B). From these curves, two reac-

tivation parameters were obtained for each lipid, namely, (a) the percentage of maximal reactivation (Gazzotti et al., 1975) or relative reactivation (Vidal et al., 1978), defined as the ratio between the maximal activity obtained with the specific lecithin and that obtained with MPL, which was set equal to 100% (Gazzotti et al., 1975) or to 1.0 (Vidal et al., 1978), and (b) the efficiency of reactivation, defined as the number of moles of PC added per mole of apoBDH subunit to obtain half-maximal reactivation (Gazzotti et al., 1975; Vidal et al., 1978). By this definition, a smaller number denotes better efficiency of activation.

As shown in Figure 1A,B, the reactivation curves obtained with the different phospholipids employed in the present study were similar for apoBDH purified from either beef heart or rat liver mitochondria. Therefore, unless otherwise stated, we shall refer to apoBDH without any specification regarding the source of enzyme. The curves presented in Figure 1 for MPL and PC(18:1) are remarkably different from those for the short-chain PC(6:0), PC(7:0), and PC(8:0). In fact, with MPL or PC(18:1) there is an increase in enzyme activity with the phospholipid concentration up to a maximum, and no significant changes in enzyme activity occur upon further increase in phospholipid concentration. The maximal specific activities as well as the reactivating efficiencies are high, i.e., 85–92 μmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$ with an efficiency of 2–4 mol of PC/mol of apoBDH with MPL and 70–78 μmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$ with an efficiency of 8–10 mol of PC/mol of apoBDH with PC(18:1). Conversely, with the short-chain lecithins the titration curves are biphasic. The enzyme activity increases with the phospholipid concentration up to a maximum; however, further increase in phospholipid results in a sharp decrease of the enzyme activity, which falls to zero near the critical micellar concentration of the lecithin used. This decrease in enzyme activity seems to be related with the concentration-dependent aggregation of the phospholipid, the enzyme being rapidly inactivated when complexed to these aggregates (Grover et al., 1975; Gazzotti et al., 1975; Vidal et al., 1978). The peak (or apparent maximal) BDH specific activities obtained with these short-chain lecithins [22–36 μmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$] are therefore artificially low. The apparent reactivating efficiencies (i.e., the amount of PC required to obtain half of the apparent maximal activities) ranged from 250 mol for PC(8:0) to 8000 mol for PC(6:0) per mol of apoBDH subunit. Extrapolation to maximal reactivation at infinite monomeric PC concentration ($1/v_0$ vs. $1/[PC]$) is not practical because of the nonlinearity of the double-reciprocal plots at low lipid concentrations.

Rate Equation for Reactivation of ApoBDH by Short-Chain Lecithins. The ascending parts of the titration curves are sigmoidal (Figure 1A,B), with inflection points at 0.06 mM for PC(8:0), 0.73 mM for PC(7:0), and 2.2 mM for PC(6:0), which suggests either positive cooperativity for lecithin binding or that the enzyme requires a certain amount of bound PC in order to obtain the active conformation. The Hill plots for beef heart and rat liver apoBDH in complexes with the different short-chain lecithins, in the range of PC concentrations corresponding to the ascending part of the curves (not shown), are approximately linear with limiting slopes ($n_{app_{max}}$) close to 2.0 even at the lowest phospholipid concentration compatible with the measurement of enzyme activity (Segel, 1975). The fact that these limiting slopes do not approach 1.0 could imply that the binding of at least two PC molecules per apoBDH subunit is required for function. If the two binding sites were highly cooperative, the velocity equation as a function of the

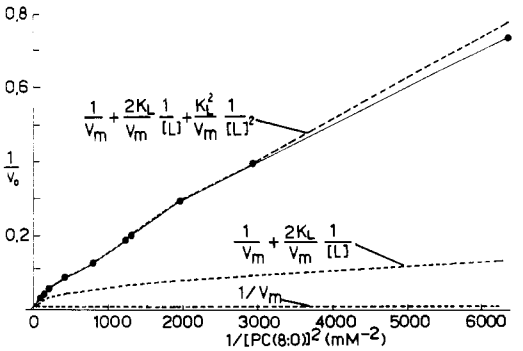


FIGURE 2: Double-reciprocal plot of initial velocities as a function of the square of the concentration of PC(8:0). The experimental data were obtained from the rising part of the titration curves shown in Figure 1. Equation 11 has been plotted (dashed lines) including all the terms (top), the first two terms (middle), and only the first term. The values of $2K_L/(V_m[L])$ and $2K_L/(V_m[L]) + K_L/(V_m[L]^2)$ have been calculated from data presented in Table I. The data fit only the full eq 11, which includes [L] to the second power.

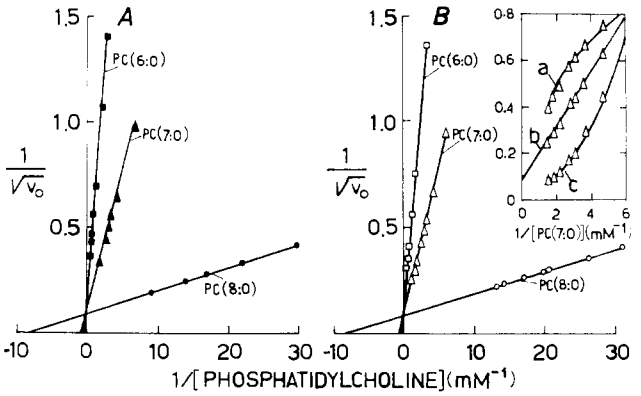


FIGURE 3: Double-reciprocal plots of the square root of initial velocities as a function of phospholipid concentration (cf. the text, eq 4). The experimental data were obtained from the rising part of the titration curves shown in Figure 1. (A) Beef heart BDH; (B) rat liver BDH. (Inset) Comparison of the results obtained from the Hill equation by plotting $1/v_0$ (curve c, $n = 1$), $1/v_0^{1/2}$ (curve b, $n = 2$), and $1/v_0^{1/3}$ (curve a, $n = 3$) as a function of the reciprocal PC(7:0) concentration.

PC concentration could be expressed by a modified Hill equation:

$$\frac{v_0}{V_m} = \frac{[L]^2/K_L'}{1 + [L]^2/K_L'} = \frac{[L]^2}{K_L' + [L]^2} \quad (1)$$

where v_0 is the initial reaction rate, V_m is the maximal velocity at $[L] = \infty$, and K_L' represents the product of K_L^n (K_L being the intrinsic dissociation constant of the PC site) and the $n - 1$ interaction factors raised to the appropriate powers. For $n = 2$, $K_L' = K_L^2 a$, where $a \ll 1$; then

$$\frac{1}{v_0} = \frac{1}{V_m} + \frac{K_L'}{V_m}\frac{1}{[L]^2} \quad (2)$$

However, as shown in Figure 2, the plot of $1/v_0$ vs. $1/[PC(8:0)]^2$ is not a straight line (as predicted by eq 2 for highly cooperative binding sites) so that this model for two interacting sites does not fit the data. In contrast, the data in Figure 2 fit a curve described by eq 11 (see below and Discussion), which is compatible with two noninteracting PC sites. In Figure 2, the intercept on the $1/v_0$ axis provides an estimate of $1/V_m$, which is 7.15×10^{-3} min (mg of protein) (μmol of NAD^+ reduced)$^{-1}$ ($V_m = 140$). Similarly, a curve was obtained when $1/v_0$ was plotted as a function of $1/[PC(7:0)]^2$, with an intercept on the $1/v_0$ axis of 7.52×10^{-3} min (mg of protein)

Table I: Kinetic Parameters for Reactivation of ApoBDH with Different Short-Chain Lecithins

	beef heart enzyme			rat liver enzyme		
kinetic parameter	PC-(6:0)	PC-(7:0)	PC-(8:0)	PC-(6:0)	PC-(7:0)	PC-(8:0)
K_L[a] × 10^3 (M)	4.36	1.56	0.13[b]	4.53	1.46[b]	0.12[b]
V_m[a] [μmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$]	122	136	143	119	129	140
no. of expts	5	4	6	5	6	8

[a] The values of K_L and V_m were obtained from $1/v_0^{1/2}$ vs. 1/[PC] plots as shown in Figure 3 and are based on eq 4, in the text. The maximal relative errors were lower than 9 and 6%, respectively. [b] Values of K_L were also obtained by competition with phospholipase A_2 (cf. Figure 4) for binding of PC(8:0) to beef heart and rat liver apoBDH (0.134 and 0.124 mM, respectively) and for binding of PC(7:0) to rat liver apoBDH (1.42 mM).

(μmol of NAD^+ reduced)$^{-1}$ (V_m = 133). These V_m values are similar to those obtained from Figure 3 (see below and Table I).

On the other hand, the plots of $1/v_0^{1/2}$ vs. the reciprocal PC concentrations were linear with either PC(6:0), PC(7:0), or PC(8:0) (Figure 3A,B). This can be explained by assuming that apoBDH contains two identical, noninteracting PC-binding sites and the complex with two PC molecules is the only catalytically active species. In fact, if the free phospholipid is always in equilibrium with the PC bound to apoBDH, the probability (P_1) of having one site of the enzyme occupied by PC will be $P_1 = \bar{X}_{EL} = 1/(1 + K_L/[L])$, where $\bar{X}_{EL}$ is the molar fraction of sites on BDH occupied by PC, K_L is the intrinsic dissociation constant of the PC site complexes, and L is the concentration of free phospholipid. Thus, the probability of having two identical and noninteracting sites simultaneously occupied by PC will be $P_2 = 1/(1 + K_L/[L])^2$.

If this is the only catalytically active species, the specific velocity will be linearly related to the molar fraction of the enzyme in the form EL_2, that is

$$\frac{v_0}{V_m} = \frac{[EL_2]}{[E_t]} = \frac{1}{(1 + K_L/[L])^2} \quad (3)$$

where v_0 is the initial rate at any given concentration of phospholipid [L], V_m is the maximal velocity at [L] = ∞, [EL_2] is the concentration of enzyme in complex with two PC molecules, and [E_t] is the total enzyme concentration. If eq 3 describes the experimental results, it may be rearranged to give

$$\frac{1}{v_0^{1/2}} = \frac{1}{V_m^{1/2}} + \frac{K_L}{V_m^{1/2}} \frac{1}{[L]} \quad (4)$$

According to eq 4, the plot of $1/v_0^{1/2}$ as a function of 1/[PC] will give a straight line with a slope $K_L/V_m^{1/2}$, an intercept at $1/v_m^{1/2}$, and an extrapolated intercept on the abscissa axis at $-1/K_L$. As shown in Figure 3 (inset), linear plots are obtained with the reciprocal square root of the velocity only. The values of K_L and V_m obtained with each lecithin from plots presented in Figure 3 are summarized in Table I. By use of the K_L and V_m values for PC(8:0), the data for PC(8:0) plotted in Figure 2 are shown to fit eq 11 (derived from eq 4, see Discussion), which expresses [L] in the power of 2. The fit to the experimental data is not obtained when the second- or first-order terms are eliminated from eq 11, i.e., $(K_L^2/V_m)\cdot(1/[L]^2)$ and $(2K_L/V_m)(1/[L])$, respectively. These results (Figure 2) are therefore compatible with two identical, noninteracting PC sites.

So that the agreement between the experimental results and those to be expected from eq 3 could be tested, the initial velocities as a function of PC(8:0) concentration were plotted and compared with the calculated values (see below) of the molar fractions of both EL_2 (i.e., $X_{EL_2} = v_0/V_m$) and EL_1 (i.e., X_{EL_1}) as a function of the $[L]/K_L$ ratio by using the values of K_L and V_m presented in Table I (not shown). The experimental data obtained up to $[L]/K_L = 0.8$ were coincident with the calculated curve for X_{EL_2}, again reflecting agreement with eq 3 in which EL_2 (both PC sites filled) is the only active form of the enzyme. The inflection point of both the experimental and calculated curves occurred at $[L]/K_L = 0.5$ and $v_0/V_m = 0.111$ (see Discussion).

The molar fraction of free enzyme E (X_E) and the species EL_1 (X_{EL_1}) can be calculated as follows

$$X_E = \frac{[E]}{[E_t]} = \frac{K_L^2/[L]^2}{(1 + K_L/[L])^2} = [1 - (v_0/V_m)^{1/2}]^2 \quad (5)$$

and

$$X_{EL_1} = \frac{2[EL_1]}{[E_t]} = 1 - (X_E + X_{EL_2}) \quad (6)$$

The relationship between the molar fractions of the enzyme species and the molar fractions of vacant ($\bar{X}_E$) and occupied ($1 - \bar{X}_E$) PC sites on BDH is

$$X_E + X_{EL_1} + X_{EL_2} = \bar{X}_E + (1 - \bar{X}_E) = 1 \quad (7)$$

Then

$$\bar{X}_E = X_E + 1/(2X_{EL_1}) = 1 - (v_0/V_m)^{1/2} = X_E^{1/2} \quad (8)$$

and

$$1 - \bar{X}_E = X_{EL_2} + 1/(2X_{EL_1}) = (v_0/V_m)^{1/2} = X_{EL_2}^{1/2} \quad (9)$$

Therefore, according to eq 3 and 9, the degree of saturation ($1 - \bar{X}_E$) of the PC sites as a function of the free phospholipid concentration will be a rectangular hyperbola described by the equation $1 - \bar{X}_E = 1/(1 + K_L/[L])$. It is clear that although eq 3 and 9 describe accurately the experimental results, they cover only a part of the activation curve. This may be because the K_L values are too close to the cmc for each of the lecithins. Therefore inactivation of BDH by micellar arrays of short-chain lecithins would occur prior to reaching the K_L values. For example, the maximal activity that can be measured without inhibition is about $0.2V_m$ at approximately $0.7K_L$. Therefore it is important to test the system by an additional complementary procedure.

Inhibition of Activity of BDH–PC Complexes by Competition with Phospholipase A_2. The ability of porcine pancreas phospholipase A_2 to bind monomeric lecithins in the absence of Ca^{2+} ions has been well documented (de Haas et al., 1971; Pieterson et al., 1974b; Volwerk et al., 1974). One lecithin molecule is bound per mole of phospholipase, and the dissociation constant of the phospholipase A_2–PC complexes has been determined by nonkinetic procedures (cf. Experimental Procedures). It is to be expected that in the presence of phospholipase A_2, the activity of the BDH–PC complexes will be inhibited, as a consequence of the decrease in the concentration of free phospholipid. An appropriate treatment of the inhibition (cf. Appendix I) will allow us to determine whether the PC-binding sites on BDH are identical and noninteracting, and in that case, it will provide us with an estimation of the dissociation constants of the BDH–PC complexes.

The plots of 1/[PL] vs. $\bar{X}_E/(1 - \bar{X}_E)$ as described in eq I5 (Appendix I) obtained with BDH–PC(8:0) complexes in the presence of two fixed concentrations of phospholipase A_2 are

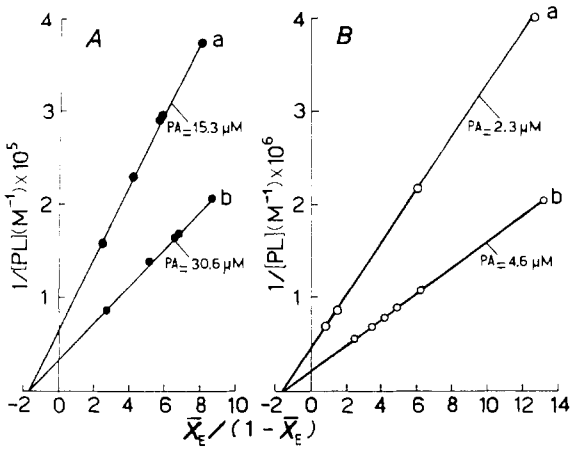


FIGURE 4: Plots of $1/[PL]$ as a function of $\bar{X}_E/(1-\bar{X}_E)$ for BDH–PC(8:0) complexes, as described in eq I5 (Appendix I), in the presence of phospholipase A_2 (PA). The values of $1/[PL]$ were calculated as described in eq II7 (Appendix II). The values of $\bar{X}_E$ and $1-\bar{X}_E$ were calculated as described in eq 8 and 9, respectively. (A) Plot of beef heart apoBDH (7.0 μg of protein) with variable amounts of PC(8:0) in the presence of two fixed concentrations of phospholipase $[P_t]$ [cf. eq I5 (Appendix I)], as indicated in the figure, is shown: (curve a) the $1/[PL]$ intercept $(1/[P_t])$ 0.67×10^5 M^{-1} and K_P/K_E ratio 1.59 ($K_L = 1.27 \times 10^{-4}$ M); (curve b) intercept $(1/[P_t])$ 0.32×10^5 M^{-1} and K_P/K_E ratio 1.68 ($K_L = 1.34 \times 10^{-4}$ M). (B) Plot of rat liver apoBDH (3.0 μg of protein) with variable amounts of PC(8:0) in the presence of two fixed concentrations of phospholipase $[P_t]$ [cf. eq I5 (Appendix I)], as indicated in the figure, is shown: (curve a) intercept $(1/[P_t])$ 0.5×10^6 M^{-1} and K_P/K_E ratio 1.5 ($K_L = 1.26 \times 10^{-4}$ M); (curve b) intercept $(1/[P_t])$ 0.21×10^6 M^{-1} and K_P/K_E ratio 1.58 ($K_L = 1.2 \times 10^{-4}$ M).

presented in parts A (beef heart BDH) and B (rat liver BDH) of Figure 4. The data, replotted according to eq I5 (Appendix I), i.e., $[P_t]/[PL]$ vs. $\bar{X}_E/(1-\bar{X}_E)$, all fit a single straight line with an intercept on the abscissa of 1 (as predicted by the equation), for either beef heart BDH or rat liver BDH (not shown). No hydrolysis of the phospholipid was detected under the experimental conditions employed. The K_L values calculated by this method (Figure 4) were similar to those obtained from the kinetic model for the activation of BDH (cf. Figure 3 and Table I).

In order to ascertain that inhibition of BDH activity in the presence of phospholipase A_2 did not involve phospholipid hydrolysis, we carried out two similar experiments with rat liver BDH with L- and D-PC(7:0). Since the latter is poorly hydrolyzed by phospholipase A_2 even in the presence of Ca^{2+} ions [cf. Bonsen et al. (1972)], while BDH does not exhibit any stereospecificity for the reactivating lecithin (Vidal et al., 1978; Isaacson et al., 1979). Similar results were obtained with the two enantiomeric PC(7:0). The plots of $1/[PL]$ as a function of $\bar{X}_E/(1-\bar{X}_E)$ in the presence of 32 and 64 μM phospholipase A_2 (not shown) were straight lines with intercepts at 0.316×10^5 and 0.154×10^5 M^{-1}, respectively. The extrapolated intercepts on the abscissa axis were 1.89 ($K_L = 1.48 \times 10^{-3}$ M) and 1.9 ($K_L = 1.45 \times 10^{-3}$ M), respectively. The plots of $[P_t]/[PL]$ as a function of $\bar{X}_E/(1-\bar{X}_E)$ as described in eq I6 (Appendix I) were linear with an intercept at 0.984 (1.010–0.959) and an extrapolated intercept on the abscissa axis at 1.85 (1.74–1.96), which results in a K_L value of 1.42 (1.39–1.50) $\times 10^{-3}$ M (the figures in parentheses represent 95% confidence limits). These values are similar to those presented in Table I. Therefore, the possible contribution of phospholipid hydrolysis to the inhibition of BDH activity in the presence of phospholipase A_2 can be ruled out under the experimental conditions employed. More important, these results suggest that the PC-binding sites on BDH are identical and noninteracting, hence the agreement between the K_L values calculated by this procedure and those obtained from direct activation experiments presented in Table I.

Table II: Thermodynamic Parameters of BDH-PC Interaction as a Function of Fatty Acyl Chain Length

lecithin	no. of methylenes	K_{assoc}[a] (M^{-1})	$\Delta G°_{assoc}$ (kJ/mol)	$\Delta G°/RT$	$\Delta H°$[b] (kJ/mol)	$T\Delta S°$[c] (kJ/mol)
PC(6:0)	8	219	−13.9	−5.39	−62.8	−48.9
PC(7:0)	10	714	−16.9	−6.57	−65.3	−48.4
PC(8:0)	12	8475	−23.3	−9.04	−67.4	−44.1

[a] When the values of K_{assoc} are expressed in unitary units, i.e., $K_{assoc} = (1-\bar{X}_E)/(\bar{X}_E X_{LW})$, where X_{LW} is the molar fraction of free phospholipid, the corresponding values of $\Delta G°/(RT)$ are −8.1 −8.18, −10.25, and −12.5 for PC(6:0), PC(7:0), and PC(8:0), respectively. These values are similar to those reported by Tausk et al. (1974) for the $\Delta G°/(RT)$ of micellization of the corresponding lecithins. [b] $\Delta H°$ was determined from Arrhenius plots ($-\log K_L$ vs. $1/T$) (cf. Results). [c] $T\Delta S$ was calculated from $\Delta G°_{assoc}$ and $\Delta H°$.

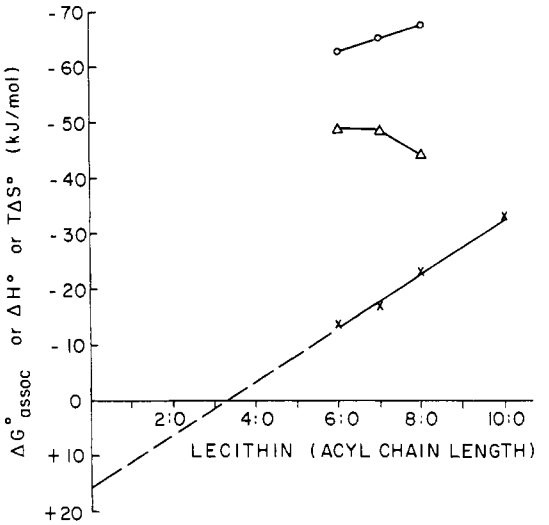


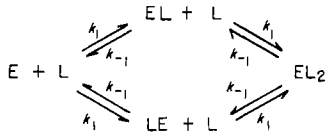
FIGURE 5: Thermodynamic parameters for binding of soluble diacyllecithins to beef heart BDH as a function of the fatty acyl chain length. $\Delta G°_{assoc}$ (×), $\Delta H°$ (○), and $T\Delta S$ (△) were obtained as described in Table II.

Thermodynamic Considerations. If the K_L values obtained are the dissociation constants of the PC site complexes on apoBDH, some thermodynamic calculations seem pertinent. The standard free energies of binding of PC(6:0), PC(7:0), and PC(8:0) to apoBDH at 37 °C are presented in Table II. The plot of $\Delta G°_{assoc}$ as a function of the fatty acyl chain length of the lecithins (Figure 5) has a slope of approximately 2.4 kJ $(0.92RT)$/mol of methylene group. It might be noted that studies with 1,2-didecanoyl-*sn*-glycero-3-phosphocholine [PC(10:0)] give $\Delta G°$ values of about 33 kJ/mol that fit on a straight line in this plot. The use of ethanol (8% by volume) was required in these studies. We found for PC(8:0) that this concentration of ethanol had no effect on $\Delta G°$. The temperature dependence of the K_L values for the different lecithins was studied in the range from 20 to 37 °C. The values of $\Delta H°$ for the binding of the different lecithins to apoBDH were calculated from the slopes of $-\log K_L$ vs. $1/T$ plots (not shown), giving values of −62.8 kJ/mol with PC(6:0), −65.3 kJ/mol with PC(7:0), and −67.4 kJ/mol with PC(8:0) (Table II and Figure 5).

Discussion

The purpose of this study was to establish, within the limitations of the assay procedures available, the kinetic mech-

anism and stoichiometry for the reactivation of D-β-hydroxybutyrate apodehydrogenase by short-chain lecithins. The reasons why monomeric lecithins were employed were indicated above and will not be discussed further. It should be noted that this study constitutes the first attempt to analyze kinetically the phospholipid–protein interaction of a membrane-bound, lipid-requiring enzyme. The simplest model that describes the experimental data obtained with both beef heart and rat liver apoBDH is that the functional unit of BDH has two identical, noninteracting PC-binding sites and requires the simultaneous occupation of both sites by PC to achieve its catalytically active conformation. The active species (EL_2) exists in equilibrium with those having a lesser degree of occupation (EL_1) and with free apoenzyme (E), both of which are catalytically inactive, according to the scheme



where k_1 and k_{-1} are respectively the intrinsic rate constants for the binding of L to a free site and for the dissociation of L from either occupied site on apoBDH. At equilibrium k_{-1}/k_1 is equal to K_L. Initial velocity patterns, in which straight lines are obtained only when $1/v_0^{1/2}$ is plotted as a function of the reciprocal PC concentrations (Figure 3A,B), are consistent with this mechanism. The plots of v_0 vs. [PC] are sigmoidal and fitted closely with the theoretical curve for X_{EL_2} as predicted by the model, which provides additional evidence regarding EL_2 (i.e., the functional enzyme with two bound lecithins) as the only active species. For systems with multiple (noncooperative) essential sites (Segel, 1975) the inflection point will occur at $[L] = [(n-1)/2]K_L$ and $v_0 = [(n-1)/(n+1)]^n V_m$, where n is the actual number of sites. In our model ($n = 2$), the inflection point will occur at $[L]/K_L = 0.5$ and $v_0/V_m = (1/3)^2 = 0.111$, and this was found experimentally (not shown).

The rate equation arising from this model (cf. eq 5) is highly restrictive, concerning both stoichiometry and cooperativity. Indeed, the only expression that gives a linear relationship is $1/v_0^{1/2}$ vs. 1/[PC] (Figure 3, inset).[2] On the other hand, it can be shown that nonlinear plots would have been obtained if the binding of the first PC molecule changed the K_L for the second site by a factor of only 0.8.

Concerning the Hill plots, if any enzyme contains noncatalytic sites that must be occupied before the substrate(s) can bind to the catalytic site, the slope of the Hill plot will increase as the ligand concentration decreases, and at very low ligand concentrations such as those employed in this study, it will approach the number of sites that must be occupied before any reaction occurs. For the model described above, the Hill plot will exhibit a limiting slope of 2.0 at low PC concentrations. In fact, eq 10 can be obtained by rearrangement of eq

$$\log [v_0/(V_m - v_0)] = -2 \log K_L + 2 \log [L] - \log [(2[L]/K_L) + 1] \quad (10)$$

[2] If it is assumed that EL_2 can generate species with higher degrees of occupancy that are (EL_3, EL_4, ..., EL_n) catalytically active, the rate equation would be

$$v_0/V_m = [[L]^2/K_L^2 + \sum_{i=3}^{n}([L]/K_L)^i]/[(1 + [L]/K_L)^2 + \sum_{i=3}^{n}([L]/K_L)^i]$$

where i is the degree of occupancy (i.e., 3, 4, ..., n) and n is the maximal degree of occupancy. The $1/v_0^{1/2}$ vs. 1/[L] plots are nonlinear already for $i = 3$.

3. At low PC concentrations, the contribution of the term log $[(2[L]/K_L) + 1]$ will be negligible (it accounts for less than 10% at $[L]/K_L = 0.4$), and the limiting slope will be 2.0, suggesting two PC sites. Only in the region of $(0.3–0.7)V_m$ will the plot be nearly linear with a slope of about 1.2, indicative of lack of cooperativity between the two PC sites. In contrast to lecithin binding, the Hill plots for both NAD^+ and D-3-hydroxybutyrate are linear with a slope of 1.0 [cf. Latruffe & Gaudemer (1974)]. A multisite enzyme with at least two strongly cooperative sites will give linear $1/v_0$ vs. $1/[L]^2$ plots, and this is not found experimentally. As shown in Figure 2, these plots are curves that can be accurately described by

$$\frac{1}{v_0} = \frac{1}{V_m} + \frac{2K_L}{V_m}\frac{1}{[L]} + \frac{K_L^2}{V_m}\frac{1}{[L]^2} \quad (11)$$

Equation 11 can be derived from the two identical, noninteracting PC site model described above (eq 3). It is worth noting that the intercept on the $1/v_0$ axis (i.e., $1/V_m$) gives V_m values of 137.9 [PC(8:0)] and 133 [PC(7:0)] μmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$, which agreed closely with the values obtained from the $1/v_0^{1/2}$ vs. 1/[PC] plots (Table I).

A critical test for the model would be to check the agreement between the K_L values and the dissociation constants of the PC–BDH complexes by a direct binding technique. Direct binding experiments performed on Bio-Gel P-20 or Sephadex G-25 columns preequilibrated and eluted with fixed concentrations of [^{14}C]PC(8:0) (not shown) did not show significant binding of PC(8:0) to the enzyme at the lipid concentrations employed for the kinetic studies [cf. Gazzotti et al. (1975)]. Attempts to increase the enzyme concentration were unsuccessful, probably due to the concentration-dependent self-aggregation of the enzyme, which results in high molecular weight species of enzyme with reduced activity in the presence of PC(8:0) (McIntyre et al., 1978b). These features prevented us from obtaining meaningful data with this technique.

BDH has been used previously in a competition assay to measure, in a qualitative manner, the binding of lecithin to other proteins or polypeptides (Fleischer et al., 1967; Jackson et al., 1973). Now, by use of soluble lecithins and the known binding characteristics of these lipids by phospholipase A_2, a quantitative treatment of BDH binding to soluble lecithins has been achieved. Inhibition of BDH activity in the presence of phospholipase A_2, due to a decrease in the effective phospholipid concentration, was sufficiently sensitive and reproducible to give a complementary method for measurement of K_L. The treatment of this type of inhibition (Appendix I) also utilizes the two-site model for reactivation of apoBDH (eq 8 and 9 are used to calculate $\bar{X}_E$ and $1 - \bar{X}_E$ for Figure 4; see below), but the K_L value is obtained from a complementary theoretical model (Appendix I) that utilizes the known values of PC binding to phospholipase A_2. The reciprocal concentration of phospholipase A_2–PC complexes can be calculated (Appendix II) from the total concentrations of PC, apoBDH, and phospholipase A_2, without any approximation concerning the relative concentrations of free and bound ligand. The only requirement to obtain linear plots is that the PC sites on apoBDH must have one single intrinsic binding constant [K_E in eq I2a (Appendix I)]. The 1/[PL] vs. $\bar{X}_E/(1 - \bar{X}_E)$ plots (Figure 4A,B) and the $[P_t]/[PL]$ vs. $\bar{X}_E/(1 - \bar{X}_E)$ plots (not shown) fulfilled the requirements stated in eq I5 and I6 (Appendix I), respectively, indicating that the two PC sites on apoBDH are identical and noninteracting. Since eq I5 and I6 (Appendix I) are obeyed, an explicit relationship between the association constants K_P and K_E may be established, and

since K_P is known, K_E (i.e., $1/K_L$) can be calculated. The calculated K_L values are in close agreement with those obtained from the $1/v_0^{1/2}$ vs. $1/[PC]$ plots (Table I).

The simple model of the active form of BDH having two identical, noninteracting PC sites describes accurately all of the experimental data, i.e., the reactivation curves, a limiting slope of 2 for the Hill plot, and the phospholipase A_2 competition experiments (Figure 4), which indicate that the PC sites on BDH are identical and noninteracting. Kinetic analysis can only be used to test consistency rather than to prove a model. We have employed two complementary kinetic approaches to evaluate the activation mechanism of BDH with soluble lecithins. The kinetic model for activation by soluble lecithins assumes two noninteracting lecithin sites on the functional unit of the enzyme. The model based on the competition with phospholipase A_2 also assumes two PC sites, but by use of a theoretical treatment based on the known PC binding to phospholipase A_2, a single association constant (K_L) for PC binding to apoBDH is obtained that is in numerical agreement with that obtained from the kinetic model for two noninteracting sites. Both complementary approaches are consistent with the simple model of two identical binding sites for lecithin that are noninteracting. This model is consistent with the data obtained with four different soluble lecithins (see discussion of thermodynamic data, below). It appears unnecessary to invoke cooperativity among sites to explain the sigmoidicity of the reactivation curves.

Two observations should be mentioned at this point: first, the V_m values obtained with PC(6:0), PC(7:0), and PC(8:0) (Table I) are similar to those obtained with long-chain lecithins, and second, the dissociation constant of the BDH–PC complexes decreases as the fatty acyl chain length increases. However, a direct comparison of the reactivating parameters with short-chain PCs and those with the long-chain, vesicle-forming lecithins is not possible. As pointed out above, "infinite lecithin concentration" may not have the same physical meaning as "infinite interface". In any case, the results presented above show that the reactivating capacity of short-chain lecithins is fairly high; the low activity observed experimentally (Figure 1) is artificial, due to the inactivation of the enzyme in complexes with micellar phospholipid.

Previous reports dealing with the binding of NADH to beef heart BDH–MPL complex (Gazzotti et al., 1974) and with the hydrodynamic properties of the BDH–PC(8:0) complex (McIntyre et al., 1978b) suggested that the active form of the enzyme is a dimer. The studies reported here suggest that the active form of the enzyme is EL_2, where E is the functional unit of the enzyme and does not pertain to an actual oligomeric size. We have analyzed the kinetic behavior of several mechanisms in which the active species are taken to be a dimer of the functional unit (see paragraph at end of paper regarding supplementary material). The results obtained suggest that the active form could also be a dimer of functional units, e.g., $(EL_2)_2$, but only if the kinetic properties of the enzyme are not affected by dimerization, the dimer being in equilibrium with the monomeric functional unit.

The thermodynamic parameters for binding of PC to apoBDH (cf. Table II) plotted as a function of chain length (Figure 5) show that the increase in $\Delta G°_{assoc}$, as the acyl chain-length of the PC increases, is about 2.4 kJ/mol per CH_2 group, i.e., $0.92RT$/mol of methylene group. It might be speculated that the two acyl chains in the short-chain PCs contributed independently of each other to the $\Delta G°$ of binding [cf. Tausk et al. (1974)] presumably by interaction with an apolar region on the enzyme. This plot extrapolates to $\Delta G°$ = 0 at about PC(3:0), while extrapolation to zero chain length results in a $\Delta G°$ of about +15 kJ/mol, suggesting that although the specificity for reactivation is determined by the chemical parameters of the polar head, a certain degree of hydrophobicity is essential for effective binding of PC to apoBDH. From these data, it can be predicted that the shortest PC able to reactivate apoBDH would be PC(4:0) ($\Delta G° \simeq -3$ to -5 kJ/mol) while PC(2:0) ($\Delta G° \simeq +5$ to +8 kJ/mol) or glycerophosphocholine ($\Delta G° \simeq +10$ to +15 kJ/mol) will be ineffective. This is in keeping with previous experimental results for BDH (Grover et al., 1975; Vidal et al., 1977b; H, G. Bock and S. Fleischer, unpublished studies). A positive $\Delta G°$ was also obtained by extrapolation to zero chain length for the activation of pyruvate oxidase from *Escherichia coli* with various amphiphiles (Blake et al., 1978). It is interesting to note that the $\Delta G°$ of association of BDH with short-chain lecithins (Table II) is similar to that for the association of these lecithins with themselves to form micelles (Tausk et al., 1974). The values of $\Delta H°$ at 37 °C decreased with the chain length with a slope of about 1.3 kJ/mol per CH_2 group. When combined with those of free energy ($\Delta G°$), $T\Delta S$ values of −48.9, −48.4, and −44.1 kJ/mol for PC(6:0), PC(7:0), and PC(8:0), respectively, are obtained. The binding of PC to apoBDH seems to be approximately 60% enthalpy and 40% entropy driven. The apparent unfavorable energy of association of the polar head group at zero methylenes with BDH must be overcome by increasing the number of methylene groups per acyl chain to obtain an energetically favorable interaction. Thus, the absolute specificity for lecithin for function by the enzyme (Isaacson et al., 1979) is not to be equated with a favorable energetic interaction with the polar moiety, per se.

Acknowledgments

We are indebted to Dr. A. C. Paladini, Dr. P. J. Garrahan, and Dr. E. A. M. Fleer for critically reviewing the manuscript.

Appendix

(*I*) *Calculation of BDH–PC Dissociation Constants from Competitive Inhibition with Phospholipase A_2.* Let $[L_t]$, $[\bar{E}_t]$, and $[P_t]$ be the total concentration of lecithin, PC sites on apoBDH, and phospholipase A_2, respectively, and $[L]$ the concentration of free phospholipid. $[\bar{E}]$ and $[\overline{EL}]$ will be the concentrations of vacant and occupied PC sites on BDH, while $[P]$ and $[PL]$ will be the concentrations of vacant and occupied sites in phospholipase A_2. It must be noted that since phospholipase A_2 binds one PC molecule per mol, the concentration of free sites equals the concentration of free enzyme. The conservation equations are

$$[\bar{E}_t] = [\bar{E}] + [\overline{EL}] \quad \text{(I1a)}$$

$$[P_t] = [P] + [PL] \quad \text{(I1b)}$$

$$[L_t] = [L] + [\overline{EL}] + [PL] \quad \text{(I1c)}$$

The intrinsic affinity constant of the PC sites on apoBDH is $K_E = 1/K_L$, defined as

$$\frac{1}{K_L} = K_E = \frac{[\overline{EL}]}{[\bar{E}][L]} = \frac{1 - \bar{X}_E}{\bar{X}_E[L]} \quad \text{(I2a)}$$

and that of the PC sites in phospholipase A_2 is

$$K_P = \frac{[PL]}{[P][L]} = \frac{[PL]}{([P_t] - [PL])[L]} \quad \text{(I2b)}$$

From eq I2a we obtain

$$\bar{X}_E = \frac{1}{1 + K_E[L]} \quad \text{(I3)}$$

On the other hand, eq I2b can be rearranged to give

$$[L] = \frac{1}{K_P\left(\frac{[P_t]}{[PL]} - 1\right)} \quad \text{(I4)}$$

Substituting eq I4 into eq I3 and solving for $1/[PL]$, we have

$$\frac{1}{[PL]} = \frac{1}{[P_t]} + \frac{K_E}{K_P[P_t]} \frac{\bar{X}_E}{1 - \bar{X}_E} \quad \text{(I5)}$$

The initial velocities (v_i) obtained with a fixed concentration of apoBDH can be measured at different lecithin concentrations in the presence of a constant amount of phospholipase A_2. The values of $\bar{X}_E$ and $1 - \bar{X}_E$ can be calculated according to eq 8 and 9, respectively. According to eq I5, the plot of $1/[PL]$ as a function of $\bar{X}_E/(1 - \bar{X}_E)$ will give a straight line with a slope of $K_E/(K_P[P_t])$; the intercept on the ordinate axis will be $1/[P_t]$, and the extrapolated intercept on the abscissa axis will be $-K_P/K_E$. Since the term $1/[P_t]$ in eq I5 affects the slope and the intercept on the ordinate axis but not the extrapolated intercept on the abscissa axis, the plots of $1/[PL]$ vs. $\bar{X}_E/(1 - \bar{X}_E)$ obtained in the presence of several fixed concentrations of phospholipase A_2 will give a family of straight lines with a common intercept on the abscissa axis at $-K_P/K_E$.

Moreover, multiplying both sides of eq I5 by $[P_t]$ gives

$$\frac{[P_t]}{[PL]} = 1 + \frac{K_E}{K_P} \frac{\bar{X}_E}{1 - \bar{X}_E} \quad \text{(I6)}$$

According to eq I6 a plot of $[P_t]/[PL]$ as a function of $\bar{X}_E/(1 - \bar{X}_E)$ will give a straight line with a slope of K_E/K_P; the intercept on the ordinate axis will be 1.0, and the extrapolated intercept on the abscissa axis will be $-K_P/K_E$. All the values of $1/[PL]$ when multiplied by the total concentration of phospholipase A_2 at which they were obtained must fit on this line.

(II) Calculation of $1/[PL]$ as a Function of Measurable Parameters. In the absence of phospholipase A_2, the intrinsic affinity constant of the PC sites on apoBDH ($K_E = 1/K_L$) can be defined as

$$K_E = \frac{1 - \bar{X}_{E_0}}{\bar{X}_{E_0}[L]_0} \quad \text{(II1)}$$

and

$$[L]_0 = [L_t] - [EL]_0 = [L_t] - [E_t](1 - \bar{X}_{E_0}) \quad \text{(II2)}$$

Combining eq II1 and II2, we have

$$K_E\{[L_t] - [E_t](1 - \bar{X}_{E_0})\} = \frac{1 - \bar{X}_{E_0}}{\bar{X}_{E_0}}$$

and

$$[\bar{E}_t] = \frac{[L_t]}{1 - \bar{X}_{E_0}} - \frac{1}{K_E\bar{X}_{E_0}} \quad \text{(II3)}$$

In the presence of phospholipase A_2

$$[L] = [L_t] - [\overline{EL}] - [PL] = [L_t] - [E_t](1 - \bar{X}_E) - [PL] \quad \text{(II4)}$$

The value of $[\bar{E}_t]$ from eq II3 can be substituted into eq II4, and this can, in turn, be substituted into eq I2b to give

$$K_E = \frac{1 - \bar{X}_E}{\bar{X}_E\left\{\left[[L_t] - \left(\frac{[L_t]}{1 - \bar{X}_{E_0}} - \frac{1}{K_E\bar{X}_{E_0}}\right)(1 - \bar{X}_E)\right] - [PL]\right\}}$$

Solving for [PL], we get

$$[PL] = [L_t] - [L_t]\frac{1 - \bar{X}_E}{1 - \bar{X}_{E_0}} + \frac{1 - \bar{X}_E}{K_E\bar{X}_{E_0}} - \frac{1 - \bar{X}_E}{K_E\bar{X}_E} \quad \text{(II5)}$$

Equation II5 has the same form as eq II4 and can be reordered to give

$$[PL] = [L_t]\left(1 - \frac{1 - \bar{X}_E}{1 - \bar{X}_{E_0}}\right) + [L]\left(\frac{\bar{X}_E}{\bar{X}_{E_0}} - 1\right) \quad \text{(II6)}$$

Substitution of the term containing [L] and rearrangement of the resulting equation yield a second-order equation of the form

$$a[PL]^2 + b[PL] + c = 0 \quad \text{(II7)}$$

where

$$a = 1.0$$

$$b = \frac{\frac{\bar{X}_E}{\bar{X}_{E_0}} - 1}{K_P} - [L_t]\left(1 - \frac{1 - \bar{X}_E}{1 - \bar{X}_{E_0}}\right) - [P_t]$$

$$c = [P_t][L_t]\left(1 - \frac{1 - \bar{X}_E}{1 - \bar{X}_{E_0}}\right)$$

and

$$[PL] = \frac{-b - (b^2 - 4ac)^{1/2}}{2a}$$

since the discriminant ($b^2 - 4ac$) is higher than zero and [PL] cannot be higher than $[L_t]$.

Supplementary Material Available

Rate equations for different mechanisms involving a dimer of the functional unit of BDH as the only active species (8 pages). Ordering information is given on any current masthead page.

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