Cooperativity in Lipid Activation of 3-Hydroxybutyrate Dehydrogenase: Role of Lecithin as an Essential Allosteric Activator†

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ABSTRACT: 3-Hydroxybutyrate dehydrogenase (BDH) is a lecithin-requiring mitochondrial enzyme which catalyzes the interconversion of 3-hydroxybutyrate and acetoacetate with NAD(H) as coenzyme. The purified enzyme devoid of lipid (i.e., the apodehydrogenase or apoBDH) can be reactivated with soluble lecithin or by insertion into phospholipid vesicles containing lecithin. Two different models have been proposed to explain the sigmoidal lipid activation curves. For both models, activation of BDH is assumed to require the binding of two lecithin molecules per functional unit. Activation of soluble enzyme (dimeric form) by short-chain (soluble) lecithin is consistent with a model in which lecithin binding is noncooperative, whereas activation of the membrane-bound enzyme (tetrameric form) indicates cooperativity between the lecithin binding sites. A new comprehensive model is presented in which lecithin is considered to be an essential allosteric activator that shifts the equilibrium between conformational states of the enzyme. Resonance energy transfer data, reflecting NADH binding to membrane-bound and soluble apoBDH, are consistent with such a lecithin-induced conformational change. Apparent dissociation constants for binding of NADH to BDH are ~ 10 μM and ~ 37 μM for BDH activated by bilayer and soluble lecithin, respectively. The maximal fluorescence resonance energy transfer (ΔF_{max}) increases with higher mole fraction of lecithin in the bilayer. The largest changes occur between mole fractions 0 and 0.13, thereby correlating with enzymic function. Essentially no binding of NADH is observed in the absence of lecithin. The allosteric model reconciles the apparently distinct activation of BDH by soluble and bilayer phospholipid and can account for the lack of cooperativity between binding sites observed previously for the activation of the enzyme with soluble phospholipid [Cortese, J. D., Vidal, J. C., Churchill, P., McIntyre, J. O., & Fleischer, S. (1982) *Biochemistry 21*, 3899–3908]. Both noncooperative and cooperative activation of BDH by lecithin are limiting cases of the more general allosteric model.

Lipid activation curves for 3-hydroxybutyrate dehydrogenase [EC 1.1.1.30, (*R*)-3-hydroxybutyrate:NAD$^+$ oxidoreductase, BDH][1] have a sigmoidal shape. Two models have been proposed to explain sigmoidal activation (Cortese et al., 1982; Sandermann et al., 1986). With regard to lecithin binding, a noncooperative model with two identical noninteracting lecithin binding sites was consistent with the activation by soluble lecithins (Cortese et al., 1982). A model involving cooperative interactions between at least two different lecithin binding sites, which follows a Hill-type equation, explained activation by bilayer phospholipids (Sandermann et al., 1986). We have recently reinvestigated the basis for the different behavior of the enzyme in these two systems and confirmed that the noncooperative behavior is characteristic of activation with soluble lecithins, whereas mixtures of activating phospholipids in the form of a bilayer obey a cooperative model (Cortese & Fleischer, 1987).

In this study, new kinetic and spectroscopic data are presented, and a generalized molecular interpretation for the two models is provided, suggesting that the phospholipid (PC) serves as an essential allosteric activator.

Experimental Procedures

Reagents. Sucrose, tris(hydroxymethyl)aminomethane (Tris), Hepes, dithiothreitol (DTT), β-NAD$^+$, sodium (*RS*)-3-hydroxybutyrate (BOH), and bovine serum albumin (BSA type F) were purchased from Sigma Chemical Co. (St. Louis, MO). Reduced nicotinamide adenine dinucleotide (chromatographically pure, NADH) was obtained from Pharmacia P-L Biochemicals (Uppsala, Sweden). 1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine [PC(8:0)], 1,2-didecanoyl-*sn*-glycero-3-phosphoethanolamine [PE(10:0)], 1,2-didecanoyl-*sn*-glycero-3-phosphate [PA(10:0)], 1,2-dioleoyl-*sn*-glycero-3-phosphocholine [PC(18:1)], and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine [PE(18:1)] were purchased from Avanti Polar Lipids (Birmingham, AL). The synthetic phospholipid PP (1-palmitoyl-2-lauroylphosphatidylpropan-1,3-diol) was obtained as described previously (Churchill et al., 1983). Thin-layer chromatography was performed on precoated silica gel thin-layer sheets (E. Merck, Darmstadt, Federal Republic of Germany). All the other reagents and solutions were of analytical grade, and solutions were prepared in double-distilled and deionized water unless otherwise stated.

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[1] Abbreviations: apoBDH, (*R*)-3-hydroxybutyrate dehydrogenase apoenzyme; BDH, (*R*)-3-hydroxybutyrate dehydrogenase; BSA, bovine serum albumin; BOH, sodium hydroxybutyrate; DPG, diphosphatidylglycerol; DTT, dithiothreitol (Cleland's reagent); EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L, phospholipid as a ligand; MPL, mitochondrial phospholipid (approximated by the mixture of PC–PE–DPG in 1.0/0.8/0.2 molar phosphorus ratio, respectively) isolated from bovine heart mitochondria; PC, phosphatidylcholine; PC(8:0), 1,2-dioctanoyl-*sn*-glycero-1-phosphocholine; PE, phosphatidylethanolamine; PE(10:0), 1,2-didecanoyl-*sn*-glycero-1-phosphoethanolamine; PP, phosphatidylpropan-1,3-diol; X_{PC}, X_{PE}, or X_{PP}, mole fraction of either PC, PE, or PP of the total phospholipid; PC–PE–PP vesicles, vesicles prepared at $X_{PP} = 0.1$ and $X_{PE+PC} = 0.9$; PE–PP vesicles, vesicles prepared at $X_{PP} = 0.1$ and $X_{PE} = 0.9$; TLC, thin-layer chromatography.

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Methods. Protein concentration was measured according to Lowry et al. (1951) with bovine plasma albumin as standard. When the samples contained DTT, the modification described by Ross and Schatz (1973) was used. Phospholipid concentrations were determined as inorganic phosphorus by the method of Chen et al. (1966) as described in Rouser and Fleischer (1967).

3-Hydroxybutyrate Dehydrogenase. 3-Hydroxybutyrate dehydrogenase was released from bovine heart mitochondria by phospholipase A treatment and then purified to homogeneity according to a modification (McIntyre et al., 1988) of the procedure described by Bock and Fleischer (1974, 1975).

Preparation of Phospholipid Vesicles. Mitochondrial phospholipids from bovine heart mitochondria (MPL) were extracted and purified as described by Rouser and Fleischer (1967) and Gazzotti et al. (1975). Phospholipid vesicles were prepared by sonication essentially as described previously (Churchill et al., 1983). Aliquots of MPL in chloroform/methanol, 2:1 (v/v), were pipetted into 5 × 50 mm glass test tubes. The samples were dried under a stream of argon and subsequently in vacuo for 1.0–1.5 h. Argon-saturated buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.1) which had been filtered through a 0.22-μm filter (type GSTF, Millipore Corp., Bedford, MA) was added to the dried lipid to give a concentration of 200 μg of P/mL. The phospholipid in buffer was sonicated in the test tube for 10–15 min at 25 °C with a Model G12PIT sonic bath (Laboratory Supplies Co., Inc., Hicksville, NY) until no further decrease in turbidity was observed. Samples were stored in gas-evacuated solutions (about 200 μg of phosphorus/mL); they were resonicated and diluted before use. Phospholipid vesicles in aqueous buffer of varying composition were prepared from mixtures of synthetic phospholipids [PC–PE–PP vesicles and PC(18:1) vesicles]. Phospholipids dispersed in aqueous buffer according to this procedure form small unilamellar vesicles (Fleischer & Fleischer, 1967; Sandermann et al., 1986). The size of the vesicles depends somewhat upon the phospholipid composition (MPL versus PC versus PC–PE–PP, 5:4:1) and ranges from 600- to 800-Å diameter as determined by quasi-elastic light scattering measurements.[2] Pure dioleoyl-PE forms hexagonal (H_{II}) phase above about 10 °C, but inclusion of ≥15 mol % PC destabilizes the hexagonal phase (Cullis & DeKruiff, 1979). Addition of either PC or negatively charged lipids (in the absence of Ca^{2+}) to PE stabilizes the bilayer structure (Cullis & DeKruiff, 1979). PE in the hexagonal phase is quite turbid compared to bilayer-phase phospholipid (E. A. Dratz, personal communication). Phospholipid vesicles of PC–PE–PP containing PP (mole fraction 0.1) and varying PC/PE ratios (PC mole fraction varied from 0.05 to 0.50) used in our studies were slightly opalescent and exhibited similar light scattering. For this reason and on the basis of the studies of Cullis and DeKruiff (1979; see above), it is unlikely that there is significant hexagonal (H_{II}) phase at low PC content.

Insertion of BDH into Phospholipid Vesicles. The procedure used is a modification of protocols described previously (Churchill et al., 1983; Sandermann et al., 1986). Appropriate aliquots of phospholipid vesicles (see above) were pipetted into a small glass tube containing 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, 100 mM NaCl, and 5 mM DTT (final volume, 57 μL). 3-Hydroxybutyrate dehydrogenase (3 μL of 1 mg/mL stock solution) was added and gently mixed with the lipid dispersion. The tube was flushed with argon and sealed with Parafilm, followed by swirling with a Vortex mixer. The mixture was then incubated sequentially for 30 min at 30 °C and 45 min at room temperature and placed on ice prior to assay of enzymic activity.

When the samples were prepared for fluorescence measurements, aliquots containing 50 μg of apoBDH inserted into phospholipid vesicles in different ratios of phospholipid/protein (up to 250 mol of phospholipid/mol of apoBDH) were diluted to a total volume of 1.25 mL (1.3 μM BDH), containing 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, 100 mM NaCl, and 5 mM DTT. Samples were incubated under argon for 30 min at 30 °C and for 50 min at room temperature for complex formation and then dialyzed for 10–12 h under argon flux in 10 mM Hepes–KOH (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 2 mM DTT. Dialysis tubing was pretreated to avoid sulfhydryl oxidation during long-term dialysis according to Brewer et al. (1974). After dialysis, DTT was added to a final concentration of 5 mM, and the contents were poured into quartz cuvettes. Samples were kept under nitrogen at 25 °C during the fluorescence measurements.

Measurement of Specific Activity of BDH. Insertion of BDH into phospholipid vesicles was carried out as described above, and an aliquot was used to measure enzymic activity. Quartz cuvettes (1.0 mL) containing 10 mM K_2PO_4–KOH (pH 7.35 at 37 °C), 0.5 mM EDTA, 0.3 mM DTT, 0.04% BSA, 1.27% ethanol, 20 mM BOH, and 5 mM NAD^+ were prewarmed to 37 °C. Aliquots of the complex (0.25–1.0 μg of apoBDH) were added to start the catalytic reaction. The reduction of NAD^+ was followed spectrophotometrically at 340 nm in a Cary 219 spectrophotometer (Varian Associates, Palo Alto, CA). The extinction coefficient of 6.22 $mM^{-1}\cdot cm^{-1}$ was used to calculate specific activity. All the measurements were performed in duplicate, and the points represent average values.

The enzyme was assayed in PC(8:0) at 25 °C under optimized conditions as described by Cortese and Fleischer (1987). Reaction mixtures contained 10 mM Hepes–KOH (pH 8.0 at 25 °C), 1 mM EDTA, 2 mM DTT, 5% ethanol, and 5 mM NAD^+. Twenty microliters of 1 M BOH was added to start the reaction, and BDH specific activity was calculated as indicated above. Absolute ethanol (5%) was added to increase the critical micellar concentration (cmc) of this lecithin and does not alter significantly the K_L for PC(8:0) activation of BDH (Cortese & Fleischer, 1987).[3] Specific activity of BDH reconstituted with MPL, measured under similar conditions, was taken to be 100% activation. The ternary mixture of three single molecular species of phospholipids (PC–PE–PP, 5:4:1 ratio by phosphorus) mimics the

[2] J. O. McIntyre, E. Chang, and S. Fleischer, unpublished studies.

[3] Kinetic definitions: v is the initial velocity; V_m is the maximal velocity extrapolated from double-reciprocal plots as described under Experimental Procedures; $[L_t]$ is the total concentration of the phospholipid ligand (expressed as moles of PL per mole of apoBDH); $[L_{10}]$ and $[L_{50}]$ are the respective ligand concentrations that give 10% and 50% of maximal activation; K_L is the *intrinsic dissociation constant* for the binding of lecithin to lecithin binding sites [i.e., $K_L = [E][L]/[EL] = [EL][L]/[EL_2]$; see also Cortese et al. (1982)]. For the studies of BDH in bilayer phospholipid, $[L_t]$ and K_L values are expressed as moles of PL per mole of BDH since this provides the ratio of the two components in the bilayer (two-dimensional system). For this reason, expressing K_L in molar units would have less meaning than for a soluble system (three-dimensional system). L is the phospholipid considered as a ligand; A (*allosteric constant*; $A = [T_0]/[R_0]$) is a parameter defined as the ratio between two unliganded forms of an allosteric protein (T_0 and R_0 forms); A' (*relative allosteric constant*; $A' = [T_0]/[R_0]$ at $[L] = K_L/10$) is a parameter defined as the ratio between unliganded forms of an allosteric protein (as for A) in the presence of a ligand concentration equal to one-tenth of the ligand dissociation constant. The value for A' is used as an experimental estimate of A. MWC (equation or model) is used as abbreviation for the Monod–Wyman–Changeaux (Monod et al., 1965) model of allosterism.

optimal reactivation of BDH obtained with MPL. PP, an acidic phospholipid, can substitute for mitochondrial diphosphatidylglycerol (Eibl et al., 1982; Churchill et al., 1983).

Fluorescence Measurements. After insertion of BDH into phospholipid vesicles or addition to soluble phospholipids, fluorescence was measured in a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a Hitachi 057 X-Y recorder (Perkin-Elmer Corp., Norwalk, CT). With an excitation wavelength of 296 nm (excitation slit width of 7 nm), tryptophan fluorescence at 340 nm and resonance energy transfer between tryptophan and NADH at 455 nm (emission slit width of 10 nm) were recorded as a function of NADH concentration. The values presented are averages of 2.5-min recordings. NADH fluorescence was measured with λ_{ex} = 340 nm and λ_{em} = 455 nm. For titration of BDH–PC(8:0) with NADH, fluorescence values were recorded for about 15 s with excitation at 290 nm and slits for excitation and emission of 8 and 16 nm, respectively. Initial and final excitation and emission spectra were recorded for each sample as controls. Corrections for inner-filter effects were calculated from measured absorbances in both optical directions at 296, 340, and 455 nm for each cuvette, as described by Lakowicz (1983). BDH specific activity was tested before and after titration with NADH. A lipid control was titrated simultaneously with the other samples, and subtracted. The blanks were tested for linearity by regression analysis and subtracted accordingly.

For the soluble enzyme, tryptophan fluorescence was measured under conditions that are in the proportional range for fluorescence and enzymic activity measurements. Tryptophan fluorescence (excitation 296 nm; emission 340 nm) is proportional to BDH concentration in the range 0–50 μg of apoBDH. Samples stored for 1 h at 25 °C retained about 90–95% of the fluorescence of the original, and after an additional hour of incubation with 0.4 M LiBr (which enhances BDH dissociation; McIntyre et al., 1978), a reduction of intrinsic fluorescence of 14% was observed. BDH activity is proportional to the protein concentrations in the range 0–10 μg of protein (not shown). In all the experiments, the enzymic activity was similar to that of the apoBDH sample kept on ice during the same time (92–98% of initial activity). There was essentially no difference in enzymic activity for apoBDH stored in the absence or presence of 0.4 M LiBr (not shown).

Results

Reactivation of 3-Hydroxybutyrate Dehydrogenase by Bilayer Phospholipids. The activation of BDH with a mixture of synthetic phospholipids (PC–PE–PP, X_{PC} = 0.50), which mimics that of mitochondrial phospholipid (X_{PC} = 0.43, X_{PE} = 0.37, X_{DPG} = 0.20; Fleischer et al., 1967), or with PC–PE–PP (X_{PC} = 0.13 or 0.05) is shown in Figure 1. Ratios of lipid concentrations that give 50% and 10% maximal activation ($[L_{50}]/[L_{10}]$ ratio) obtained from the activation curves shown in Figure 1 are summarized in Table I. Hill plots were drawn with an estimated maximal velocity (V_m) of 120 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ (obtained from comparison of double-reciprocal plots of v^{-1} vs $[L]^{-1}$ and $v^{-1/2}$ vs $[L]^{-1}$, not shown) for MPL and for PC–PE–PP vesicles (PC mole fractions of 0.5, 0.13, and 0.05) and 103 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ for PC(18:1). The activation of BDH by MPL or PC alone or mixtures of phospholipids containing lecithin (X_{PC} = 0.13–0.50) is cooperative as judged by the $[L_{50}]/[L_{10}]$ values, which are between 4.0 and 6.87. These values are significantly lower than 9.0, the ratio pertaining to a noncooperative (hyperbolic) activation curve. Likewise, $n_{H(app)}$ values for activation by phospholipid vesicles with X_{PC} between 0.13 and 1.00 ranged from 1.75 to 1.48,

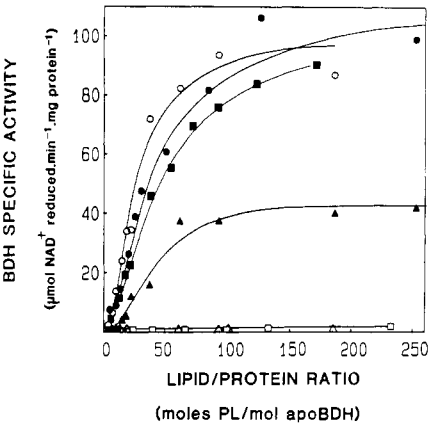

FIGURE 1: Reactivation of BDH with phospholipid vesicles of varying mole fraction of lecithin. Activation of BDH with MPL (solid circles), pure PC(18:1) (X_{PC} = 1.00, solid squares), or PC–PE–PP vesicles (the molar fraction of PP was 0.10 with the remainder being PC + PE) containing the following: (a) $X_{PC(18:1)}$ = 0.50 (open circles), or (b) $X_{PC(18:1)}$ = 0.13 (solid triangles), or (c) $X_{PC(18:1)}$ = 0.05 (open squares), or PE–PP vesicles (X_{PC} = 0, open triangles). BDH specific activity is expressed as μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ and the rato lipid/protein as moles of phospholipid per mole of apoBDH. Double-reciprocal plots of v^{-1} as a function of $[L]^{-1}$ curved upward as expected from the noncooperative model presented previously (Cortese et al., 1982), and the V_m obtained was 120 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ for PC–PE–PP vesicles (X_{PC} = 0.50, 0.13, or 0.05) and 103 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ for PC vesicles.

indicating significant cooperative behavior. When the molar fraction of lecithin was reduced to 0.05, the activation became more hyperbolic, i.e., $[L_{50}]/[L_{10}]$ [calculated according to Sandermann (1982), see footnote *d* of Table I] is about 9 and $n_{H(app)}$ is 1.05.

We have previously proposed a noncooperative mechanism for reactivation of soluble apoBDH by short-chain phospholipids [see Cortese et al. (1982)]. This mechanism considers EL_2 to be the only form of the enzyme which is catalytically active and that two identical intrinsic dissociation constants (K_L) are involved in the formation of a maximally liganded enzyme–lipid species. The equation obtained is

$$v/V_m = 1/(1 + K_L/[L_t])^2 \quad (1)$$

where v, V_m, K_L, and $[L_t]$ have been defined (see footnote 3). This equation is consistent with the activation curves for short-chain phospholipids interacting with apoBDH [see Cortese et al. (1982) and Cortese and Fleischer (1987)]. Data obtained with PC–PE–PP vesicles and MPL (Figure 1) do not fit into the simple model described by eq 1. They give upward-curved plots of $v^{-1/2}$ versus $[L]^{-1}$ (not shown).

The data obtained for bilayer phospholipid with varying PC concentration [PC–PE–PP and PC(18:1) vesicles] can be fit to a simple cooperative model proposed by Monod et al. (1965), which includes two forms of the enzyme (an inactive or T form and an active or R form; see Figure 5B). We consider that the active form requires two molecules of the ligand (lecithin) to be in the catalytically active form (RL_2) and that there is a simple equilibrium between unliganded forms (T_0 and R_0) of the enzyme, regulated by a constant A; i.e., $A = [T_0]/[R_0]$. This ratio is referred to as the *allosteric constant*, according to the formalism of Monod et al. (1965). The relative velocity equation obtained is

$$v/V_m = \frac{1}{\left(1 + \frac{K_L}{[L_t]}\right)^2 + A'\left(\frac{K_L}{[L_t]}\right)^2} \quad (2)$$

where A' is the apparent allosteric constant in the presence

Table I: Parameters for the Lipid Activation and Influence on NADH Binding Properties of 3-Hydroxybutyrate Dehydrogenase[a]

phospholipid complexes of apoBDH	kinetic parameters of activation			NADH binding (resonance energy transfer)	
	$n_{H(app)}$[b,c]	$[L_{50}]/[L_{10}]$[d]	A'[e]	ΔF_{max} (290 nm to 455 nm)	K_D (μM)[f]
(A) MPL	1.67[b] 1.62[c]	4.75	28.5 (39.2; 20.8)	65 ± 2	8.1 ± 1.0
(B) PC–PE–PP vesicles ($X_{PC(18:1)}$)					
1.00	1.48[b] 1.25[c]	5.00	33.0 (48.7; 22.4)	79 ± 1	6.1 ± 0.3
0.50	1.64[b] 2.05[c]	4.00	29.4 (40.1; 21.5)	66 ± 4	6.2 ± 0.6
0.13	1.75[b] 2.00[c]	6.87	23.1 (32.6; 16.4)	57 ± 4	11 ± 1
0.05	1.05[b] 1.15[c]	9.87	ND	12 ± 1	26 ± 5
0.00	ND	ND	ND	0	ND
(C) soluble apoenzyme (no lipid)	ND	ND	ND	ND	ND
apoBDH + PC(8:0) (100 μM)	1.81[b] 1.92[c]	4.29	0.18 (0.97; 0.03)	42 ± 4	37 ± 6
apoBDH + PE(10:0) (10 μM)	ND	ND	ND		

[a] Values for parameters of the cooperative reactivation of BDH by bilayer phospholipids are included here (parentheses indicate 95% confidence limits calculated for some of these values). Experimental information regarding fluorescence measurements carried out as described under Experimental Procedure is also provided. ND indicates a nondetectable parameter. [b] Hill slopes ($n_{H(app)}$) were calculated from the midsaturation region of Hill plots for each set of experimental data. The numbers obtained for experiments shown in Figure 1 are as follows: (a) for MPL, $y = 1.670x - 2.740$, $r = 0.990$ ($n = 8$), $K_{Hill} = 43.8$ (105.1; 18.1) mol of PL/mol of apoBDH (values between parentheses are 95% confidence limits); (b) for $X_{PC(18:1)} = 1.00$, $y = 1.301x - 2.180$, $r = 0.997$ ($n = 18$), $K_{Hill} = 47.35$ (188.1; 11.9) mol of PL/mol of apoBDH; (c) for $X_{PC(18:1)} = 0.50$ (PC–PE–PP vesicles), $y = 1.643x - 2.572$, $r = 0.992$ ($n = 9$), $K_{Hill} = 36.7$ (102.4; 13.2) mol of PL/mol of apoBDH; (d) for $X_{PC(18:1)} = 0.13$, $y = 1.749x - 3.492$, $r = 0.992$ ($n = 8$), $K_{Hill} = 99.3$ (243.3; 40.5) mol of PL/mol of apoBDH, and for $X_{PC(18:1)} = 0.05$, $y = 1.047x - 4.160$, $r = 0.862$ ($n = 13$), $K_{Hill} = 9.81 \times 10^3$ (2.56×10^4; 3.76×10^3) mol of PL/mol of apoBDH. The values for $n_{H(app)}$ are dependent on the V_{max} values, which are estimated as described in the legend to Figure 1. [c] Additional estimates of the Hill coefficients were obtained according to the graphic method described by Kurganov (1982). The values are calculated from plots of $1/v$ versus log [L]: three points ($[L_1]$, $[L_2]$, and $[L_3]$) are selected in a way that $\log([L_3]/[L_1]) = \log([L_1]/[L_2]) = \log k$; then n_H (a Hill coefficient) is obtained from the reciprocal initial velocities obtained for these phospholipid concentrations with the equation: $n_H = \log[(1/v_2 - 1/v_1)/(1/v_1 - 1/v_3)]/\log k$. We repeated this calculation for several sets of points; averages of them are shown in this table. [d] Values of $[L_{50}]/[L_{10}]$ were calculated from direct plots of experimental data (see Figure 1). This ratio of ligand concentrations is equal to 9.0 for a hyperbolic curve; lower values are obtained for sigmoidal curves. The measured $[L_{50}]/[L_{10}]$ ratios are dependent on the V_{max} values which were estimated from double-reciprocal plots (see legend of Figure 1). The $[L_{50}]/[L_{10}]$ ratios for $X_{PC} = 0.13$ and 0.05 were calculated from ratios measured at lower percentage of maximal activation (V_{max}) since the measured activity did not reach 50% of V_{max} (see Figure 1). For PC–PE–PP vesicles with $X_{PC} = 0.13$, the measured $[L_{25}]/[L_{10}]$ ratio was 2.29, compared with a theoretical value of 3 for a hyperbola. From eq 4 of Sandermann (1982), $[L_{50}]/[L_{10}] = 3([L_{25}]/[L_{10}])$, giving a calculated $[L_{50}]/[L_{10}]$ of 6.87 for $X_{PC} = 0.13$. For PC–PE–PP vesicles with $X_{PC} = 0.05$, the measured $[L_{0.86}]/[L_{0.5}]$ was 1.9, compared with a theoretical value of 1.732 for a hyperbola. Since $[L_{50}]/[L_{10}] = 5.196([L_{0.86}]/[L_{0.50}])$, the calculated $[L_{50}]/[L_{10}]$ ratio is 9.87 for $X_{PC} = 0.05$. For activation of BDH by these vesicles, we also analyzed the departure from hyperbolic shape by comparing the ratios of activities at consecutive points (different lipid/protein ratios) in the activation curve with the theoretical ratio expected for a hyperbola [calculated according to Sandermann (1982)]. For activation by vesicles with $X_{PC} = 0.05$, we found an average deviation of +8.5% as compared with a hyperbolic curve and calculated the experimental $[L_{50}]/[L_{10}]$ ratio to be 9.77 (1.085 times 9, the theoretical value for an hyperbola). This value is comparable to the $[L_{50}]/[L_{10}]$ value of 9.87 obtained from the $[L_{0.86}]/[L_{0.50}]$ ratio. [e] Values of A' were obtained according to the procedure of Horn and Börnig (1969; see Figure 2), and expressed as mean (upper and lower 95% confidence limits in parentheses). For activation with pure PC(18:1) vesicles ($X_{PC} = 1.00$), the data could also be fit to the noncooperative model [eq 1; see Cortese et al. (1982)] by use of a nonlinear regression analysis program (VMS-SAS, SAS Institute Inc., Cary, NC) with two variables (V_{max} and K_L). This analysis gave a V_{max} value [91 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$] comparable to that obtained from double-reciprocal plots [103 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$] (see legend of Figure 1) and used for analysis of the data according to the allosteric model (see legend of Figure 2). But a significantly higher value of K_L (26 mol of PL/mol of BDH) was obtained by nonlinear regression analysis on the basis of the noncooperative model than the K_L value (4.75 mol of PL/mol of BDH) calculated previously (Cortese & Fleischer, 1987) and used in the Horn–Börnig plot for the allosteric model (see Figure 2). For activation by pure PC(18:1) vesicles ($X_{PC} = 1.00$), the data are therefore consistent with both allosteric and noncooperative models for activation. By contrast, for activation by MPL or by PC–PE–PP vesicles ($X_{PC} = 0.50$ or 0.13), the data are not consistent with the noncooperative model [see Sandermann et al. (1986) and Cortese and Fleischer (1987)]; nonlinear regression analyses of these data gave poor fits to the noncooperative model as reflected in both large values for the sum of the squares of the residuals and nonrandom distributions of errors in plots of residuals (Motulsky & Ransnas, 1987). [f] Apparent dissociation constants for fluorescence resonance energy transfer (K_D) were calculated from titrations with NADH of complexes prepared with several phospholipid vesicles (Figure 3A) at phospholipid/protein ratios of 100 mol of phospholipid/mol of apoBDH. A nonlinear regression program was used to fit a hyperbolic binding curve (Duggleby, 1981): $F_{cor} = \Delta F_{max}/(1 + K_D/[NADH])$, where ΔF_{max} is the maximal change in fluorescence corrected by inner-filter effect (see Experimental Procedures). Values of K_D and ΔF_{max} for soluble lecithins are also included (Figure 4).

of an essential activator (i.e., lecithin). This constant is a function of the ligand concentration [$A' = A/(1 + [L_t]/K_L)^2$; see Dixon and Webb (1979)]. The allosteric constants (A') that were obtained for the data represented in Figure 1 are presented in Table I (with 95% confidence limits). They were obtained from a linear form of the Monod–Wyman–Changeaux (MWC) equation for exclusive binding [Horn & Börnig, 1969; see Figure 2 and footnote *e* of Table I]. A' values were calculated for $[PC] = K_L/10$ by linear regression analysis of the data (Figure 2): values in the range of 30 are obtained for mixtures of phospholipid containing $X_{PC} \leq 0.13$ and for activation by PC(18:1). These values are to be compared with a value of zero for the noncooperative mechanism, which is a limiting case [a value near zero was obtained for PC(8:0), see Figure 2 inset and Table I].

For activation by PC(18:1) vesicles ($X_{PC} = 1.00$), the data are consistent with both the allosteric and noncooperative models (see Table I, footnote *e*). The fits to the two models are obtained with slightly different values for V_{max} but markedly different values for K_L (26 and 4.75 mol of PL/mol

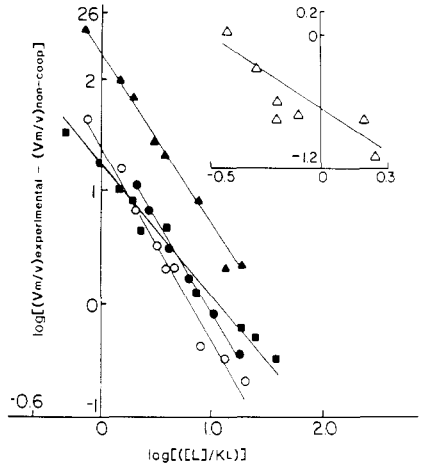

FIGURE 2: Horn–Börnig plots for the activation of BDH with bilayer and soluble phospholipid. log $[(V_m/v)_{exptl} - (V_m/v)_{noncoop}]$ is plotted as a function of log $([L]/K_L)$. In the main figure, the experimental data are for bilayer phospholipid taken from Figure 1. Values of the relative allosteric constant A' (for exclusive binding of the R form) have been calculated with the procedure of Horn and Börnig (1969), modified for an essential allosteric activator and two ligand binding sites (see Results). The equation obtained is log $[(V_m/v)_{exptl} - (V_m/v)_{noncoop}] = \log A' - 2 \log [([L]/K_L)]$, where $(V_m/v)_{exptl}$ is defined as the reciprocal of the reduced BDH specific velocity (v/V_m) as plotted in Figure 1 and $(V_m/v)_{noncoop}$ is the reciprocal reduced BDH specific velocity, obtained for the noncooperative model as previously discussed (Cortese et al., 1982) with K_L as an apparent dissociation constant of lecithin binding [K_L is 4.75 mol of PL/mol of apoBDH for phospholipid vesicles containing PC or MPL (Cortese & Fleischer, 1987), V_m is 120 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ for PE–PC–PP vesicles and MPL, and V_m is 102.9 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ for pure PC vesicles; see legend to Figure 1]. Values of A' were calculated with linear regression analysis of log $[(V_m/v)_{exptl} - (V_m/v)_{noncoop}]$ plotted as a function of log $[([L]/K_L)]$. We calculated A' for the ordinate axis at $x = \log (K_L/10)$ using the equation $\log A' = \log [(V_m/v)_{exptl} - (V_m/v)_{noncoop}] + 2 \log ([L]/K_L X_{PC})$. The results are as follows: (a) for MPL, $y = -1.624x + 1.5090$, $r = -0.987$ ($n = 7$), $\log A' = 1.455$ [SD (y intercept) = 0.138]; (b) for $X_{PC(18:1)} = 1.0$ [PC(18:1) vesicles], $y = -1.144x + 1.149$, $r = 0.963$ ($n = 9$), $\log A' = 1.519$ (SD = 0.169); (c) for $X_{PC(18:1)} = 0.5$ (PC–PE–PP vesicles), $y = -1.785x + 1.429$, $r = -0.991$ ($n = 9$), $\log A' = 1.469$; (d) for $X_{PC(18:1)} = 0.13$, $y = -1.628x + 2.281$, $r = -0.994$ ($n = 8$), $\log A' = 1.364$ (SD = 0.149). Values obtained for $X_{PC(18:1)} = 0.05$ were not sufficiently accurate and are not included here. Values of A' (A for [L] = $K_L/10$), expressed as mean (with upper and lower 95% confidence limits), are given in Table I. (Inset) Activation with PC(8:0) follows the noncooperative model (linear double-reciprocal plots of $v^{-1/2}$ versus $[PC(8:0)]^{-1}$; Cortese et al., 1982), and the procedure for estimation of A' [V_m = 23.6 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$ and K_L = 28.84 μM PC(8:0)] gives small values for this parameter: $y = -1.215x - 0.635$, $r = -0.710$ ($n = 7$; most values from 20 determinations were negative or zero).

of BDH for noncooperative and allosteric models, respectively). Therefore for activation by PC(18:1) vesicles, the data provide no basis to discriminate between the two models. By contrast, the data for activation by soluble PC(8:0) are consistent with the noncooperative model while activation by MPL or PC–PE–PP vesicles (X_{PC} = 0.50 or 0.13) is consistent with the allosteric model (eq 2) but not the noncooperative model (eq 1) (Sandermann et al., 1986; Cortese & Fleischer, 1987).

Fluorescence Characteristics of 3-Hydroxybutyrate Dehydrogenase. Measurements of resonance energy transfer between tryptophan and NADH of apoBDH inserted into phospholipid vesicles are shown in Figure 3A. The mole fraction of PC was varied, but the phospholipid/protein ratio was kept constant at 100 mol of PL/mol of apoBDH. Energy transfer was not detected in phospholipid vesicles lacking PC [Figure 3A, open triangles; see also Gazzotti et al. (1974)]. With X_{PC} = 0.05, there was significant energy transfer, ~15%

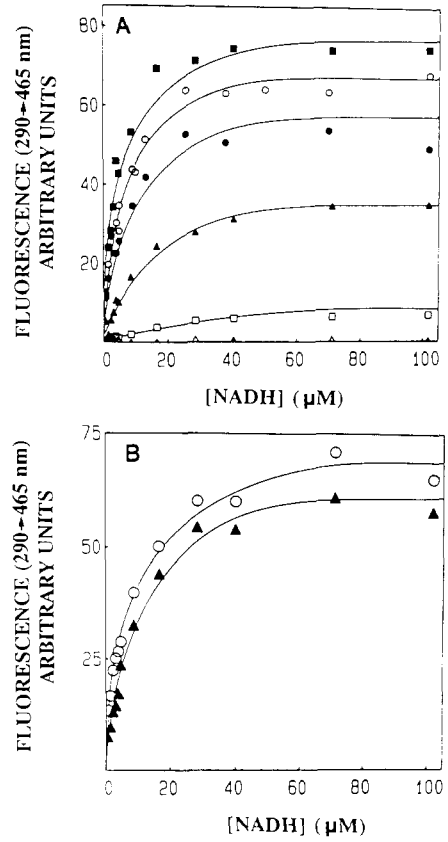

FIGURE 3: Resonance energy transfer measurements of NADH binding for BDH activated with bilayer phospholipid containing lecithin (excitation at 296 nm, emission at 455 nm). The apoBDH was reconstituted into bilayer phospholipid. The bilayer phospholipid included PE–PP–PC vesicles, PC vesicles, and MPL, prepared as described in Figure 1. (A) Direct plot of energy transfer data obtained with (a) $X_{PC(18:1)}$ = 1.00 (solid squares), (b) $X_{PC(18:1)}$ = 0.50 (open circles), (c) $X_{PC(18:1)}$ = 0.13 (solid triangles), (d) $X_{PC(18:1)}$ = 0.05 (open squares), (e) $X_{PC(18:1)}$ = 0.00 (PE–PP vesicles; open triangles), and (f) MPL (solid circles). In each case, the complexes were prepared at a phospholipid/BDH ratio of 100 mol of PL/mol of apoBDH with 1.3 μM BDH (see Table I for calculated K_D values). (B) Direct plot of energy transfer data obtained with PC–PE–PP–apoBDH complexes prepared [$X_{PC(18:1)}$ = 0.50 (open circles) and $X_{PC(18:1)}$ = 0.13 (solid triangles)] at phospholipid/protein ratios (15 and 60, respectively) that give the same BDH specific activity [approximately 38 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$, see Figure 1] and with 1.3 μM BDH. The calculated K_D's from nonlinear regression analysis are 9.7 and 4.8 μM NADH for X_{PC} = 0.13 and 0.50, respectively.

of that with X_{PC} = 1.00 (Figure 3A and Table I). The maximal energy transfer increases with $X_{PC(18:1)}$ (Figure 3A and Table I). In these and other studies, the tryptophan and NADH fluorescence was also measured for a more complete analysis. In the absence of NADH, the tryptophan fluorescence (λ_{max} = 296 nm, λ_{em} = 340 nm) of BDH at 13 μM, reconstituted in PC–PE–PP vesicles with $X_{PC(18:1)}$ of 0.05 and 0.5, was the same (not shown). For the enzyme reconstituted into phospholipid vesicles containing the different mole fractions of PC shown in Figure 3A, the tryptophan fluorescence of BDH at 1.3 μM was similar (not shown). Therefore, the lower energy transfer with lower X_{PC} cannot be attributed to a change in tryptophan fluorescence. For the enzyme reconstituted in PC–PE–PP with X_{PC} = 0.5, the NADH fluorescence intensity (λ_{ex} = 340 nm, λ_{em} = 465 nm) in the presence of 8 μM NADH was enhanced ~3-fold by 13 μM BDH, whereas BDH reconstituted in PC–PE–PP with X_{PC} = 0.05 gave no significant enhancement of the NADH fluorescence (14 μM NADH titrated with up to 13 μM BDH) (Q.-C. Chen, J. O. McIntyre, and S. Fleischer, unpublished studies). This

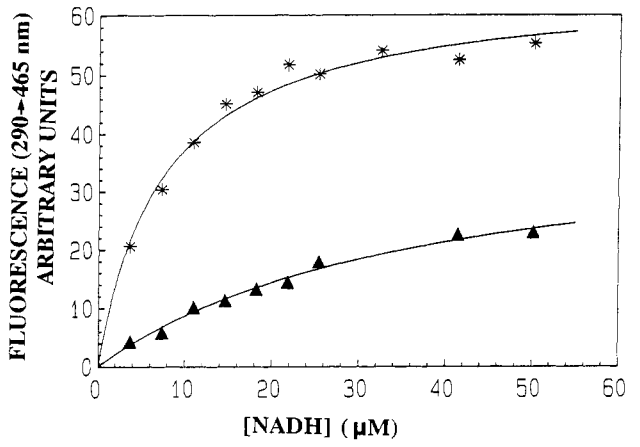

FIGURE 4: Comparison of resonance energy transfer for NADH binding to BDH reactivated with either MPL or soluble lecithin. The excitation was at 290 nm, and emission was at 465 nm. BDH (10 μg/mL, 0.32 μM) in 10 mM Hepes–KOH (pH 8.0), 1 mM EDTA, 50 mM NaCl, and 5 mM DTT was reconstituted either with MPL (asterisks, 100 mol of phospholipid/mol of apoBDH) or in the presence of 100 μM PC(8:0) (closed triangles). The samples were titrated with NADH, and the fluorescence of each sample together with appropriate blank (no BDH) was measured. Fluorescence was corrected for dilution, and values are plotted after subtraction of the fluorescence obtained in the absence of BDH. The data were fit with a hyperbolic binding curve to determine the ΔF_{max} and K_D values (see Table I, footnote *f*, and Experimental Procedures). Each titration of sample and blank was carried out in parallel and was completed within 40 min so as to minimize loss of enzymic activity of BDH–PC(8:0). The apparent K_D for NADH binding to BDH in PC(8:0) was 37 μM (see Table I).

indicates only minimal binding of NADH at X_{PC} of 0.05. With 1.3 μM BDH (experiment shown in Figure 3A), the NADH fluorescence intensities were similar with varying $X_{PC(18:1)}$ (not shown) whereas the resonance energy transfer increased markedly with increasing X_{PC}. The resonance energy transfer data, taken together with NADH and tryptophan fluorescence data, suggest that the NADH binding increases with lecithin content (see Discussion).

Energy transfer between tryptophan and NADH for BDH in the presence of soluble lecithins was detected but had a lower ΔF_{max} (Figure 4 and Table I), under experimental conditions of catalytic activity of PC(8:0)–apoBDH complexes. Apparent dissociation constants (K_D) calculated from nonlinear regression analysis of data shown in Figures 3A and 4 are presented in Table I.

We attempted to correlate BDH activity with NADH binding, detected by resonance energy transfer (Figure 3B). ApoBDH was inserted into phospholipid vesicles containing different mole fractions of PC(18:1) ($X_{PC(18:1)}$ = 0.50 or $X_{PC(18:1)}$ = 0.13), and with the molar ratio of lipid/protein adjusted to give the same measurable BDH specific activity [38 μmol of NAD^+ reduced·min^{-1}·(mg of protein)$^{-1}$; see also Figure 1]. Although the K_D values were somewhat different (Figure 3B), the maximal energy transfer was the same for both. Analysis of data presented in Figure 1 and Table I shows that the relationship between the specific activity obtained at a ratio of 100 mol of PL/mol of apoBDH and the maximal resonance energy transfer measured for that lipid/protein ratio (Figure 3A) is approximately proportional [resonance energy transfer = 0.844(BDH specific activity) + 1.534, r = 0.967 (n = 5), not shown]. These studies indicate that the role of lipid (lecithin) for enzymic activity confers to the enzyme the ability to bind coenzyme (NADH).

Discussion

An allosteric model (Monod et al., 1963, 1965) can explain

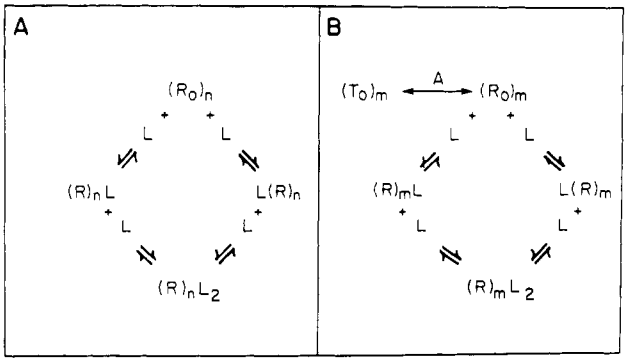

FIGURE 5: Different multiple equilibria for lecithin binding to soluble and membrane-bound 3-hydroxybutyrate dehydrogenase. (A) Activation of apoBDH in aqueous solution. The model presented previously for soluble lecithins is shown (Cortese et al., 1982). The activatable oligomer is indicated as $(R_0)_n$, where n is the number of oligomer subunits for soluble apoBDH [i.e., n = 2; the dimer is the predominant molecular species in solution (McIntyre et al., 1978)]. (B) Activation of apoBDH in the bilayer. A transition between enzyme conformations is shown for membrane-bound forms of 3-hydroxybutyrate dehydrogenase (the symbol ↔ is used for the transition regulated by the *allosteric constant* $A = [T_0]/[R_0]$). The actual stoichiometry for the ratio between protein subunits and lipid molecules bound to them is still unknown, and we indicate this by a different subscript, m, in $(R)_mL_2$ [i.e., distinct active forms are possible: R_4L_2, R_4L_4, or $[(R_2)L_2]_2$]. According to this scheme, the nonactivatable form of the enzyme $(T_0)_m$ is unable to bind ligand (L). The latter assumption does not modify our conclusions (see Discussion); the difference between R_0 and T_0 could be at the level of kinetic behavior or a consequence of the insertion into membranes of the enzyme (R and T forms may have a different degree of penetration in the bilayer and accessibility to the lipid ligand). This model accounts for linear Hill plots obtained with mixtures of long-chain phospholipids (Cortese & Fleischer, 1987) and for a behavior similar to that exhibited by the soluble apoenzyme.

the lecithin–BDH interactions for both soluble and bilayer phospholipids, as studied by kinetic activation and correlated with NADH binding as studied by spectroscopic methods. The MWC model provides a simple conceptual framework to interpret the experimental data available for BDH (Figure 5). Basically, this theory assumes a structural role for the ligand (Fersht, 1985). The kinetics of activation and coenzyme binding are consistent with this model. The model can now be used as a working hypothesis to consider experiments related to structural aspects.

A basic assumption of the MWC model is that there is more than one structural form for a cooperative enzyme [usually referred to as R and T states; for reviews, see Koshland (1969), Wyman (1972), and Baldwin (1975)]. Such forms have been identified for some proteins (Fersht, 1985), but it is not clear at present for BDH and many allosteric enzymes whether the transition between them in an oligomeric structure is (a) conservative (or concerted), i.e., all the oligomers simultaneously change their conformations from one state to the other (original MWC model; Monod et al., 1965), (b) pseudoconservative, where some subunits in the oligomer can be present in the R state, and others in the T state (Viratelle & Seydoux, 1975), or (c) sequential, where each subunit is able to undergo stepwise transition between R and T states [Adair–Koshland–Nemethy–Filmer model or AKNF model; Koshland et al., 1966; see also Dixon and Webb (1979)]. Additional distinctions are possible with regard to exclusive binding (binding of a ligand to either R or T states) versus nonexclusive binding (binding to both R and T states) [Rubin & Changeaux, 1966; for more extensive reviews, see Dixon and Webb (1979), Fersht (1985), and Palmer (1985)].

3-Hydroxybutyrate dehydrogenase exhibits important differences in its multiple equilibria for lecithin binding depending

on whether it is inserted into a mixture of bilayer phospholipid containing lecithin (highly cooperative mechanism; Sandermann et al., 1986; Cortese & Fleischer, 1987) or in a soluble form (noncooperative activation; Cortese et al., 1982; Cortese & Vidal, 1983). For activation by phospholipid in bilayers containing only lecithin [i.e., PC(18:1), $X_{PC} = 1.00$], the data are consistent with either model and do not decisively discriminate between the two mechanisms (see Table I, footnote *e*). The composition of the bilayer and/or the physical state of phospholipid (soluble short-chain lecithins versus phospholipid mixtures in bilayers) exerts a marked effect on the kinetic behavior of this enzyme. This effect may be related to differences in the association of BDH, i.e., a dimer in solution (McIntyre et al., 1978) and a tetramer when inserted in membranes (McIntyre et al., 1983). For both models of lecithin binding, the assumed stoichiometry is two lecithin binding sites per active unit (Cortese et al., 1982; Cortese & Fleischer, 1987). This is analogous to the case of hemoglobin; $\alpha\beta$ dimers are not cooperative (Edelstein, 1975), but the tetramer ($\alpha_2\beta_2$) follows a MWC behavior with only one value for the allosteric constant A (Edelstein, 1971).

In an early study (Gazzotti et al., 1974), equilibrium dialysis and resonance energy transfer gave a dissociation constant of 6–16 μM for NADH binding to the enzyme in mitochondrial phospholipid vesicles, with 0.5 mol of NADH bound per BDH monomer. The binding of NAD(H) to BDH was reinvestigated recently (Fritzsche et al., 1984; Dubois et al., 1986b; Rudy et al., 1989) by ultrafiltration, equilibrium dialysis, and EPR spectroscopy and confirmed that lecithin is essential for binding of NADH to the enzyme. In this study, we reconfirm that NADH binds to BDH in the presence of PC (K_D is $\sim$10 μM) using resonance energy transfer. This is the first study in which resonance energy transfer was used to measure NADH binding for the soluble enzyme reactivated with soluble lecithin ($K_D \sim 37$ μM).

Two experimental findings are especially worth noting. The enzyme in the absence of lecithin does not exhibit resonance energy transfer (Figure 3). We have previously correlated both enzymic activity and NADH binding, as studied by resonance energy transfer, with the presence of lecithin in the bilayer (Gazzotti et al., 1974). We now also find NADH binding by resonance energy transfer for the enzyme reactivated with soluble lecithin. There is no energy transfer for the enzyme inserted into vesicles devoid of PC [Figure 3A and Gazzotti et al. (1974)]. The essential role of lecithin for this lipid-requiring enzyme (Figures 1 and 3A) can be correlated with conferring enhanced NADH binding.

A second observation is that the extent of energy transfer increases with the mole fraction of lecithin in the bilayer vesicles (cf. Figure 3 and Table I; see Results), but the apparent dissociation constant K_D obtained is approximately the same, in the range of 10–30 μM. The reduction in ΔF_{max} (maximal resonance energy transfer) for NADH binding to BDH reconstituted in vesicles with lower mole fractions of PC does not appear to derive from differences in the intrinsic tryptophan fluorescence of BDH which did not vary markedly with X_{PC}. The fluorescence of NADH is enhanced by binding to the BDH–MPL complex (Gazzotti et al., 1974; McIntyre et al., 1984) or to the BDH–PC–PE–PP complex ($X_{PC} = 0.5$). The lack of apparent enhancement of the NADH fluorescence by BDH in PC–PE–PP vesicles ($X_{PC} = 0.05$) implies, for this form of the enzyme with low activity, either that the fluorescence of bound NADH is not enhanced or that the amount of bound NADH is small compared with that of the fully active enzyme in PC–PE–PP ($X_{PC} = 0.5$). We did not find a simple relationship between the energy transfer data and the quenching of tryptophan fluorescence by NADH, indicating that the quenching may reflect complex processes perhaps arising from the presence of 16 tryptophans in each BDH tetramer (four per monomer; Bock & Fleischer, 1975). The reduction in ΔF_{max} for resonance energy transfer with decreasing mole fraction of PC (Figure 3A) appears to reflect a decrease in the amount of bound NADH rather than changes in the fluorescence characteristics of either the bound NADH or the tryptophan of the enzyme. The simplest interpretation of the resonance energy transfer data is that the fraction of BDH molecules that bind NADH depends on the molar fraction of PC. That is, as the PC mole fraction of the vesicles is increased, the amount of the enzyme in the form exhibiting resonance energy transfer is higher. Therefore, there may be a ligand-induced conformational change in BDH as it binds lecithin, and one (or more) conformation(s) obtained in the presence of lecithin give(s) enhanced NADH binding with the proper alignment of dipoles (for tryptophan and NADH) to give the energy transfer process. At $X_{PC} = 0.05$, the K_D is significantly increased, and no energy transfer is detected in the absence of PC. The binding of NADH to BDH reconstituted in phospholipid both in the presence and in the absence of PC has recently been reinvestigated with an ultrafiltration method to quantitate binding directly (Rudy et al., 1989). Those studies show little or no NADH binding to BDH in the absence of PC in the range up to $\sim$100 μM NADH, consistent with the absence of resonance energy transfer found in these studies. Hence, PC is essential for the binding of NADH.

We find here that a single allosteric constant (A) is obtained, according to the procedure of Horn and Bornig (1969; see Table I) for activation of apoBDH with mixtures of bilayer phospholipid containing PC (PE–PC–PP vesicles) or PC alone, as expected if a MWC model applies to membrane-bound BDH (Edelstein, 1971). The MWC equation for lecithin as an allosteric activator (eq 2, Results) implies a simple equilibrium between R and T forms (regulated by $A = [T_0]/[R_0]$) and that only the RL_2 form of liganded BDH is active (see Figure 5). This active form is considered to be RL_2, according to Cortese and Fleischer (1987). For vesicles with low PC content (PC–PE–PP with 5% and 13% PC), the response approaches hyperbolic curves (i.e., $[L_{50}]/[L_{10}]$ is closer to 9.0; see Table I) with higher K_D for NADH (measured by resonance energy transfer, Table I) and lower activity. These results imply that there is a conformational equilibrium, which is shifted by PC toward the activatable form (RL_2). Thus, PC can be considered to be a heterotropic allosteric activator of BDH and appears to function by stabilizing the active RL_2 form [see Kurganov (1982)]. Lecithin will affect the ratio between R and T forms of the enzyme and the fraction of BDH molecules that are functional in substrate binding and catalysis. Accordingly, we observe hyperbolic NADH binding curves measured by resonance energy transfer (Figures 3 and 4). Sigmoidal NADH binding curves would indicate either changes in BDH affinity for NADH (with NADH serving as a homotropic allosteric effector that provides additional thermodynamic stability to the R form) or differences in NADH binding affinity between subunits of the BDH tetramer.

We consider that the present kinetic evidence regarding differences between soluble apoBDH and the enzyme reconstituted into bilayers containing mixtures of phospholipids (Cortese et al., 1982; Sandermann et al., 1986; Cortese & Fleischer, 1987) suggests a distinct kinetic behavior for the enzyme removed from a bilayer environment. However, the

data for activation by bilayers containing only lecithin [PC-(18:1), X_{PC} = 1.00] is consistent with both models for activation, so that the difference in kinetic mechanism between the two extremes may reflect, in part, the effect of other phospholipids in the bilayer as well as the different physical state of the phospholipid and/or the oligomeric structure of the enzyme (tetramer in bilayers versus dimer in soluble form). For activation by bilayers containing only lecithin, the lack of discrimination between the two models might be accounted for, in part, by the experimental design in that, at high protein to phospholipid ratios, the insertion of BDH into the bilayer may have been limiting (McIntyre et al., 1979). This same limitation may also pertain to the activation by mixtures of phospholipids in bilayers. However, it should be noted that this limitation also pertained to the data (Fleischer et al., 1979; Churchill et al., 1983) which originally indicated site–site interaction for lecithin activation of BDH (Sandermann, 1984), yet similar results were obtained with alternative experimental approaches (Sandermann et al., 1986). Due to both the experimental limitations in the studies reported here and the necessity to determine an apparent allosteric constant (A' in the presence of lecithin), rather than the true allosteric constant (A, in the absence of lecithin), we consider the values for A' (Table I) to be approximations of the allosteric constant. Further, it should be noted that the data for activation by bilayer phospholipids reported here are also consistent with the previous studies in which the data were analyzed with a Hill-type equation and which were interpreted in terms of site–site interaction for activation of BDH by lecithin (Sandermann et al., 1986). The nonlinearized Hill equation [$v/V_{max} = 1/(1 + K_{coop}/[L]^{n_H})$; Sandermann, 1982; Sandermann et al., 1986] has three variables (i.e., V_{max}, K_{coop}, and n_H) with n_H and K_{coop} being somewhat dependent on the value V_{max}. Likewise, the allosteric model described here (eq 2) also has three variables (i.e., V_{max}, K_L, and A) but differs from the Hill-type analysis in that the equation is derived from a specific mechanistic model for activation of the enzyme by lecithin, i.e., an MWC model with exclusive binding of lecithin to the R form and the catalytically active form having two molecules of lecithin bound (RL_2) (see Results).

We suggest that the MWC model represented by eq 2 from this paper encompasses the limiting case (eq 1) for the kinetic behavior of the enzyme reactivated by soluble lecithin (see Figure 5). A shift between R and T forms can be a consequence of the free energy for lecithin binding, which is proportional to the acyl chain length (Cortese et al., 1982). If we assume that the T form is more stable in a membrane environment [as indicated by a large value of A' (about 30)] and that the enzyme exists mainly as R form in the soluble phase (as shown with short-chain lecithins to activate soluble apoBDH), we can then conclude that lecithin stabilizes the R form (activatable with two identical noninteracting sites for PC) in the bilayer.

A number of observations regarding the function and structure of the enzyme can now be summarized:

(1) The enzyme has an absolute lipid requirement, which is specific for *lecithin* in the form of either soluble or bilayer phospholipid (Gazzotti et al., 1975; Fleischer et al., 1983; this study).

(2) The activation of BDH by soluble lecithins can be described by a model with two noninteracting lecithin sites (noncooperative; Cortese et al., 1982), whereas activation by a mixture of bilayer phospholipids (mimicking MPL) behaves cooperatively and follows a Hill-type equation (Sandermann et al., 1986; Cortese & Fleischer, 1987). Both models assume two lecithin binding sites, suggesting that this characteristic is intrinsic to the enzyme.

(3) The enzyme in the soluble form activated by soluble lecithins is a dimer (McIntyre et al., 1978), whereas in the presence of a mixture of bilayer phospholipids the enzyme is a tetramer (McIntyre et al., 1983).

(4) Resonance energy transfer from tryptophan to NADH requires the presence of lecithin in either bilayer or soluble form (Figures 3 and 4, respectively). There is no resonance energy transfer in the absence of lecithin. The activation of enzymic activity by lecithin is closely related to conferring the enzyme with the ability to bind coenzyme (NADH).

The allosteric model can explain a number of these characteristics and is compatible with each. Characteristic 2 can be derived directly from comparison of eq 1 and 2 (see Results). The equation for the noncooperative lecithin binding model [eq 1; see Cortese et al. (1982)] is a limiting case of an MWC model that considers L as a ligand with properties of an essential allosteric activator that only binds to an activatable R form of the enzyme (eq 2). With short-chain lecithins, the second term in the denominator in eq 2 approaches zero, so that eq 1 is a limiting case of eq 2.

Equation 2 also accounts for the linear Hill plots obtained over a wide range of PC molar fractions (Cortese & Fleischer, 1987). When only pure PC(18:1) is present in the bilayer (Figure 1), the Hill coefficient is lower than 2 (see Table I). These lower values of $n_{H(app)}$ may be a consequence of the second-power term on the right of eq 2, i.e., $A'K_L^2/[L]^2$, and of a similar term hidden within the binomial expression in eq 2, i.e., $(1 + K_L/[L])^2 = 1 + 2K_L/[L] + K_L^2/[L]^2$. The combined contribution of these second-power terms accounts for the shape obtained for the Hill plots. In excess lecithin (PC vesicles), the contribution of the quadratic terms is lower, and the Hill slope also is a lower value.[4]

Characteristics 3 and 4 can be related to the existence of more than one form of the enzyme (i.e., R_0 and T_0 forms), with different kinetic and binding properties. They also imply that distinct three-dimensional structures exist for apoBDH inserted into phospholipid vesicles, as expected for a system that undergoes conformational transition between states (characterized by different ligand binding properties). The lipid requirement is at least in part related to the ability of the enzyme to bind coenzyme, as reflected by characteristic 2.

Evidence presented here and previously (Cortese et al., 1982; Sandermann et al., 1986; Cortese & Fleischer, 1987) does not preclude other complex cooperative models. Other types of heterotropic modulation (interaction between BDH with other phospholipids, e.g., diphosphatidylglycerol [see Wang et al. (1988)] or lipid molecules present in the inner mitochondrial membrane) can be considered a direct consequence of our proposed model. The cooperative model presented here serves as a working hypothesis for consideration of the physiological significance for the allosteric behavior of lecithin and other phospholipids in this interaction and may have general rele-

[4] The actual Hill equation [log $(v/V_m - v)$ versus log [L]], for the MWC model (see eq 2), is

$$\log (v/V_m - v) = 2 \log (X_{PC}[L]/K_L) - \log [2X_{PC}([L]/K_L) + (A' + 1)] \quad \text{(i)}$$

Compared with the common Hill equation, this equation has an additional negative term which is a function of [L] and decreases the actual Hill slope ($n_{H(app)}$) that is obtained. For K_L = 4.75 (mol of PL/mol of apoBDH) and A' of ~10, that term accounts for a change in $n_{H(app)}$ of −0.5 unit (calculations not shown). This effect depends on X_{PC}, in a nonlinear fashion, and is substantial at low molar fractions of lecithin.

vance for other lipid-requiring enzymes and lipid binding proteins.

Registry No. BDH, 9028-38-0; NADH, 58-68-4.

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