Coenzyme Binding by 3-Hydroxybutyrate Dehydrogenase, a Lipid-Requiring Enzyme: Lecithin Acts as an Allosteric Modulator To Enhance the Affinity for Coenzyme†

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ABSTRACT: The role of phospholipid in the binding of coenzyme, NAD(H), to 3-hydroxybutyrate dehydrogenase, a lipid-requiring membrane enzyme, has been studied with the ultrafiltration binding method, which we optimized to quantitate weak ligand binding (K_D in the range 10–100 μM). 3-Hydroxybutyrate dehydrogenase has a specific requirement of phosphatidylcholine (PC) for optimal function and is a tetramer in the membrane. The binding of NAD(H) to the enzyme, purified from bovine heart mitochondria, was quantitated both for the apodehydrogenase, which is devoid of phospholipid, and for the enzyme reconstituted into phospholipid vesicles in either the presence or absence of PC. We find that (i) the stoichiometry for NADH and NAD binding is 0.5 mol/mol of enzyme monomer (2 mol/mol of tetramer); (ii) the dissociation constant for NADH binding is essentially the same for the enzyme reconstituted into the mixture of mitochondrial phospholipids (MPL) (K_D = 15 ± 3 μM) or into dioleoyl-PC (K_D = 12 ± 3 μM); (iii) the binding of NAD^+ to the enzyme–MPL complex is more than an order of magnitude weaker than NADH binding ($K_D \sim 200$ μM versus 15 μM) but can be enhanced by formation of a ternary complex with either 2-methylmalonate (apparent K_D = 1.1 ± 0.2 μM) or sulfite to form the NAD–SO_3^- adduct (K_D = 0.5 ± 0.1 μM); (iv) the binding stoichiometry for NADH is the same (0.5 mol/mol) for binary (NADH alone) and ternary complexes (NADH plus monomethyl malonate); (v) binding of NAD^+ and NADH together totals 0.5 mol of NAD(H)/mol of enzyme monomer, i.e., two nucleotide binding sites per enzyme tetramer; and (vi) the binding of nucleotide to the enzyme reconstituted with phospholipid devoid of PC is weak, being detected only for the NAD^+ plus 2-methylmalonate ternary complex (apparent $K_D \sim 50$ μM or ~50-fold weaker binding than that for the same complex in the presence of PC). The binding of NADH by equilibrium dialysis or of spin-labeled analogues of NAD^+ by EPR spectroscopy gave complementary results, indicating that the ultrafiltration studies approximated equilibrium conditions. In addition to specific binding of NAD(H) to 3-hydroxybutyrate dehydrogenase, we find significant binding of NAD(H) to phospholipid vesicles. An important new finding is that the nucleotide binding site is present in 3-hydroxybutyrate dehydrogenase in the absence of activating phospholipid since (a) NAD^+, as the ternary complex with 2-methylmalonate, binds to the enzyme reconstituted with phospholipid devoid of PC and (b) the apodehydrogenase, devoid of phospholipid, binds NADH or NAD–SO_3^- weakly (half-maximal binding at ~75 μM NAD–SO_3^- and somewhat weaker binding for NADH). We conclude that the role of PC for activation of 3-hydroxybutyrate dehydrogenase is related, at least in part, to enhancing the nucleotide binding by at least an order of magnitude. Thus, PC appears to serve as an allosteric activator of this lipid-requiring enzyme.

3-Hydroxybutyrate dehydrogenase [(*R*)-3-hydroxybutanoate:NAD^+ oxidoreductase, EC 1.1.1.30] is a lipid-requiring enzyme that has a specific requirement of phosphatidylcholine (PC)[1] for enzymic activity. The enzyme, purified from beef heart mitochondria, is devoid of phospholipid and is referred to as apodehydrogenase. The apodehydrogenase is inactive but can be reactivated by forming an enzyme–phospholipid complex. Activation can be achieved with soluble lecithins below the critical micellar concentration or by reconstitution (unidirectional insertion) into phospholipid vesicles containing lecithin (Fleischer et al., 1983; Fleischer & McIntyre, 1985; Maurer et al., 1985; McIntyre et al., 1979). The membrane-bound enzyme is a tetramer (McIntyre et al., 1983).

In our earlier studies using equilibrium dialysis, phosphatidylcholine was found to be essential for NADH binding; a stoichiometry of 0.5 equiv of NADH bound per enzyme monomer was obtained with a K_D of about 15 μM (Gazzotti et

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[1] Abbreviations: DPG, diphosphatidylglycerol from bovine heart; PE, phosphatidylethanolamine; PC, phosphatidylchloline; DMPE, *N,N*-dimethylphosphatidylethanolamine; MPL, mitochondrial phospholipids; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MMA, 2-methylmalonic acid (sodium salt); MMM, malonic acid monomethyl ester (sodium salt); Tris, tris(hydroxymethyl)aminomethane; BDH-MPL, 3-hydroxybutyrate dehydrogenase reconstituted with mitochondrial phospholipid vesicles; DTNB, 3,3′-dithiobis(6-nitrobenzoate); CNTP-BDH, (carboxynitrophenyl)thio derivative of BDH (BDH derivatized with DTNB); NTCB, 2-nitro-5-thiocyanobenzoate; CN-BDH, BDH cyanylated by derivatization with NTCB; N^6-SL-NAD^+, N^6-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl) derivative of NAD^+; C8-SL-NAD^+, 8-[(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)amino] derivative of NAD^+; DSL, perdeuterated spin-label.

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al., 1974). We did not previously detect NADH binding to the apodehydrogenase (i.e., in the absence of phospholipid) either by equilibrium dialysis or by fluorescence resonance energy transfer. Binding of NADH by the enzyme reconstituted into phospholipid vesicles devoid of PC also could not be detected by resonance energy transfer. Subsequent EPR studies of ternary enzyme–coenzyme–inhibitor complexes, utilizing spin-labeled NAD derivatives, indicated two types of tight coenzyme binding sites in the active enzyme (Fritzsche et al., 1984). In both the equilibrium dialysis and EPR studies, weak binding of NAD(H) would not have been detected since the upper concentration of ligand was ~50 μM. Therefore, on the basis of previous studies, we were unable to resolve whether PC binding to 3-hydroxybutyrate dehydrogenase results in the induction of the nucleotide binding site or whether PC modulates the binding characteristics of a preexisting weak binding site. To address this question, we have optimized the ultrafiltration binding assay so as to detect and quantitate weak binding (K_D in the range 10–100 μM) of ligands to membrane proteins and have reinvestigated the binding of nucleotide to 3-hydroxybutyrate dehydrogenase. The ultrafiltration method, which we describe here, provides accurate binding data over a broader concentration range and more rapidly than equilibrium dialysis. Complementary binding studies were carried out by conventional equilibrium dialysis as well as by EPR spectroscopy with N^6-SL-NAD$^+$(H) or C8-SL-NAD$^+$(H), active coenzyme derivatives (Fritzsche et al., 1984; Philipp et al., 1984), and by fluorescence resonance energy transfer. With the more sensitive ultrafiltration method, the binding of NADH or NAD$^+$, either alone (binary complexes)[2] or as ternary complexes, was investigated for the enzyme in phospholipid vesicles of varying composition as well as for the apodehydrogenase devoid of lipid.

Experimental Procedures

Materials. NAD$^+$ and NADH were obtained from Boehringer Mannheim or Pharmacia (Piscataway, NJ). Buffer compounds and substrates were of analytical grade from various commercial sources. Phospholipids, diphosphatidylglycerol (DPG, bovine heart), and synthetic 1,2-dioleoylphosphatidylcholine and 1,2-dioleoylphosphatidylethanolamine (PE) were from Avanti Polar Lipids (Birmingham, AL). 1,2-Dioleoylphosphatidyl-*N*,*N*-dimethylethanolamine (DMPE) was a gift of Dr. Hansjorg Eibl (Max Planck Institute, Gottingen, West Germany). N^6-SL-NAD$^+$ and C8-SL-NAD$^+$ and their perdeuterated isomers (N^6- and C8-DSL-NAD$^+$) were synthesized as described previously (Wenzel & Trommer, 1977; Gloggler et al., 1982) and purified by HPLC according to Gloggler et al. (1982). [*adenine*-2,8-$^3H_2$]NADH for the equilibrium dialysis experiments was prepared from [*adenine*-2,8-$^3H_2$]NAD$^+$ (2.8 Ci/mmol, New England Nuclear, Boston, MA) by enzymic reduction with ethanol and alcohol dehydrogenase (Rafter & Colowick, 1957). [*adenosine*-^{14}C-(U)]NAD$^+$ (600 mCi/mmol, New England Nuclear), used for the ultrafiltration binding studies, was diluted with unlabeled NAD$^+$ (5.6 mM, final concentration) to yield a stock solution with specific radioactivity of ~1.8 Ci/mol in 40% (v/v) ethanol, which was stored at −20 °C. The purity of the [^{14}C]NAD$^+$ was tested by reduction with alcohol dehydrogenase (10 μL of 5.6 mM [^{14}C]NAD$^+$ stock solution in 500 μL of 0.1 M glycine–0.5 M ethanol, pH 9.5) followed by chromatography [in the presence of excess unlabeled NAD$^+$ (nonreduced)] over a Fractogel TSK 650M (EM Science, Cherry Hill, NJ) DEAE column (Cl$^-$ form, 2 × 30 cm). The column was eluted with a linear gradient of 500 mL of H_2O/500 mL of 0.1 M LiCl to separate NADH from NAD$^+$; 99% of the counts were found in the NADH fraction with the remainder eluting with NAD$^+$.

Preparation of 3-Hydroxybutyrate Dehydrogenase (Apodehydrogenase) and Phospholipid Vesicles. 3-Hydroxybutyrate dehydrogenase (M_r ~31 000; Bock & Fleischer, 1975) was purified to homogeneity from beef heart mitochondria (Bock & Fleischer, 1974; McIntyre et al., 1988) and was stored frozen in a liquid nitrogen refrigerator (1–2 mg/mL in 0.4 M LiBr, 5 mM Hepes, and 5 mM dithiothreitol, pH 7.0). Phospholipid vesicles were prepared as described previously (Cortese et al., 1989).

Reconstitution of 3-Hydroxybutyrate Dehydrogenase with Phospholipid Vesicles. For equilibrium dialysis studies, the apodehydrogenase (2.5 mg) was reconstituted by incubation with phospholipid vesicles (500 μg of lipid phosphorus) for 1 h at room temperature in a final volume of 10 mL of 10 mM Hepes–KOH or Tris-HCl buffer (pH 8.0), containing 1 mM EDTA and 5 mM dithiothreitol. Under these conditions, the enzyme inserts unidirectionally into the phospholipid vesicles and becomes activated by the phosphatidylcholine when present (McIntyre et al., 1979; Sandermann et al., 1986). For the ultrafiltration and EPR binding experiments, the enzyme (0.25 mg/mL) was reconstituted with either activating (MPL or PC), partially activating (DMPE/PE/DPG, at a ratio of 5/4/1 by phosphorus), or nonactivating (PE/DPG, 9/1 by phosphorus) phospholipid vesicles [25 or 50 μg of P/mL to give lipid/protein molar ratios of 100 (for MPL, DMPE/PE/DPG, and PE/DPG) or 200 (for PC)] in 20 mM Tris-HCl, 1 mM EDTA, and 5 mM DTT, pH 8.1 (total volume of 1.76 mL). Control samples (no enzyme) were prepared in parallel, and the samples were dialyzed for 1–2 h versus 250 volumes of 10 mM Hepes, 1 mM EDTA, 0.1 M NaCl, and 1 mM DTT, pH 7.5 or 8.0 for NAD$^+$ or NADH binding studies, respectively (see below). The specific activity of the enzyme reconstituted in MPL (BDH–MPL complex) used in these studies ranged from 110 to 140 μmol min^{-1} (mg of protein)$^{-1}$ at 37 °C. For the enzyme reconstituted with PC, the specific activity was 90% of that obtained when enzyme was reconstituted with MPL (Gazzotti et al., 1975; Churchill et al., 1983).

Derivatization of 3-Hydroxybutyrate Dehydrogenase with 3,3'-Dithiobis(6-nitrobenzoate) (DTNB) or 2-Nitro-5-thiocyanobenzoate (NTCB). Derivatization of the reactive sulfhydryl of 3-hydroxybutyrate dehydrogenase (reconstituted into MPL) with DTNB to yield the CNTP-modified enzyme was carried out under argon essentially as described previously (Dubois et al., 1986) except that the reaction was carried out at room temperature (~24 °C). Direct cyanylation of the reactive sulfhydryl of the enzyme was obtained with a 50-fold molar excess of NTCB, yielding a preparation with properties similar to those of the cyanylated enzyme obtained by the two-step procedure described previously (Dubois et al., 1986) (B. Rudy and W. E. Trommer, unpublished studies).

Assays. Assays for protein and phospholipid were described previously (Dubois et al., 1986; McIntyre et al., 1988). 3-Hydroxybutyrate dehydrogenase was assayed spectrophoto-

[2] For consistency with literature terminology and our previous studies (Fritzsche et al., 1984), we refer to complexes of the enzyme with NAD(H) as binary complexes whether or not the enzyme is already reconstituted with phospholipid. Thus, the binary complex of NAD(H) with 3-hydroxybutyrate apodehydrogenase has two components (ligand and enzyme) whereas the binary complex of NAD(H) with the enzyme–phospholipid complex has three components (ligand, enzyme, and phospholipid). Likewise, ternary complexes contain enzyme (with or without phospholipid) and NAD(H) plus an additional ligand (e.g., 2-methylmalonate or monomethyl malonate or sulfite).

metrically at 340 nm, as the rate of reduction of NAD^+ with 20 mM 3-hydroxybutyrate (racemic mixture) as substrate (McIntyre et al., 1983). The reaction was started by addition of the enzyme–phospholipid complex to the assay mixture preequilibrated at 37 °C.

Coenzyme Binding Studies. (*A*) *Preparation and Quantitation of* [^{14}C]*NAD(H) for Binding Studies.* For the ultrafiltration binding experiments with NAD^+ or NAD–SO_3^-, an appropriate aliquot (usually 30–40 μL) of the [^{14}C]NAD^+ stock solution (5.6 mM, specific radioactivity ~1.8 Ci/mol; see Materials) was added directly to the sample in the Amicon cell (see below). For [^{14}C]NADH binding studies, an aliquot of [^{14}C]NAD^+ was reduced with alcohol dehydrogenase as follows: to 50–200 μL of [^{14}C]NAD^+ (5.6 mM in 40% EtOH) was added 0.5 volume of glycine buffer (0.1 M glycine, 0.5 M EtOH, pH 9.5) and 2% (v/v) alcohol dehydrogenase (20 mg/mL). To confirm that complete reduction was approached, an aliquot (2.5 μL) was diluted in 1 mL of buffer (10 mM Hepes–KOH, pH 8.0) and the absorbance spectrum measured to determine the 260 nm/340 nm ratio (the ratio for NADH is 2.35–2.4, as specified by Boehringer for crystalline NADH; samples with ratios ≤2.8 were considered acceptable). For NAD–SO_3^-, formation of the adduct in the presence of 100 mM sodium sulfite was confirmed by the characteristic absorption at 320 nm (Pfleiderer et al., 1956; Fritzsche et al., 1984). For radioactivity determination, samples (40 μL of aqueous solution) were diluted with 0.5 mL of H_2O and 5 mL of ACS (aqueous counting scintillant, Amersham Corp., Arlington Heights, IL) and counted to ≤1% 2σ (i.e., for at least 10 min or until a minimum of 40 000 counts had been measured) with either a Searle Analytic Mark III Model 6880 (Des-Plaines, IL) or a Beckman LS 7000 (Palo Alto, CA) liquid scintillation counter.

(*B*) *Ultrafiltration Method for NAD(H) Binding to 3-Hydroxybutyrate Dehydrogenase.* Ultrafiltration studies were carried out on an Amicon stirred cell (Model M-3, Amicon Corp., Bedford, MA) with a type PM-10 (10 000 M_r cutoff) membrane and a narrow-bore effluent tube (0.25-mm inner diameter) to minimize the void volume. Buffer (2 mL), with the same composition as that of the enzyme sample, was added to the cell, which was then pressurized with argon (~5 psi) so as to fill the dead volume of the cell. The buffer in the cell was then replaced with the 2000 μL of appropriate sample (either enzyme–phospholipid complex or phospholipid alone) prepared as described above and diluted with dialysis buffer to 0.2 mg of protein/mL (or equivalent phospholipid concentration). For studies of NADH binding, ethanol (0.5% v/v) was added to the buffer to ensure that the NADH was maintained in the reduced form by the alcohol dehydrogenase added in the NADH stock solution. Ternary complexes of 3-hydroxybutyrate dehydrogenase with NAD(H) were prepared by addition of either monomethyl malonate (monomethyl ester of malonic acid, MMM) (pH 7.0), 2-methylmalonic acid (MMA, pH 7.5), or sodium sulfite (100 mM each) to the buffer as indicated in the figure legends. MMA and MMM are competitive inhibitors of 3-hydroxybutyrate dehydrogenase (Tan et al., 1975; Fritzsche et al., 1984). [^{14}C]NAD(H) was then added to the cell to give a concentration of ~100 μM, which was quantitated by triplicate determination of the radioactivity in 40-μL aliquots. The cell was then pressurized (5–8 psi argon to give a drop rate of ~10 per min) while the solution was mixed with the magnetic stirrer. About half of the volume was forced through the membrane and the effluent collected in 8–12 fractions (~8 drops equivalent to ~100 μL per fraction). The volume of each fraction was determined by weighing, and the concentration of unbound [^{14}C]NADH ($[NADH]_{free}$) in each fraction was determined by counting duplicate aliquots. The $[NADH]_{free}$ at the end of the run was determined by linear regression analysis of the values for $[NADH]_{free}$ in each of the last five fractions collected. Pressure was then released from the cell, and aliquots were removed for scintillation counting (total NADH concentration) and for enzymic activity measurements. For the enzyme reconstituted in "nonactivating" phospholipid (PE/DPG), enzymic activity (~2% of that for BDH–MPL) was measured at high nucleotide (10 mM NAD^+) and substrate (200 mM 3-hydroxybutyrate) concentrations (McIntyre and Fleischer, unpublished studies). The residual volume in the cell at the end of the run was determined in two ways, which gave corresponding values: first, by difference between the initial volume minus the total volume of effluent; second, by dilution of the sample with a measured volume of buffer to a final volume of 2.0 mL (guided by a mark on the cell) and quantitation of the concentration of ligand in the cell by radioactivity counting before and after dilution so that the volume before dilution could be calculated. A second binding study was carried out after the cell had been filled to 2.0 mL with appropriate buffer. Aliquots (40 μL in triplicate) were removed to redetermine the [^{14}C]NAD(H) concentration at the start of the second run, and the cell was then repressurized to collect fractions. This procedure was repeated for up to six times. For experiments with NADH, the absorption spectrum of the nucleotide was measured again to verify the stability of the nucleotide during the experiment. Six runs took up to 3 h. Within experimental error (±5%), the total amount of the [^{14}C]NAD(H) in the fractions and in the cell at the end of the experiment corresponded to the amount of [^{14}C]NAD(H) added to the cell. In some experiments, additional NAD(H) was added after the second or third run so as to increase the NAD(H) concentration in the cell. The measured NAD(H) binding characteristics to 3-hydroxybutyrate dehydrogenase were not significantly different whether the ligand concentrations decreased stepwise or increased stepwise in the experiment. Although the removal of aliquots from the cell at the end of each run changed the total enzyme concentration as the binding study proceeded, the results were normalized by expressing binding as moles of ligand bound per mole of enzyme. In the absence of enzyme and phospholipid, the ultrafiltration membranes (either type PM10 or YM10) were permeable to [^{14}C]NAD(H) since the maximum difference between the concentration inside the cell and that in the effluent was 2% or 1% of the total ligand for [^{14}C]NAD(H) and NAD–SO_3^-, respectively (see Figure 5).

(*C*) *Equilibrium dialysis* to measure NADH binding to the enzyme reconstituted with MPL (see above) was carried out in a microdialysis apparatus (Gazzotti et al., 1975; Englund et al., 1969) with a volume of 25 μL per half-cell for 24–30 h at 3 °C. The enzyme concentration was 50 μM, and NADH was varied from 2.2 μM to 1.25 mM. A 115 μM [^{3}H]NADH stock solution, 29 Ci/mol, was diluted appropriately with 40 mM unlabeled NADH. After completion of the dialyses, 95% of the solution was removed by means of a syringe (Hamilton Co., Reno, NV) and counted to determine the NADH concentration on both sides of the dialysis membrane. The specific activity of the enzyme was assayed prior to and after the experiment and was unchanged during this time. NADH concentration was determined by enzymatic oxidation with lactate dehydrogenase and pyruvate as substrate (Bergmeyer, 1974).

(*D*) *Fluorescence Titrations.* For NADH binding by fluorescence, 3-hydroxybutyrate dehydrogenase was activated by reconstitution with phospholipid vesicles and diluted to 4–12 μM protein (was 0.12–0.37 mg/mL). Prior to the experiments, the enzyme–phospholipid complex was dialyzed at 2–4 °C against 10 mM Tris-HCl/1 mM EDTA or Hepes–KOH/1 mM EDTA buffer (pH 8.1), in the presence or absence of 100 mM NaCl, and filtered through a 5-μm filter (Millipore Corp., Bedford, MA) to decrease light scattering. The enzyme–phospholipid complex (2 mL) was titrated with NADH by adding small aliquots of 5, 10, or 37 mM NADH stock solutions to a final concentration of 400 μM, fluorescence being measured at 20 °C with a Perkin-Elmer spectrofluorometer, Model LS5. Titrations were carried out in cuvettes sealed with silicon rubber stoppers in an argon atmosphere. NADH binding was followed by resonance energy transfer from tryptophan of the protein to bound NADH (excitation at 290 nm with 10-nm slit width; emission was integrated from 420 to 480 nm). The measured fluorescence was corrected for the absorption of NADH at 290 nm (pre-inner-filter effect) according to Holbrook (1972):

$$I_{cor} = I_m \times 10^{Ad} \quad (1)$$

where I_m = measured fluorescence, A = NADH absorbance at 290 nm, and d = effective path length of 0.5 cm for 1-cm cell.

(*E*) *EPR Measurements.* X-band EPR spectra were recorded with a Bruker B-ER 420 or ESP-300 spectrometer in micro flat cells (Wenzel et al., 1976) under argon in a total volume of 50 μL at 4 or 24 °C with 0.7- or 2.0-G modulation and a microwave power of 9.5 mW. The enzyme–phospholipid complex was concentrated under argon to about 10 mg/mL in a microdialysis concentrator (Prodicon, Model 310, Bio-Molecular Dynamics, Beaverton, OR) with 10 mM Hepes–KOH/1 mM EDTA buffer (pH 8.2). Concentrations of nucleotide (N^6-SL-NAD$^+$ or C8-SL-NAD$^+$) and inhibitors (2-methylmalonate or sulfite) are given in the legends to the figures. For binding studies by EPR titration, the SL-NAD$^+$ was added in small aliquots by means of syringes equipped with platinum needles and Teflon-tipped plungers (Hamilton Co., Reno, NV) to avoid reduction of the spin-label (Wenzel et al., 1976).

Results

Ultrafiltration Procedure To Measure Binding. The ultrafiltration binding procedure has been optimized to study weak binding (K_D = 10–100 μM) of ligands to enzymes in membranes. This method has been applied to study the binding of NADH and NAD$^+$ to 3-hydroxybutyrate dehydrogenase reconstituted into phospholipid vesicles. The same enzyme sample is used to obtain binding of ligand over a 50-fold concentration range.

The ligand and membrane vesicles are placed inside the ultrafiltration cell. The vesicles are retained whereas unbound ligand, NAD(H), passes through the ultrafiltration membrane. In the first three fractions of effluent, a fast rise in $[NADH]_{free}$ is obtained as the dead volume is displaced, and then a plateau is approximated (Figure 1). The concentration of the free NADH at the end of the run is determined by linear regression analysis of the measured free NADH in the fractions representing the plateau region (Figure 1). The bound ligand concentration, calculated from the difference between the measured total concentration of ligand inside the cell at the end of the run and the $[NADH]_{free}$, is expressed as the ratio with respect to the concentration of the enzyme–phospholipid complex. In principle, bound and free ligand concentration

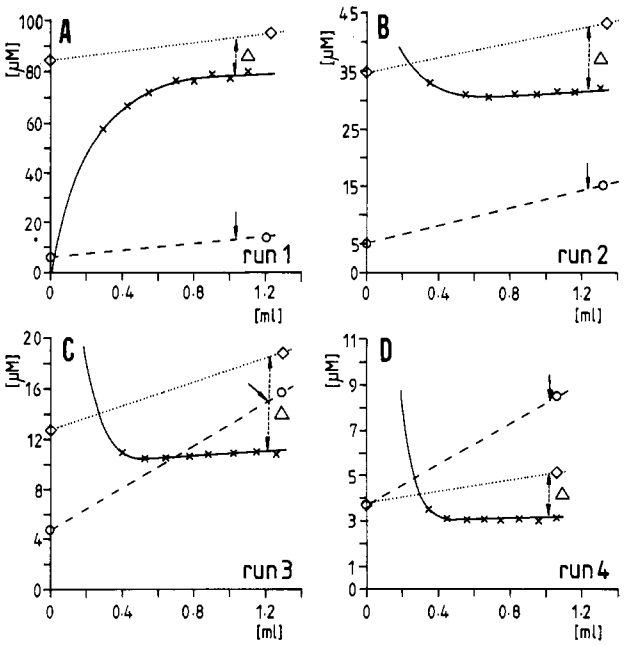

FIGURE 1: Ultrafiltration measurement of [^{14}C]NADH binding to 3-hydroxybutyrate dehydrogenase (BDH) reconstituted with MPL. The four panels show data obtained in a typical experiment in which the concentration of unbound NADH is measured in the effluent from an ultrafiltration cell (3-mL size) at sequentially four different concentrations of NADH in the cell (see Experimental Procedures). The sample initially contained 6.5 μM BDH–MPL (0.2 mg of protein/mL with a lipid/protein molar ratio of 100) and 86 μM [^{14}C]NADH in a total volume of 2.00 mL of 10 mM Hepes–KOH, 1 mM EDTA, 0.1 M NaCl, 0.5% (v/v) ethanol, and 1 mM DTT (pH 8.0). The initial and final concentrations of NADH [$[NADH]_{total}$ (◇)] and enzyme (○) in the cell were measured in each run. The concentration of unbound [^{14}C]NADH [$[NADH]_{free}$ (×)] was measured in the ultrafiltrate, which was collected in 10 fractions. In this experiment, the first two fractions from each run were not counted. The $[NADH]_{free}$ at the end of the run was calculated from linear regression analysis of the measured NADH concentration in each of the last five fractions collected. At the end of the first run (panel A), the sample was diluted to 2.0 mL and the process repeated three more times (runs 2–4, shown in panels B–D, respectively). The $[NADH]_{total}$ decreases progressively due to filtration to final concentrations of ~43, 19, and 5 μM NADH at the completion of runs 2–4, respectively. Note that the scales for the ordinate are different in each panel. The concentration of bound NADH (△), calculated from the difference between $[NADH]_{total}$ and $[NADH]_{free}$ determined at the end of each run, is then plotted as a function of [NADH] (see Figure 2).

as well as the enzyme concentration can be calculated at intermediate points between the start and end of the run although only values obtained from the end point of each run are presented. At the end of the first binding measurement, the contents of the cell are diluted, and the nucleotide concentration is measured both before and after dilution. The binding measurement is repeated three to six times sequentially at different ligand concentrations (panels B–D of Figure 1, respectively).

Controls are carried out to measure binding of ligand to phospholipid under comparable conditions. The values for ligand bound by phospholipid vesicles are then plotted on a scale comparable to that for binding to the enzyme–phospholipid complex (Figure 2). The difference between the two curves gives specific binding of ligand to the enzyme obtained for up to six different concentrations of ligand. The data for several different experiments (Figure 3) is then analyzed by nonlinear regression to provide the B_{max} and K_D values for the specific binding.

The binding of NADH and NAD$^+$ to 3-hydroxybutyrate dehydrogenase, reconstituted into phospholipid vesicles of

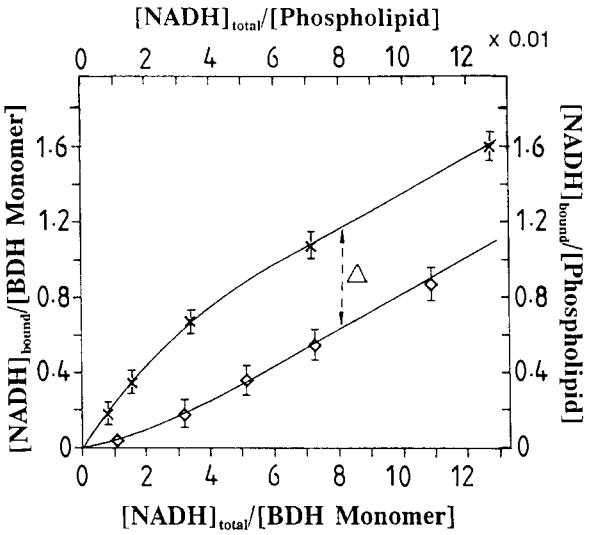

FIGURE 2: Binding of [^{14}C]NADH to 3-hydroxybutyrate dehydrogenase (BDH)–MPL complex (×) or MPL alone (◇) measured by ultrafiltration. Data from an experiment similar to that shown in Figure 1 was obtained for BDH–MPL and in the absence of BDH with a comparable amount of MPL. Bound NADH was calculated from the difference between total and free NADH and is plotted as the ratio of $[NADH]_{bound}/[BDH\ monomer]$ versus $[NADH]_{total}/[BDH\ monomer]$. The specific binding of NADH to BDH (△) was determined by subtraction of the measured binding of NADH to a comparable amount of phospholipid (◇) (100 mol of phospholipid/mol of BDH monomer). The 0.01 factor refers to both the upper and right axes. Data averaged from three separate experiments are shown in Figure 3 (panel A).

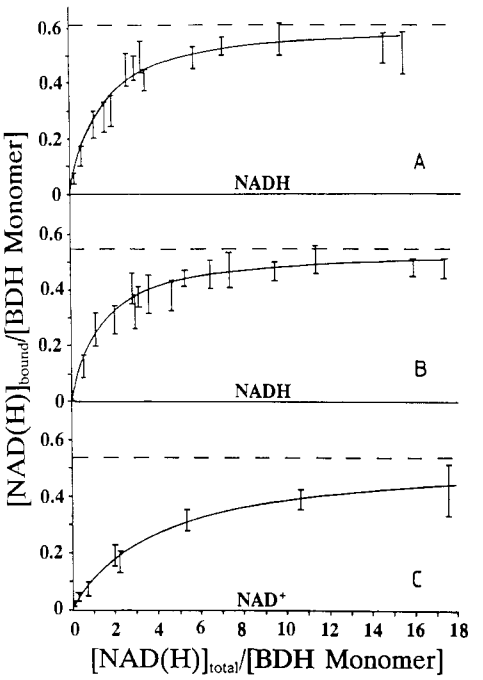

FIGURE 3: Binding of NAD(H) to 3-hydroxybutyrate dehydrogenase (BDH) reconstituted into phospholipid vesicles of different composition as measured by ultrafiltration. For each of these experiments, the BDH concentration varied from 8 to 16 μM (expressed per BDH monomer). (A) Binding of [^{14}C]NADH to the BDH–MPL complex. The specific binding of NADH to BDH was determined as described in the legend to Figure 2 [the values for (△)], in three separate experiments similar to those shown in Figures 1 and 2 and in each of which four or five dilutions were carried out. The maximal NADH concentration was 177 μM. (B) Binding of [^{14}C]NADH to the BDH–PC complex. The specific binding of NADH to BDH reconstituted in PC (200 mol of PC/mol of BDH) was determined as for the BDH–MPL complex (see panel A and Figures 1 and 2). Data shown were obtained from four separate experiments with a maximum NADH concentration of 163 μM. (C) Binding of [^{14}C]NAD^+ to the BDH–PE/DPG complex in the presence of 2-methylmalonate. The specific binding of NAD^+ to BDH reconstituted in PE/DPG (100 mol of PE/DPG per mol of BDH) was determined as the ternary complex with 2-methylmalonate (100 mM). Data shown were obtained from two separate experiments with a maximum NAD^+ concentration of 212 μM. For each of panels A–C, the data (expressed per enzyme tetramer, rather than per monomer as in the figure) were fit (solid lines) by nonlinear regression analysis (Peters & Pingoud, 1976) to determine the K_d and B_{max} for NAD(H) binding to the enzyme (see Table I). For the regression analysis, the zero NADH concentration was constrained to zero NADH bound, with both K_D and B_{max} being allowed to vary. For panels A–C, the values for B_{max} (expressed as mol/mol of BDH monomer) were 0.6 ± 0.1, 0.50 ± 0.05, and 0.50 ± 0.08 and K_d values were 15 ± 3, 12 ± 3, and 48 ± 5 μM, respectively (see Table I).

different composition, was studied under a variety of conditions. The results are summarized in Table I. The K_D for NADH binding to the enzyme, reconstituted either in MPL or in PC, is similar (15 μM and 12 μM, respectively) with maximal binding (B_{max}) of ~0.5 mol/mol of enzyme monomer for both (Figure 3, panels A and B). The same B_{max} for NADH binding is obtained in the presence of monomethyl malonate which enhances the NADH binding ($K_D \sim 2$ μM) by forming a ternary complex (see footnote 2) with the enzyme (Fritzsche et al., 1984). Binding of NAD^+ to BDH–MPL by itself is weak ($K_D \sim 190$ μM) but is much tighter as ternary complexes with either 2-methylmalonate ($K_D \sim 1$ μM) or sulfite ($K_D \sim 0.5$ μM). The B_{max} for NAD^+ binding as either of the ternary complexes is also 0.5 mol of NAD^+/mol of enzyme monomer. When the binding of equimolar amounts of NAD^+ and NADH to the enzyme–MPL complex in the presence of 2-methylmalonate was measured, we found that the stoichiometry remained a total of 0.5 mol of nucleotide/mol of enzyme monomer (Table I). Thus, there are 0.5 equiv of nucleotide binding sites (two per tetramer) which can be occupied by any combination of NAD^+ and NADH, although mutually exclusive allosteric sites cannot be precluded.

We do not detect binding of NAD(H) to 3-hydroxybutyrate dehydrogenase in the absence of PC in the phospholipid vesicles (see PE/DPG column in Table I). However, in the absence of activating phospholipid, weak binding of NAD^+ can be detected as the ternary complex with 2-methylmalonate ($K_D \sim 50$ μM, about 50-fold weaker than that in the presence of PC with the BDH–MPL complex) albeit the stoichiometry of 0.5 is the same as for the enzyme in MPL (Figure 3, panel C, and Table I). We conclude that the presence of PC converts the binding of coenzyme from weak to tight binding.

Nucleotide binding studies were also carried out with the enzyme cyanylated at the reactive sulfhydryl since we had observed previously (Dubois et al., 1986) that such modification increases the apparent K_m for substrate. Results (summarized in Table I, column CN-BDH–MPL) show that cyanylation of the enzyme markedly decreases the nucleotide binding with no NADH binding being detected and only weak binding for NAD^+ as the ternary complex with 2-methylmalonate. The data for binding of $NAD–SO_3^-$ to the cyanylated enzyme were best fit with a model with two dissociation constants as were data for $NAD–SO_3^-$ binding to the enzyme derivatized with DTNB (CNTP-BDH–MPL) (Table I, footnote *f*). For both the DTNB-treated and cyanylated enzyme in MPL, the binding of nucleotide is significantly weaker than binding of nucleotide to the native enzyme reconstituted in MPL.

For the purified apodehydrogenase, in the absence of phospholipid, we detected binding of both $NAD–SO_3^-$ and NADH (Figure 4). The binding of $NAD–SO_3^-$ to the apoenzyme is weak binding (half-maximal binding at ~75 μM $NAD–SO_3^-$). This is in the range observed for NAD^+–2-

Table I: [^{14}C]NAD(H) Binding to 3-Hydroxybutyrate Dehydrogenase As Measured by Ultrafiltration[a]

nucleotide		BDH–lipid complex[b] MPL	PC	PE/DPG	CN-BDH-MPL[c]
NADH	B_{max}	0.60 ± 0.10	0.50 ± 0.05	no binding[d]	no binding[d]
	K_D (μM)	15 ± 3	12 ± 3		
	L_{max} (μM)	177	163	116	83
NADH + monomethyl malonate	B_{max}	0.50 ± 0.05	0.50 ± 0.08	no binding[d]	no binding[d]
	K_D	2.3 ± 0.3	1.9 ± 0.2		
	L_{max}	116	82	124	88
NAD^+	B_{max}	0.11[e]	ND	ND	ND
	K_D	190 ± 55			
	L_{max}	132			
NAD^+ + 2-methylmalonate	B_{max}	0.50 ± 0.03	ND	0.50 ± 0.08	0.1[e]
	K_D	1.1 ± 0.2		48 ± 5	370 ± 90
	L_{max}	19		212	103
NAD–SO_3^-	B_{max}	0.50 ± 0.03	ND	ND	0.57 ± 0.10[f]
	K_D	0.50 ± 0.1			K_{D1} = 1.0 ± 0.3
					K_{D2} = 38 ± 10
	L_{max}	135			112
NADH + NAD^+ (equimolar) + 2-methylmalonate	B_{max}	0.5 ± 0.05[g]	ND	ND	ND
	L_{max}	201 (total)			

[a] ND refers to no data being obtained for these conditions. 3-Hydroxybutyrate dehydrogenase (BDH) was reconstituted with phospholipid vesicles (either MPL, PC, or PE/DPG; see Experimental Procedures) and NAD(H) binding measured by ultrafiltration (see Figures 1–3). The specific binding of NAD(H) to BDH was determined after subtraction of the nonspecific binding to the phospholipid, and the data were analyzed by nonlinear regression analysis (Peters & Pingoud, 1976) to obtain the maximal binding (B_{max}, expressed as moles of NAD(H) per mole of BDH monomer) and dissociation constant (K_D) for data obtained up to the maximal ligand concentrations tested (L_{max}). The error values given are computed on the basis of the statistical error in the fit to the binding curve as determined by the regression analysis program. [b] The binding of NAD–SO_3^- or NADH to the apodehydrogenase (devoid of lipid) is shown in Figure 4. [c] CN-BDH-MPL refers to BDH, reconstituted in MPL and cyanylated at the reactive sulfhydryl with 2-nitro-5-thiocyanobenzoate (NTCP) (see Experimental Procedures). [d] For experimental conditions listed as no binding, the amount of binding to the BDH–phospholipid complex was comparable to the nonspecific binding to the phospholipid alone. Such results do not preclude weak binding of nucleotide to BDH that is masked by the nonspecific binding to phospholipid. [e] For NAD^+ binding to BDH-MPL or to the cyanylated enzyme (CN-BDH-MPL in the presence of 2-methylmalonate), the "B_{max} values" given in the table are the measured ligand bound at L_{max} and not valid B_{max} values. For these data, the error in the analysis of the binding curves is appreciable since the maximal ligand concentration tested (L_{max}) was less than the estimated K_D. For these samples, the K_D values given were estimated by nonlinear regression analysis of the binding data with B_{max} constrained to a value of 0.5 mol/mol of BDH monomer, on the basis of the value obtained under conditions of tight binding. [f] The data for NAD–SO_3^- binding to cyanylated BDH in MPL (CN-BDH-MPL) were more consistent with a model having two dissociation constants [see Peters and Pingoud (1976) and eq 2 in the text] rather than a single K_D (cf. Table III). The B_{max} value refers to the total maximal binding for K_{D1} and K_{D2} combined. The nonlinear regression analysis program (Peters & Pingoud, 1976) assumes that the number of both types of site is equal. Similar results were obtained for binding of NAD–SO_3^- to the enzyme (in MPL) derivatized with DTNB (i.e., CNTP-BDH-MPL) [B_{max} = 0.55 ± 0.1 (total binding) with K_{D1} = 1.5 ± 0.3 μM and K_{D2} = 55 ± 30 μM]. [g] For measurements in the presence of equimolar concentrations of NADH and NAD^+, K_D values are not given since the experiments were designed to determine B_{max} by measuring binding at high ligand concentrations.

methylmalonate binding to the enzyme–PE/DPG complex (devoid of PC) (see Figure 3, panel C, and Table I). However, the binding to the apodehydrogenase is more complicated than the binding to the enzyme–phospholipid complexes and does not fit a simple binding curve, although the maximum measured binding (~0.5 mol/mol of enzyme monomer) is similar in the absence versus presence of phospholipid. A small amount of NADH binding to the apodehydrogenase was also detected (~0.2 mol of NADH bound/monomer at 120 μM NADH) (Figure 4) and was not significantly enhanced by the addition of 0.1 M monomethylmalonate (not shown). The Hummel and Dryer (1962) gel-exclusion column chromatography method was used to study NADH binding to the apodehydrogenase; at 50 μM NADH, we found 0.1–0.2 mol of [^{3}H]NADH bound per mole of apodehydrogenase (data not shown), comparable to that obtained by the ultrafiltration procedure (see Figure 4). The difference between the NADH binding to the apodehydrogenase (weak binding) and the enzyme in nonactivating phospholipid (no binding detected) may be due, at least in part, to the large amount of NADH binding to phospholipid alone (~10% of NADH added, see Figure 5, panel C), which could obscure detection of very weak binding to the enzyme. For both NADH and NAD–SO_3^- binding to the apodehydrogenase, the limited range of ultrafiltration binding data that could be obtained, together with the relatively large error in the values for bound ligand, precluded a detailed analysis of the complex binding curves. Studies to further characterize the details of the binding of nucleotide to the apodehydrogenase by utilizing higher protein concentrations were considered impractical due to concentration-dependent self-association of the enzyme with increasing protein concentration (McIntyre et al., 1978). Indeed, the unusual binding of nucleotide to the apohydrogenase may, in part, be complicated by changes in protein self-association during the course of the experiment. The binding of nucleotide to the apohydrogenase is at least 1 order of magnitude weaker than nucleotide binding to the active enzyme–phospholipid complex in the presence of PC (see Table I).

We found that the binding of NAD(H) to phospholipid vesicles alone (see Figure 2) could be appreciable (up to 10% of added NADH) and varied somewhat depending on the conditions (Figure 5). The binding of NAD(H) to lipid is nearly proportional to the NADH/lipid ratio and is equivalent to partitioning of NADH into the lipid bilayer. The slope of the binding is a quantitative index of the partitioning. Binding of NADH to MPL vesicles was about 2-fold higher than that for NAD^+ (panel A versus panel D of Figure 5). The binding of both NAD^+ and NADH to phospholipid was diminished about 2-fold in the presence of added salt (100 mM monomethyl malonate or 2-methylmalonate) but remained significantly greater than nonspecific binding to the filter in the absence of phospholipid and enzyme (~2% and 1% of the total NADH and NAD^+, repectively, in a concentration range up to 200 μM nucleotide; see Figure 5A,D). The binding of NAD–SO_3^- to phospholipid (Figure 5D) was comparable to that for NADH in the presence of added salt (Figure 5A). The binding of NAD(H) did not vary appreciably with the composition of the phospholipid vesicles [compare panels A

Table II: Binding Stoichiometry of DSL-NAD$^+$ to 3-Hydroxybutyrate Dehydrogenase (BDH)–Phospholipid Complexes in the Presence of Inhibitors As Studied by EPR Spectroscopy[a]

NAD$^+$ analogue	inhibitor	phospholipid vesicles	enzyme (monomer) concn (μM)	total concn of NAD$^+$ analogue (μM)	concn of bound NAD$^+$ analogue (μM)	stoichiometry, NAD$^+$ bound/BDH monomer[b]	resolved av hyperfine splitting (G)
N^6-DSL-NAD$^+$	2-methylmalonate	MPL	26	32	12	0.46 ± 0.05	67.0 ± 0.5
N^6-DSL-NAD$^+$	sulfite	MPL	26	32	14	0.54 ± 0.05	67.0; 70.0
C8-DSL-NAD$^+$	2-methylmalonate	MPL	26	58	13	0.50 ± 0.05	69.0 ± 0.5
C8-DSL-NAD$^+$	sulfite	MPL	26	58	13	0.50 ± 0.05	56 ± 2; 68 ± 0.5
N^6-DSL-NAD$^+$	2-methylmalonate	DMPE/PE/DPG	53	44	7	0.13 ± 0.03	65.5 ± 0.5

[a] Data for N^6-DSL-NAD$^+$ binding are from Figures 6 and 7. Data for C8-DSL-NAD$^+$ binding (not shown) were obtained as for N^6-DSL-NAD$^+$ binding (see Figure 6). [b] The stoichiometry values given are measured directly from the EPR spectra. For the BDH–MPL complexes (100 mol of phospholipid/mol of BDH monomer), these values approximate B_{max} since the ligand concentrations are significantly greater than the K_D values for NAD–SO_3^- or NAD$^+$ in the presence of 2-methylmalonate (0.5 and 1.1 μM, respectively; see Table I) and the spin-label analogues of NAD(H) used in these studies bind to BDH with K_D's similar to those obtained with NAD(H) (Fritzsche et al., 1984). For BDH–DMPE/PE/DPG (100 mol of phospholipid/mol of BDH monomer; Figure 7), an apparent K_D of 103 μM is obtained for N^6-DSL-NAD$^+$ binding, assuming a B_{max} of 0.5 mol/mol of BDH monomer. K_D was calculated from $K_D = [(n - B)F]/B$, where K_D is the dissociation constant, n is the number of sites per enzyme monomer, B is bound ligand (expressed as mol/mol of BDH monomer), and F is the concentration of free ligand.

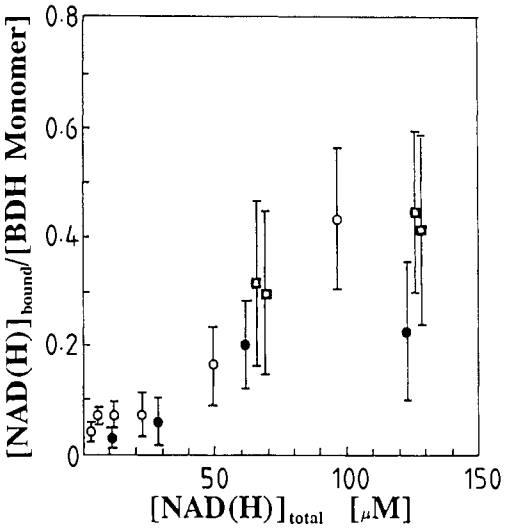

FIGURE 4: Binding of NADH or NAD–SO_3^- to 3-hydroxybutyrate apodehydrogenase (apo-BDH) measured by ultrafiltration. Binding studies of [^{14}C]NADH (●) with apo-BDH were carried out as described in Figure 1 except in the absence of phospholipid. For binding of NAD–SO_3^-, experiments were carried out with somewhat different enzyme concentrations (○, □). The purified apo-BDH was diluted to 0.25 mg/mL in 10 mM Hepes, 1 mM EDTA, 0.1 M NaCl, and 1 mM DTT (pH 7.5) and dialyzed (30 min) versus the same buffer (500 volumes), and then sodium sulfite (100 mM, final concentration) and [^{14}C]NAD$^+$ were added. For one experiment (○), the enzyme concentration varied from 10.9 to 5.8 μM, and the amount of NAD–SO_3^- bound to apo-BDH ranged between 6% and 9% of the total ligand in the cell which varied from 100 to 3 μM total NAD–SO_3^- for the first to sixth runs, respectively. Data from two separate experiments (□) with 6.5 and 4.5 μM apo-BDH and ~130 or ~65 μM NAD–SO_3^- are also shown. Data obtained up to 250 μM NAD–SO_3^- (not shown) indicated that the binding plateau value was at ~0.5 mol/mol of apo-BDH, albeit the errors were proportionately larger at the higher ligand concentrations. Data are plotted after subtraction of the values for nonspecific retention of ligand in the absence of enzyme (2% and 1% of the total ligand concentration for NADH and NAD–SO_3^-, respectively), which, for these studies, were measured, with the same filter and solutions, immediately prior to the samples containing apo-BDH.

and D (MPL) with panel B (PC) and with panel C (PE/DPG) of Figure 5]. It should be noted that, at the highest concentrations of NADH used in these studies (up to 200 μM), greater than 1 NADH per 100 phospholipid molecules binds to the phospholipid bilayer.

Nucleotide Binding As Studied by EPR. We have previously reported that both N^6- and C8-DSL-NAD$^+$ are active coenzymes for 3-hydroxybutyrate dehydrogenase, exhibiting similar K_m's but with 30% or 15% V_{max}, respectively, compared with NAD$^+$ itself (Fritzsche et al., 1984). For these SL-NAD analogues bound to the enzyme, highly immobilized EPR spectra are observed, and hence, the decrease in the signal amplitude of freely tumbling DSL-NAD$^+$ in the presence of enzyme versus its absence can be used as a measure of binding.

EPR spectra were recorded for ternary complexes composed of enzyme, spin-labeled coenzyme, and an inhibitor (Figure 6). As summarized in Table II, the maximum binding stoichiometry (B_{max}) of DSL-NAD$^+$ was found to be 0.5 per monomer (two per tetramer) in all enzyme–MPL ternary complexes studied, thereby confirming the stoichiometry obtained by ultrafiltration.

The EPR results also confirmed that cyanylation of the enzyme with NTCB resulted in weaker nucleotide binding (see Table I) since the EPR signal referable to bound nucleotide was almost completely abolished after treatment of the enzyme with NTCB (not shown, see legend of Figure 6A). Likewise, the EPR signal referable to bound SL-NAD–SO_3^- was diminished more than 10-fold by the addition of excess NADH (not shown, see legend of Figure 6B). This demonstrates that the NAD–SO_3^- and NADH binding sites are mutually exclusive, consistent with the ultrafiltration binding study which showed that NAD$^+$ and NADH together give a total of 0.5 equiv/enzyme monomer.

An EPR spectrum of N^6-DSL-NAD$^+$ in the presence of 3-hydroxybutyrate dehydrogenase reconstituted with lipids which partially activate the enzyme (~6% of the activity at 25 °C obtained with MPL activation), i.e., a mixture of DMPE/PE/DPG (5:4:1) (McIntyre & Fleischer, 1986), is shown in Figure 7. The spectrum shows some bound coenzyme as revealed by the highly immobilized component for enzyme in the absence of PC but partially activated by the DMPE in the mixture. Thus, even for the partially reactivated enzyme, binding of SL-NAD can be detected by EPR albeit with weaker binding (K_D ~ 100 μM) and lower measured binding stoichiometry than that in the presence of PC under comparable conditions (Table II). It may be noted that the resolved averaged hyperfine splitting of the EPR spectrum for N^6-DSL-NAD$^+$ bound to the partially active enzyme (in DMPE/PE/DPG) is significantly smaller (65.5 ± 0.5 G) than that for the same nucleotide bound to the fully active enzyme–MPL complex (67.0 ± 0.5 G). Thus, for the partially active enzyme, the binding of SL-NAD$^+$ is weaker (higher K_D), and the spin-label is less immobilized. For the enzyme reconstituted with PE/DPG, we were unable to detect for this inactive form of enzyme a powder-like EPR component referable to SL-NAD bound to the enzyme; either the binding was too weak (see Table I) to detect the bound component,

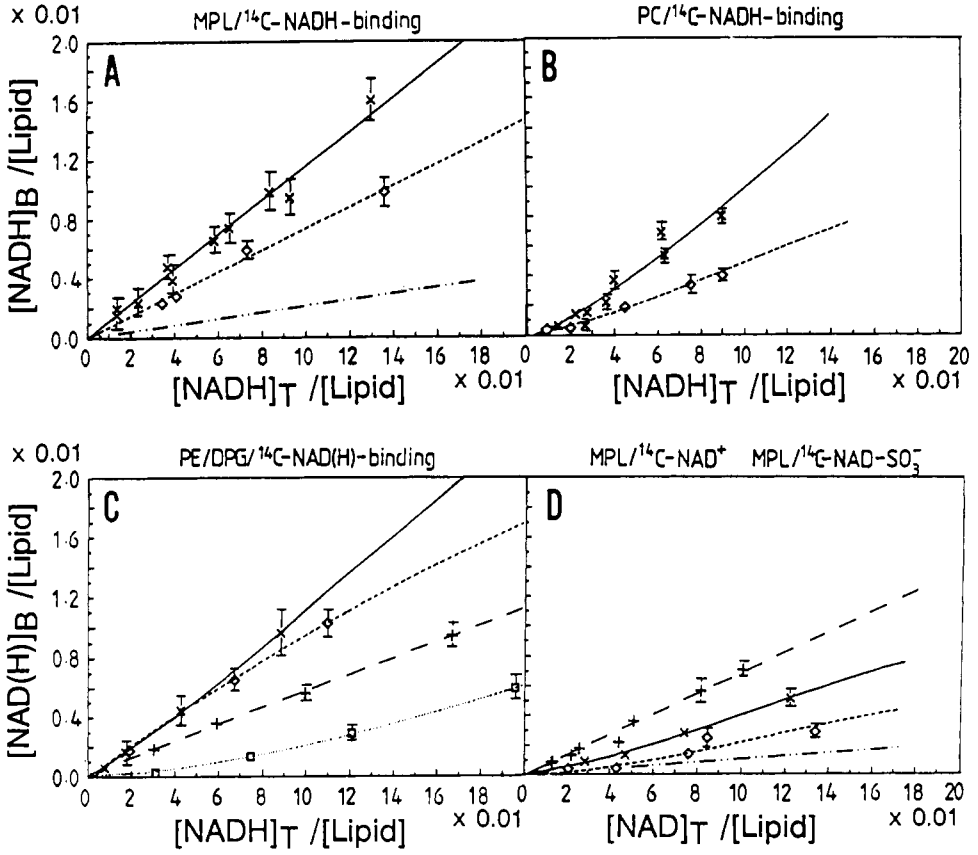

FIGURE 5: Effect of lipid composition and added salt (sodium monomethyl malonate or sodium 2-methylmalonate) on the binding of NADH or NAD^+ to phospholipid vesicles. Binding of [^{14}C]NAD(H) to phospholipid vesicles of either MPL (panels A and D) or PC (panel B) or PE/DPG (panel C) was measured by ultrafiltration (see Figures 1 and 2). The initial concentration of phospholipid was comparable to that used in the binding studies with enzyme–lipid complexes (i.e., 650 μM MPL, 650 μM PE/DPG, or 1.3 mM PC). NADH binding to phospholipid is shown in panels A (MPL), B (PC), and C (PE/DPG) either in the absence (×) or in the presence of added salt (0.1 M sodium monomethyl malonate, MMM) (◇). NAD^+ binding to phospholipid, in both the absence and presence of 0.1 M 2-methylmalonate, is shown in panels C (PE/DPG) [(+) and (□), respectively] and D (MPL) [(×) and (◇), respectively]. The binding of NAD–SO_3^- to MPL is shown in panel D (+). Nonspecific binding of nucleotide to the filter (in the absence of phospholipid and enzyme) is shown (–··–) for NADH (panel A) and NAD^+ (panel D) and is equivalent to 2% or 1% of the total nucleotide concentration, respectively. Note that the maximal ranges of the ordinates and abscissas are to 0.02 and 0.2, respectively, since they are multiplied by 0.01.

or the enzyme as the PE/DPG complex was unstable during the dialysis and concentration of the sample prior to the EPR measurements.

A titration of 3-hydroxybutyrate dehydrogenase–MPL complex with C8-DSL-NAD^+ in the presence of 2-methylmalonate (Figure 8A) reflects binding of nucleotide to the enzyme as well as some binding to the phospholipid. The binding of SL-NAD to the enzyme–MPL complex was measured by the decrease in the amplitude of the high-field EPR line of free signal compared with the amplitude in buffer (Griffith & Waggoner, 1969). The EPR spectrum of DSL-NAD^+ added to lipid bilayers in the absence of enzyme is broadened somewhat, resulting in a reduction in the EPR spectral amplitude (~2% for C8-SL-NAD^+ in MPL) (not shown). With phospholipid alone, a powder-type spectrum, as is observed for DSL-NAD^+ bound to the enzyme (Figures 6 and 7), was not detected. For the enzyme–MPL complex, the binding to ~150 μM SL-NAD^+ accounts for about 0.6 equiv bound per enzyme monomer, which includes some binding to the phospholipid (Figure 8A). These data confirm the stoichiometry of two binding sites per enzyme tetramer. The increase in the bound fraction at higher excess of C8-DSL-NAD^+ is due to binding to the phospholipid and appears to be similar to that obtained by equilibrium dialysis (see Figure 8B).

In previous studies, we observed that EPR spectra of DSL-NAD(H) bound to 3-hydroxybutyrate dehydrogenase (either NADH alone or as NAD–sulfite or NADH–monomethyl malonate ternary complexes) exhibited two immobilized components indicative of different environments for the coenzyme bound to the enzyme (Fritzsche et al., 1984; Philipp et al., 1984). To evaluate the stoichiometry for the two kinds of binding observed by EPR, we have now quantitated the binding to the enzyme of DSL-NAD^+ (added in excess) in the presence of either sulfite or 2-methylmalonate, which enhance the binding of coenzyme. In the spectra of the DSL-NAD^+–2-methylmalonate complexes, which has not previously been studied, only a single broadened component is observed even at the high resolution obtained with the deuterated spin-labels (Figure 6A and Table II). The two broadened components are again observed for the DSL-NAD–sulfite complex (Figure 6B and Table II). Sulfite adds to position 4 of the nicotinamide ring with formation of a dihydropyridine-like structure as revealed by its characteristic absorption at 320 nm (Pfleiderer et al., 1956). Hence, two components are observed in EPR spectra of SL-NADH (Fritzsche et al., 1984) or NADH-like (i.e., NAD–SO_3^-) complexes (Fritzsche et al., 1984; Philipp et al., 1984; this study) but not with SL-NAD^+ (this study). The two components do not appear to be an artifact due to the spin-label since the stoichiometry for binding is a total of 0.5 per monomer (two per tetramer) (see Table II) as in the ultrafiltration binding studies.

The existence of two distinct binding sites for NADH, or of two distinct conformations in which NADH can bind to the enzyme, is corroborated by fluorescence resonance energy

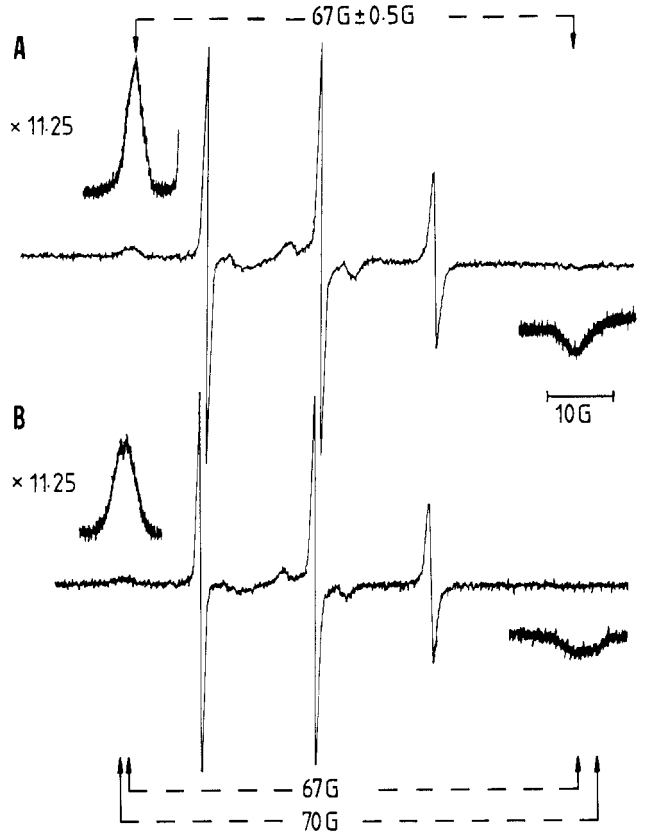

FIGURE 6: X-band EPR spectra of N^6-DSL-NAD$^+$ complexes with 3-hydroxybutyrate dehydrogenase (BDH) reconstituted with MPL. The samples contained 32 μM N^6-DSL-NAD$^+$ and 26 μM BDH–MPL (100 mol of phospholipid/mol of BDH monomer) in 10 mM Hepes–1 mM EDTA buffer, pH 8.0, at 24 °C. For both (A) and (B), the spectra were recorded at an amplifier gain of 2.0×10^5 with the low- and high-field regions also shown at a gain of 2.25×10^6 to depict the line shape of the broadened component referable to coenzyme bound to the enzyme. In spectrum A, recorded in the presence of 100 mM 2-methylmalonate, the broadened spectral component (resolved averaged hyperfine splitting of 67 ± 0.5 G) accounts for 12 μM enzyme-bound N^6-DSL-NAD$^+$ on the basis of the amplitude reduction of the free signal compared with the amplitude of the EPR spectrum in the absence of enzyme. The addition of 3 mM NTCB to this sample resulted in loss of detectable bound SL-NAD$^+$ and commensurate increase in free signal (not shown) indicative of weaker binding of the SL-NAD$^+$ resulting from cyanylation of the enzyme with this reagent. In spectrum B, recorded in the presence of 100 mM sulfite, the broadened spectral component [which consists of two spectrally distinct components with 67- and 70-G hyperfine splittings; see Fritzsche et al. (1984)] accounts for 14 μM enzyme-bound N^6-DSL-NAD$^+$. The addition of 7 mM NADH to this sample resulted in ~20-fold reduction in the amplitude of the broadened spectral component and commensurate increase in the amplitude of the signal referable to free DSL-NAD–SO_3^- (not shown).

transfer studies of NADH binding (see below) in the absence of added salt, which can best be described by two interacting sites with different K_D values.

Equilibrium Dialysis. Binding of NADH by 3-hydroxybutyrate dehydrogenase–MPL complex was measured by equilibrium dialysis using [*adenine*-2,8-$^3H_2$]NADH in the presence of 100 mM sodium chloride (Figure 8B). The data show specific binding of NADH to the enzyme ($K_D \sim 25 \pm 5$ μM and $B_{max} = 0.5$ mol/mol of enzyme monomer) together with binding to the phospholipid per se of about 8% of the added [^{3}H]NADH. Similar results were obtained from a number of equilibrium dialysis experiments in which the salt concentration was varied in the range from 50 to 120 mM sodium chloride (not shown). The results from equilibrium dialysis are consistent with those obtained by the ultrafiltration method (Figures 1–3) and confirm that, in the latter studies,

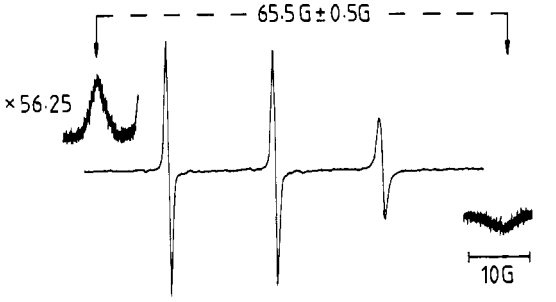

FIGURE 7: X-band EPR spectrum of N^6-DSL-NAD$^+$ as a ternary complex with 3-hydroxybutyrate dehydrogenase (BDH) reconstituted with a weakly activating mixture of phospholipids (DMPE/PE/DPG, 5/4/1). The sample contained 44 μM N^6-DSL-NAD$^+$ and 53 μM BDH–DMPE/PE/DPG (100 mol of phospholipid/mol of BDH monomer) with 100 mM 2-methylmalonate in 10 mM Hepes–1 mM EDTA buffer, pH 8.0, at 24 °C. The specific activity of the enzyme reconstituted with these phospholipid vesicles was ~3 μmol min^{-1} (mg of BDH)$^{-1}$ at 25 °C. The spectrum was recorded at a gain of 4.0×10^4, and the broadened spectral component (also shown at a gain of 2.25×10^6, as in Figure 6) accounts for 7 μM enzyme-bound N^6-DSL-NAD$^+$. These data give a calculated K_D of 103 μM for N^6-DSL-NAD$^+$ binding to this BDH–lipid complex, assuming that the number of binding sites per enzyme monomer is 0.5 (based on results with other BDH–lipid complexes, see Table I).

Table III: Binding of NADH to the 3-Hydroxybutyrate Dehydrogenase–Mitochondrial Phospholipid Complex As Quantitated by Resonance Energy Transfer from Tryptophan to Bound NADH[a]

binding model	NaCl (mM)	dissociation constant (μM) K_{D1}	K_{D2}
independent	0	12 ± 4	32 ± 10
negative cooperativity	0	10 ± 3	36 ± 12
independent	100	5 ± 2	5 ± 2
negative cooperativity	100	4 ± 2	6 ± 2

[a] Data were obtained from experiments similar to those shown in Figure 9. Each binding curve was analyzed independently by nonlinear regression analysis, allowing for two dissociation constants (see text for details). Data are given as the mean ±SEM of the K_D values obtained from up to five separate titrations.

conditions approaching equilibrium were obtained.

Resonance Energy Transfer. Resonance energy transfer from tryptophan of 3-hydroxybutyrate dehydrogenase to bound NADH provides a quantitative measure of NADH binding (Gazzotti et al., 1974; Fritzsche et al., 1984; McIntyre et al., 1984). We studied NADH binding to the enzyme–MPL complex both in the presence and in the absence of 100 mM NaCl (Figure 9). Binding of NADH is enhanced in the presence of the added salt. The binding curves were analyzed by a model with two a priori identical but interacting binding sites (negative cooperativity) for NADH per enzyme tetramer according to [see Peters and Pingoud (1976)]

$$K_1 = \frac{[LE] + [EL]}{[E][L]} \qquad K_2 = \frac{[LEL]}{([LE] + [EL])[L]} \quad (2)$$

where $[E]_{total} = [E] + [EL] + [LE] + [LEL]$ and $[L]_{total} = [L] + [EL] + [LE] + 2[LEL]$, with [E] and [L] representing the concentrations of free enzyme or ligand, respectively, and [EL], [LE] and [LEL] representing the concentrations of the various enzyme–ligand complexes. K_1 and K_2 are the macroscopic binding constants. In addition, a model with two independent sites was evaluated. The data are consistent with either model (negative cooperative or independent sites; see Table III). However, two distinct dissociation constants could be resolved in the absence of added NaCl. In the presence of 100 mM NaCl, the resonance energy tranfer data are

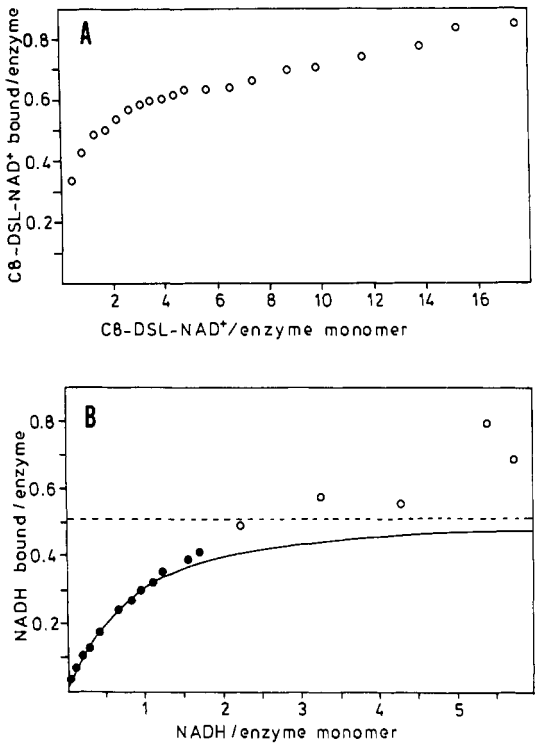

FIGURE 8: Binding of nucleotide to 3-hydroxybutyrate dehydrogenase–MPL complex as studied by EPR spectroscopy (A) or equilibrium dialysis (B). (Panel A) Binding of C8-DSL-NAD$^+$ to the enzyme–MPL complex in the presence of 200 mM 2-methylmalonate as studied by EPR spectroscopy. The initial enzyme concentration was 32 μM (based on monomer) in 50 μL and decreased to 15.8 μM at the end of the titration (total volume 101 μL) as the C8-SL-NAD$^+$ was varied from 13.9 to 275 μM. Binding to the phospholipid (see Figure 5) is observed at high C8-DSL-NAD$^+$ concentrations (DSL-NAD$^+$/enzyme ratio > 5, equivalent to DSL-NAD$^+$ > 150 μM). (Panel B) Equilibrium dialysis data for [^{3}H]-NADH binding to the enzyme–MPL complex (50 μM enzyme monomer) in 10 mM Hepes–KOH, 1 mM EDTA, 5 mM dithiothreitol, and 100 mM NaCl. Dialysis time was 24–30 h at 3 °C. The binding data were analyzed with the iterative nonlinear least-squares program LIGAND (Munson & Rodbard, 1980), which resolved two binding components: specific binding (K_D = 25 ± 5 μM, depicted by the solid curve) and nonspecific binding (8 ± 1% of the total ligand added). At higher NADH concentrations (100–300 μM), and up to 500 μM NADH (not shown), the binding is approximately proportional to the NADH concentration (open circles), reflecting binding to the phospholipid vesicles.

consistent with a single dissociation constant, since K_{D1} and K_{D2} are similar.

Discussion

The ultrafiltration method has been improved and extended to quantitate weaker binding of ligands to proteins. The special features of the method are that it is rapid and can be used to measure weaker binding in the submillimolar range. Direct measurement of the binding stoichiometry is achieved with ligand concentrations more than 10-fold in excess of the K_D. We have applied this method to investigate the binding of coenzyme, NAD(H), to 3-hydroxybutyrate dehydrogenase, a lipid-requiring membrane enzyme, and obtained the following results: (i) The stoichiometry for NADH and NAD binding is 0.5 mol/mol of enzyme monomer (2 mol/mol of tetramer). (ii) The dissociation constant for NADH binding is essentially the same for the enzyme reconstituted into MPL, the mixture of phospholipids in mitochondria (K_D = 15 ± 3 μM), or into dioleoyl-PC (K_D = 12 ± 3 μM). These values are more similar than the estimate of binding of NADH measured by kinetic analysis, which gave nucleotide K_i values

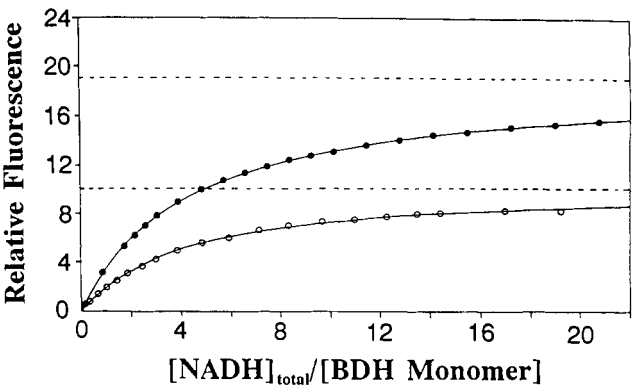

FIGURE 9: Binding of NADH to 3-hydroxybutyrate dehydrogenase (BDH) reconstituted with MPL as studied by fluorescence resonance energy transfer. Resonance energy transfer (arbitrary units) from tryptophan (λ_{ex} = 290 mm) to bound NADH (λ_{em} = 450 nm) in the absence (●) or presence of 100 mM NaCl (○) was measured as the [NADH] was varied either up to 109 μM with BDH–MPL (5.3 μM enzyme monomer) in the absence of added salt or up to 30 μM with 1.4 μM BDH–MPL in the presence of 100 mM NaCl. The solid lines were calculated (Peters & Pingoud, 1976) for negatively cooperative binding to two sites per tetramer with K_{D1} = 10 μM and K_{D2} = 44 μM in the absence of salt or K_{D1} = 4.2 μM and K_{D2} = 4.3 μM with added salt present and limiting values as indicated by the dashed lines. For a tetrameric model with independent binding, the dissociation constants (K_{D1} and K_{D2}) were 17 and 28 μM and 4.4 and 4.4 μM in the absence and presence of salt, respectively. For a dimer model, K_{D1} and K_{D2} were, for negatively cooperative binding, 9.1 and 45 μM and 3.6 and 4.3 μM or, for independent binding, 13 and 32 μM and 4.1 and 4.1 μM in the absence or presence of salt, respectively. Average values for K_D obtained from a number of independent experiments are given in Table III.

2–3-fold lower for the enzyme reconstituted in PC compared to the enzyme in MPL (Nielsen et al., 1973; Churchill et al., 1983). (iii) The binding of NAD$^+$ is more than 1 order of magnitude weaker than NADH binding (~190 μM versus 15 μM) (consistent with the ~15-fold difference in binding of NAD$^+$ and NADH obtained from kinetic analysis; Nielsen et al., 1973). (iv) The binding of NAD$^+$ to BDH–MPL is enhanced by forming a ternary complex with 2-methylmalonate (apparent K_D = 1.1 ± 0.2 μM) or by addition of sulfite to form the NAD–SO_3^- adduct (K_D = 0.5 ± 0.1). (v) In the absence of activating lecithin (i.e., in PE/DPG), the binding of nucleotide to the enzyme was detected only for the NAD$^+$ plus 2-methylmalonate ternary complex for which the apparent K_D for NAD$^+$ was ~50-fold higher (weaker binding) than that in the presence of PC. This study extends our earlier equilibrium dialysis studies which showed that phosphatidylcholine confers to this lipid-requiring enzyme the ability to bind coenzyme with a K_D of ~15 μM for NADH and a binding stoichiometry of 0.5 mol of NADH/mol of enzyme monomer (Gazzotti et al., 1974). In addition, we confirm by the ultrafiltration binding method our previous fluorescence and EPR studies, which showed that monomethyl malonate enhances the binding of NADH by forming a ternary complex (Fritzsche et al., 1984). We now find that the binding stoichiometry for NADH is the same (0.5 mol/mol) in the presence and absence of competitive inhibitor. In the studies reported here, NAD$^+$ binding has been measured directly. The binding of NAD$^+$ is weak but enhanced by formation of a ternary complex with 2-methylmalonate, a competitive inhibitor (Tan et al., 1975).

In previous studies, we had shown that the binding of NAD$^+$ and NADH to 3-hydroxybutyrate dehydrogenase is competitive as indicated by kinetic analysis of enzyme activity data (Nielsen et al., 1973) as well as by fluorescence studies which showed that bound NADH was displaced by addition of

NAD^+ (Gazzotti et al., 1974). We have now demonstrated by direct binding, using the ultrafiltration method as well as EPR, that the total binding stoichiometry, obtained with an equimolar mixture of NAD^+ and NADH in the presence of 2-methylmalonate to enhance NAD^+ binding, is 0.5 mol/mol of enzyme monomer (Table I). Likewise, bound SL-NAD–SO_3^-, measured by EPR, was displaced by the addition of excess NADH (legend of Figure 6). Thus, we detect only a total of two nucleotide binding sites per enzyme tetramer.

An important new observation is that nucleotide binding to the enzyme can be detected in the absence of activating phospholipid. Weak binding of coenzyme ($K_D \sim 50$ μM for NAD^+ as the ternary complex with 2-methylmalonate) was observed for the enzyme reconstituted into PE/DPG vesicles, i.e., in the absence of activating phospholipid. In the presence of PC, NAD^+ binding as the ternary complex with 2-methylmalonate is about 50-fold tighter (apparent $K_D = 1.1 \pm 0.2$ μM) than that to the enzyme reconstituted in PE/DPG. Likewise, we were able to demonstrate, using the ultrafiltration method, that the apoenzyme, devoid of phospholipid, binds NADH or NAD–SO_3^- (see Figure 4), albeit very weakly (half-maximal binding at ~75 μM NAD–SO_3^-) as compared with the active enzyme reconstituted into MPL vesicles which contain PC ($K_D = 0.5 \pm 0.1$ μM for NAD–SO_3^-). Thus, we conclude that the nucleotide binding site exists in the inactive enzyme. Binding of nucleotide by the apodehydrogenase was suggested by some of our previous chemical modification studies which showed that high concentrations of NAD(H) reduced the rate of derivatization by cyclohexanedione for both the apodehydrogenase and the reconstituted enzyme (Fleer & Fleischer, 1983). Studies of the quenching of intrinsic fluorescence of the rat liver apodehydrogenase by NADH also suggested binding of the nucleotide but in a concentration range similar to that observed with the enzyme–phospholipid complex (El Kebbaj et al., 1986). However, the direct binding studies reported here demonstrate that the binding of nucleotide to the apodehydrogenase is much weaker than binding of nucleotide to the active enzyme. The activation by PC enhances the nucleotide binding, reducing the K_D by at least 1 order of magnitude. In this context, PC can be considered to be an allosteric activator of 3-hydroxybutyrate dehydrogenase since PC modulates the nucleotide binding. A recent kinetic analysis of the activation of 3-hydroxybutyrate dehydrogenase by phospholipid suggested that lecithin serves as an essential allosteric activator of the enzyme (Cortese et al., 1989). The results of the studies reported here provide direct experimental evidence for allosteric modulation of nucleotide binding by lecithin.

The finding that NAD(H) binds to phospholipid bilayers is new as far as we are aware and may have physiological relevance. Control experiments, in the absence of protein, revealed significant binding of NAD(H) to phospholipid vesicles (up to 10% of added NADH and about half this amount for NAD^+) (Figure 5). At about 200 μM NADH, the concentration of NADH in the lipid is approximately 1 NADH per 100 phospholipid molecules. The binding of NAD(H) was similar for three vesicle preparations of different phospholipid composition, i.e., negatively charged (MPL and PE/DPG) or zwitterionic (PC) phospholipid vesicles, and was reduced about 2-fold in the presence of added salt. The binding to phospholipid was also detected at higher NADH concentration (100–500 μM) by equilibrium dialysis as well as by EPR (Figure 8). It is likely that NAD^+ and NADH bind to the mitochondrial membrane. If such binding is similar to that which we find in pure phospholipid vesicles (about 2-fold higher for NADH versus NAD^+), the ratio of NADH/NAD^+ in the mitochondrial membrane would be 2-fold higher than that in the mitochondrial matrix (300 μM free NADH and 4.1 mM free NAD^+; Tischler et al., 1977). Such a change in ratio of reduced/oxidized nucleotide may influence the reduction/oxidation rates of NAD(H) by membrane-bound enzymes.

Ultrafiltration binding methodology, based on the partitioning of free ligand (Gilbert & Jenkins, 1959; Hirs & Timasheff, 1985), has been used extensively to study binding of ligands to proteins [see, for example, Sophianopoulos and Sophianopoulos (1985)]. In our procedure, the precision of the method has been improved by measuring the total ligand and enzyme concentrations (in the cell) at the end of each run and after dilution before the start of the subsequent run. Further, collecting and quantitating the free ligand concentration in a series of effluent fractions provides the necessary precision in the determination of the free ligand concentration (see Figure 1). This improved precision enables quantitation of weak ligand binding (e.g., NAD^+ binding). The ultrafiltration method as described here permits several binding measurements to be made sequentially over a wide range of ligand concentrations with the same sample and thereby obtains a complete binding curve with the same limited amount of starting enzyme and in a relatively short time (~2 h versus 24–30 h by equilibrium dialysis). In addition, appropriate controls (i.e., in the absence of enzyme) can be run with both the same ligand preparation and filtration membrane either immediately before or after the binding experiment. Obtaining data rapidly is particularly important for samples with limited stability such as 3-hydroxybutyrate apodehydrogenase in the absence of phospholipid. In ligand binding studies, measurement of K_D is usually not problematic, but the quantitation of binding stoichiometry has heretofore been difficult especially for relatively weak binding such as that of NAD(H) to 3-hydroxybutyrate dehydrogenase. In the studies presented here, we have used equilibrium dialysis and EPR spectroscopy to measure the binding of NAD(H) to the active 3-hydroxybutyrate dehydrogenase–MPL complex and obtained the same binding stoichiometry as by the ultrafiltration method, i.e., 2 equiv of nucleotide bound per enzyme tetramer. Thus, we have demonstrated experimentally that the ultrafiltration method, as applied here, gives reliable binding data approximating equilibrium conditions.

EPR methodology provides a complementary approach for quantitating ligand binding. For 3-hydroxybutyrate dehydrogenase, N^6-SL-NAD(H) and C8-SL-NAD(H) both serve as functional analogues of NAD(H) (Fritzsche et al., 1984) and are therefore suitable paramagnetic probes for studying ligand binding by EPR. One advantage of the EPR method versus ultrafiltration is that the EPR signal referable to the bound ligand is detected as a spectral component separate from the signal referable to free ligand (see Figures 6 and 7). EPR not only provides quantitation of the binding stoichiometry and K_D but also allows evaluation of the motional characteristics of ligand bound to the protein from the shape of the EPR spectrum. For weak binding of nucleotide to the partially active enzyme (in DMPE/PE/DPG), we found that the EPR spectrum of bound DSL-NAD^+ was characterized by a smaller resolved averaged hyperfine splitting (less immobilized) than that for DSL-NAD^+ bound to the fully active enzyme (in MPL) (see Table II). In the ultrafiltration studies, the specific binding to the protein requires subtraction of the binding to the phospholipid. By contrast, in the EPR studies, ligand bound to the phospholipid results only in a slight am-

plitude reduction of the EPR spectrum referable to free ligand and does not contribute in the spectral region referable to ligand bound to the protein. Thus, a direct measure of only the specific binding of nucleotide to 3-hydroxybutyrate dehydrogenase is obtained by EPR under conditions of tight or weak binding (Figures 6 and 7, respectively). In our studies, the results of the EPR and ultrafiltration binding studies were consistent.

The problem of weak binding can usually be overcome by increasing the protein concentration (Englund et al., 1969; Buller et al., 1976). In our ultrafiltration studies, we find binding of coenzyme to the phospholipid vesicles that is proportional to the lipid concentration so that only modest improvement in sensitivity could be obtained by increasing the concentration of the enzyme–phospholipid complex. The ratio of enzyme to lipid has been optimized for function so that the protein concentration cannot be increased very much without increasing the amount of lipid. Due to the binding of NAD(H) to the phospholipid, the direct binding data were of insufficient precision to discriminate between different models for binding of the ligand to the enzyme tetramer; i.e., the simplest model with a single K_D provided an adequate fit of the experimental data for NAD(H) binding to the unmodified enzyme. However, for the enzyme derivatized with either CN or CNTP at the rapidly reacting sulfhydryl, the data for NAD–SO_3^- binding were fit significantly better by a model with two dissociation constants (Table I). The fluorescence resonance energy transfer studies of NADH binding to the enzyme–MPL complex provided more precise data regarding the shape of the binding curve. In the presence of 100 mM NaCl, the resonance energy transfer data are consistent with a single dissociation constant, but in the absence of added salt, two dissociation constants can be resolved (Table III). We had previously observed, and confirm in the studies reported here, that the EPR spectra of SL-NADH or SL-NAD–SO_3^- bound to 3-hydroxybutyrate dehydrogenase exhibited two spectral components (Fritzsche et al., 1984). The two components, which are resolved by utilizing perdeuterated spin-labels that give enhanced spectral resolution, appear to be referable to ligand bound at sites with slightly different polarity (Philipp et al., 1984). We now find that only a single spectral component is observed with SL-NAD^+, the binding of which was enhanced by formation of a ternary complex with 2-methylmalonate. This suggests that the orientation of SL-NAD^+ bound to the enzyme differs somewhat from that for SL-NADH or SL-NAD–SO_3^-. The EPR and fluorescence data both indicate that the two nucleotide binding sites per enzyme tetramer are not equivalent.

For the 3-hydroxybutyrate dehydrogenase tetramer, the two nucleotide binding sites could either arise from sites formed by two monomers [the enzyme in the membrane behaving as a dimer of dimers with a total of two NAD(H) sites per tetramer] or reflect strong negative cooperativity in which the binding of NAD(H) to two sites (one site per monomer) precludes the binding of NAD(H) to two other sites [total of four NAD(H) sites per tetramer]. We were limited by the binding of NAD(H) to the phospholipid to assess whether there is any additional very weak binding of NAD(H) to the enzyme at sites other than the two per tetramer detected in these studies. However, in no case did we find a binding stoichiometry greater than ~0.5 mol of NAD(H)/mol of enzyme monomer even though we could detect and quantitate weak binding of nucleotide to some forms of the enzyme, e.g., NAD^+ binding to the enzyme in PE/DPG (Figure 3C), NAD^+ or NAD–SO_3^- binding to the cyanylated enzyme in MPL (Table I), and NAD^+ or NAD–SO_3^- binding to the apodehydrogenase (Figure 4). Under conditions of tight binding (e.g., NADH plus monomethyl malonate or NAD–SO_3^- binding to the enzyme–MPL complex), we did not detect additional weak binding even with a large excess of nucleotide (more than 100-fold the K_D for tight binding, Table I). Thus, there appears to be only 0.5 equiv of nucleotide binding per monomer of 3-hydroxybutyrate dehydrogenase. These studies support our previous observation that a sulfhydryl in 3-hydroxybutyrate dehydrogenase exhibits half-site reactivity with respect to derivatization by *N*-ethylmaleimide (McIntyre et al., 1984). Taken together with the NAD(H) binding stoichiometry (0.5 mol/mol of enzyme monomer) reported here, the results indicate that there are two polypeptides per functional unit, although strong negative cooperativity in nucleotide binding to 3-hydroxybutyrate dehydrogenase cannot be precluded.

Since the active site of 3-hydroxybutyrate dehydrogenase, identified by probes attached to the rapidly reacting sulfhydryl (McIntyre et al., 1984; Yamaguchi et al., 1986), is immersed below the surface of the bilayer (Dalton et al., 1987), binding of NAD(H) to the phospholipid bilayer may contribute to the binding of this ligand to the enzyme as has been postulated for some amphipathic ligand–receptor interactions (Herbette et al., 1986). This mechanism probably does not pertain with 3-hydroxybutyrate dehydrogenase since the K_D's for NAD(H) binding (measured with respect to the concentration of nucleotide in solution) are within the range (albeit somewhat weaker) reported for soluble dehydrogenases (0.2–1.2 μM for NADH; Srivastava & Bernhard, 1987) and about 10-fold lower than the concentrations of NAD^+ and NADH in mitochondria (4.1 and 0.3 mM free nucleotide, respectively; Tischler et al., 1977). Binding of nucleotide to the phospholipid bilayer does not explain the role of PC for activation of the enzyme since such binding is similar in the presence and absence of PC, i.e., although NAD(H) binds to PE/DPG, the binding of nucleotide to the enzyme, reconstituted into PE/DPG, is weak, in a range similar to that for binding to the apodehydrogenase (devoid of lipid). Rather, the role of PC is related, at least in part, to enhancing the binding of nucleotide to a preexisting site on the enzyme by reducing the K_D for nucleotide at least 1 order of magnitude. Thus, PC appears to serve as an allosteric activator of 3-hydroxybutyrate dehydrogenase.

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Registry No. NADH, 58-68-4; NAD, 53-84-9; MMM, 16695-14-0; MMA, 516-05-2; BDH, 9028-38-0; SO_3^-, 14265-45-3; dioleoyl-PC, 4235-95-4.

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