# Hydrodynamic Properties of D-β-Hydroxybutyrate Dehydrogenase, a Lipid-Requiring Enzyme†

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ABSTRACT: The hydrodynamic properties of purified D-β-hydroxybutyrate dehydrogenase (BDH), a lecithin-requiring enzyme isolated from bovine heart mitochondria, have been studied using the active-enzyme sedimentation technique. An active complex of BDH with dioctanoyl-L-α-phosphatidylcholine, PC(8:0), was sedimented under assay conditions, and the sedimenting boundary was followed by observing the reduction of $NAD^+$. Between 2.4 and 30 μg of BDH/mL, the $s_{20,w}$ increased and was inversely related to the enzymatic activity. The most active species has the smallest apparent $s_{20,w}$ (3.3 ± 0.2 S). The diffusion coefficient of the BDH-PC(8:0) complex at 2.4 μg/mL, measured by the free diffusion method, was found to be $4.9 \pm 0.5 \times 10^{-7}$ cm²/s; at higher protein concentrations, $D_{20,w}$ decreased. The hydrodynamic studies both of the active BDH-PC(8:0) complex and of the apoenzyme demonstrate that the protein undergoes concentration-dependent self-association. At the lowest protein concentration at which meaningful measurements could be made (the layered concentration of 2.4 μg/mL), the apparent molecular weight of the active BDH-PC(8:0) complex was calculated from $s_{20,w}$ and $D_{20,w}$ to be 61 600, which corresponds to a dimer. Using these values, the Stokes radius and frictional ratio ($f/f_0$) of the BDH-PC(8:0) complex were estimated to be 28 Å and 1.56, respectively, implying an axial ratio ($a/b$) of 9 for a prolate ellipsoid or 11 for an oblate ellipsoid. The circular dichroic spectrum of apo-BDH between 205 and 240 nm suggests the presence of nearly 30% α helicity but only limited β structure. The addition of mitochondrial phospholipid had no significant effect on the circular dichroic spectrum between 215 and 240 nm.

Biological membranes are macromolecular complexes consisting of proteins and phospholipids. D-β-Hydroxybutyrate dehydrogenase, normally bound to the mitochondrial inner membrane, has been purified from bovine heart mitochondria to homogeneity as a soluble, phospholipid-free protein, apo-BDH.[1] It has a subunit molecular weight of 31 000 estimated by $NaDodSO_4$-polyacrylamide gel electrophoresis (Bock and Fleischer, 1974, 1975). Apo-BDH is inactive in the absence of lipid but forms an active complex (BDH-PC) upon the addition of aqueous dispersions of lecithin or phospholipids containing lecithin (Gazzotti et al., 1975). This paper presents a study of the hydrodynamic properties of the active enzyme. A preliminary communication has appeared (McIntyre et al., 1976).

## Materials and Methods

Solutions were prepared with deionized water. Chemicals were of reagent grade unless otherwise specified. Sucrose, special enzyme grade, ultrapure, was obtained from Schwarz/Mann Co. (Orangeburg, N.Y.). DL-β-hydroxybutyric acid (sodium salt), bovine serum albumin (fraction V, powder), equine cytochrome *c* (grade III), ovalbumin (grade VI), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Equine γ-globulin was obtained from Mann Research Laboratories (New York, N.Y.). Bovine plasma albumin solution, used as the protein standard, and crystalline plasma albumin were obtained from Armour Pharmaceutical Co. (Chicago, Ill.). $NAD^+$ was obtained from P-L Biochemicals Inc. (Milwaukee, Wis.). LiBr, obtained from Matheson, Coleman and Bell (Norwood, Ohio), was prepared as a 4 M stock solution which was purified by filtration through an activated-carbon column and a 0.22-μm filter (Millipore Corp., Bedford, Mass.).

*Preparation and Measurement of BDH and Lipids.* The apo-BDH used in these studies was purified to homogeneity as described by Bock and Fleischer (1974, 1975) and was stored frozen in a liquid nitrogen refrigerator. The lipids were prepared as described previously (Gazzotti et al., 1975), except that PC(8:0) was stored at −20 °C as a 1 mM stock solution in water and was maintained at 40 °C for the studies described below. [$^{14}$C]PC(8:0), synthesized from [1-$^{14}$C]octanoic acid as described previously (Gazzotti et al., 1975), was used, after dilution with unlabeled PC(8:0), for measurement of the concentration of unbound PC(8:0) in the presence of BDH (see below). Protein was determined by the procedure of Lowry et al. (1951) with a BSA standard. The assay was carried out as described by Ross and Schatz (1973) using iodoacetate to carboxymethylate the DTT which would otherwise interfere with the assay for protein. Phosphorus was measured using a modification (Rouser and Fleischer, 1964) of Chen et al. (1956).

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[1] Abbreviations used: BDH, D-β-hydroxybutyrate dehydrogenase; apo-BDH, lecithin-free and enzymatically inactive BDH; BSA, bovine plasma albumin; CD, circular dichroic; CMC, critical micelle concentration; DTT, dithiothreitol; GdmCl, guanidinium chloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MPL, mitochondrial phospholipid; PC, phosphatidylcholine; PC(8:0) and PC(18:1 cisΔ9), dioctanoyl- and dioleoyl-L-α-phosphatidylcholine, respectively; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-Cl, 2-amino-2-hydroxymethyl-1,3-propanediol chloride.

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*Enzymatic Assay.* BDH activity was measured as described previously (Bock and Fleischer, 1974). With PC(8:0) activation of apo-BDH, the assay was modified as follows. The assay was carried out at 25 °C, and both BSA and ethanol were omitted from the assay mixture. All components except for PC(8:0), enzyme, and substrate were incubated at 25 °C for 5 min and then an aliquot of a 1 mM PC(8:0), stock solution, maintained at 40 °C, was added; after a 5-min incubation, the enzyme was added. Following a further 15-min incubation to form the active BDH-PC(8:0) complex, the reaction was started by the addition 20 μL of 1.0 M DL-β-hydroxybutyrate.

*Measurement of the Concentration of Unbound PC(8:0) in the Presence of BDH.* The free concentration of PC(8:0) in the presence of various concentrations of BDH was measured both by equilibrium dialysis and rapid filtration. Equilibrium dialysis was carried out as described previously (Gazzotti et al., 1974) using chambers which contained 100 μL of sample on each side of the dialysis membrane. BDH was diluted to 40, 120, or 390 μg/mL in 100 mM sodium phosphate buffer, pH 7.35, containing 0.5 mM EDTA, 5 mM DTT, and 125 μM [$^{14}$C]PC(8:0) (specific radioactivity 660 cpm/nmol) and was dialyzed at 4 °C for 14 h against the same solution but without BDH; the enzyme maintained greater than 80% of its activity during dialysis. Aliquots (35 μL) were removed and counted in a Packard Tri-Carb scintillation counter to measure the concentration of PC(8:0) on both sides of the dialysis membrane and thus determine whether, in solution, there is an excess of free (unbound) PC(8:0) available for activation of the enzyme. The concentration of PC(8:0) not bound to BDH was also measured using the rapid-filtration method described by Paulus (1969). For this technique, BDH was diluted to final concentrations of protein, varying from 1.6 to 160 μg/mL, in 0.5 mL of 100 mM potassium phosphate (pH 7.35), 0.5 mM EDTA, 0.2 mM DTT, and 125 μM [$^{14}$C]PC(8:0) (specific radioactivity 110 cpm/nmol). The enzyme was incubated with the PC(8:0) for 15 min at room temperature (ca. 25 °C) to obtain optimum activation. Each sample was then filtered at room temperature through a PM-10 ultrafiltration membrane (Amicon Corp., Lexington, Mass.); the filtration time for each sample was about 10 min. The filtrate from each sample was collected in two fractions and the PC(8:0) concentration determined by counting 50-μL aliquots as described above. There was no detectable difference between the concentration of PC(8:0) in the two fractions of filtrate collected from each sample. The filters were also counted to measure the retention of PC(8:0).

*Sedimentation Velocity.* The sedimentation coefficient of apo-BDH was measured using a Model E ultracentrifuge with scanner optics. On the day before each sedimentation run, the stock apo-BDH (~2 mg/mL) was centrifuged at 130 000*g* for 1 h. The supernatant was recovered and an aliquot diluted to the appropriate protein concentration for the sedimentation velocity run. The diluted sample was stored frozen overnight in a liquid nitrogen refrigerator and was thawed just prior to loading. The protein was loaded into one side of a 1.2-cm path-length double-sector cell and centrifuged at 52 000 rpm in an AN-H rotor equilibrated at 20 °C. The cell was scanned every 250 s, monitoring protein absorbance at 280 nm vs. buffer blank, until the principal boundary had sedimented two-thirds of the distance down the cell. A control sample of apo-BDH, which was maintained at room temperature during the course of the sedimentation run, lost less than 10% of its enzymatic activity. In addition to the protein which gives rise to the principal boundary, some protein sediments more rapidly and as a separate heterogeneous species. The amount of protein present in this rapidly sedimenting species was estimated from the difference between the initial absorbance and the absorbance at the principal boundary after the two species had separated. The rapidly sedimenting species is probably denatured protein and is not in rapid equilibrium with the majority of the protein, which gives rise to the principal sedimentation boundary. The radial position ($r_b$) of the midpoint of the principal boundary was determined for each time that the cell was scanned, and the apparent $s_{20}$ was determined from the plot of ln $r_b$ vs. time; such plots were nearly linear, although some slight curvature was noted. This is expected with this self-associating system due to radial dilution as the protein sediments (Cox, 1969). The apparent $s_{20}$ of the rapidly sedimenting protein was estimated in a similar manner. The sedimentation coefficient of apo-BDH at various initial layered concentrations was also measured by the sucrose density gradient centrifugation method described by Martin and Ames (1961).

The sedimentation coefficient of the BDH-PC(8:0) complex was determined using the active-enzyme sedimentation method of Cohen and Mire (1971). A Vinograd-type double-sector centerpiece (Beckman part no. 331359) was used in an AN-H rotor with the Model E ultracentrifuge. The enzyme was activated with PC(8:0) below its CMC, in a solution containing 10 mM potassium phosphate (pH 7.35), 2 mM DTT, 0.5 mM EDTA, 125 μM PC(8:0), and 2–30 μg/mL apo-BDH; 10 μL of this BDH-PC(8:0) complex was placed in the sample well in the centerpiece of the cell and the rotor equilibrated at 20 °C inside the centrifuge. As the rotor accelerated to 52 000 rpm, the active-enzyme complex layered onto the surface of an assay solution in the main cell compartment; the assay solution contained 0.1 M potassium phosphate (pH 7.35), 2 mM DTT, 0.5 mM EDTA, 125 μM PC(8:0), 20 mM DL-β-hydroxybutyrate, and 2 mM $NAD^+$. The sedimentation of the enzyme band was monitored by the production of NADH from the enzymatic reaction, by observing the 340-nm absorption of this product. The cell was scanned at 250-s intervals, and the radial position ($r$) of the scanner trace half-height (the midpoint of the reaction boundary) was determined for each scan. The data were analyzed using an approximation method (Kemper and Everse, 1973) which computes $s_{20}$ from the plot of ln $r$ vs. time; these plots were linear within experimental error. The enzymatic activity of a control sample of the BDH-PC(8:0) complex, maintained at room temperature during the course of each sedimentation run, remained constant when assayed in the presence of either MPL or 125 μM PC(8:0).

*Partial Specific Volume Determinations.* The partial specific volume ($\bar{\nu}$) of the protein was calculated to be 0.735 mL/g from the apparent specific volumes of the component amino acids (Cohn and Edsall, 1943; McMeekin and Marshall, 1952) and the amino acid composition of apo-BDH (Bock and Fleischer, 1975). Although $\bar{\nu}$ does not equal $\bar{\nu}$ of the active BDH-PC(8:0) complex, the effective partial specific volume of the complex ($\phi'$) in the 0.1 M potassium phosphate buffer used for the active enzyme sedimentation experiments is close to this value. This is demonstrated by using Cassassa and Eisenberg's (1964) equation

$$M(1 - \phi'\rho) = M[(1 - \bar{\nu}\rho) + \delta_D(1 - \bar{\nu}_D\rho)] \qquad (1)$$

where $\rho$ is the solution density, $\delta_D$ the lipid to protein weight ratio, $\bar{\nu}_D$ the partial specific volume of the lipid, and $\phi'$ the "effective partial specific volume" of the complex. Since $\bar{\nu}_D$ = 0.987 for PC(8:0) below the CMC (Tausk et al., 1974), $(1 - \bar{\nu}_D\rho)$ = 0.0029. Thus, if 1 g of PC(8:0) was bound/g of apo-BDH, $\phi'$ would equal 0.732 as compared to 0.735, which

is the partial specific volume of apo-BDH. The amount of PC(8:0) bound to the apo-BDH was estimated by Gazzotti et al. (1975) to be about 10 mol/mol of subunit (0.17 g/g of apo-BDH), using equilibrium binding with gel-exclusion chromatography. Therefore, the contribution of the lipid to the effective partial specific volume of the BDH-PC(8:0) complex in the buffer solution used is considered negligible for purposes of the calculations.

The densities of the solutions used for the sedimentation experiments were determined using a Mettler-Parr DM02A density meter. The viscosities of the solutions were measured relative to water in a U-tube viscometer at 20 °C. The apparent $s_{20,w}$ was calculated from the measured $s_{20}$ using the formula:

$$s_{20,w} = s_{20}\left[\frac{\eta_{20}(1-\bar{\nu}\rho)_{20,w}}{\eta_{20,w}(1-\bar{\nu}\rho)_{20}}\right] \quad (2)$$

with $\bar{\nu} = 0.735$.

*Diffusion Methods.* Diffusion coefficients were determined using the free diffusion method (Gosting, 1956). Apo-BDH was activated with PC(8:0) in a solution containing 0.1 M sodium phosphate (pH 7.35), 5 mM DTT, 0.5 mM EDTA, 125 μM PC(8:0), and 5% (w/v) ultrapure sucrose. The solution was saturated with nitrogen gas prior to the addition of the PC(8:0), and finally the apo-BDH was added. This solution (2.5 mL), which contains the BDH-PC(8:0) complex, was pipetted into a 2 × 0.5 in. cellulose nitrate centrifuge tube (Beckman, part no. 305050) which was fixed vertically in a test-tube rack. An equal volume of a "light" solution of the same composition, but without the protein or the sucrose, was carefully overlayed to form a sharp initial boundary. After diffusion for between 20 and 30 h at 25 ± 0.5 °C, fractions of equal volume (by drop counting) were collected at 4 °C using a Beckman "fraction-recovery system" to expel the contents through the top of the tube. The BDH activity in each fraction was measured using the standard assay with MPL activation to determine the distribution profile of active enzyme. The sucrose distribution was measured by using a refractometer. A series of standard proteins, run in separate tubes, was analyzed as follows: cytochrome *c* and human hemoglobin by light absorbance measured at 415 nm; ovalbumin, bovine plasma albumin, and equine γ-globulins by protein analysis using the modified method of Lowry et al. (1951).

The distribution profile, at time = $t$, of a single species diffusing from a semiinfinite region terminated by a sharp initial boundary at $X = 0$ is described by the equation

$$C_X = \frac{C_0}{2}\left[1 - \frac{2}{\sqrt{\pi}}\int_0^{x/2\sqrt{Dt}} e^{-y^2}dy\right] \quad (3)$$

where $C_0$ is the initial concentration, $C_X$ the concentration $X$ cm from the initial boundary, $t$ the diffusion time in seconds and $D$ the diffusion coefficient. This equation is derived by an integration of Fick's second law of diffusion as described by Gosting (1956). The form of eq 3 is obtained directly from eq 70, 72 and 73 of Gosting (1956) with the lower concentration $C_a$ being zero for our experimental conditions. The initial boundary position was taken as the value of $X$ at which $C_X = 0.5\ C_0$; when this was determined from both the sucrose and protein distributions, the same initial boundary position was found within experimental error. For each $C_0$ and $t$, $C_X$ vs. $X$ was computed for various values of $D$. For several values of $X$, on both sides of the initial boundary position, $C_X$ was measured for the experimental data and $D$ determined by interpolation from the computer-simulated values of $C_X$. $D_{obsd}$ was taken as the average of these $D$ values determined at various positions. It was assumed that the protein diffusion is not significantly modified by the diffusion of the sucrose or other interacting flows.

The diffusion constants ($D_{obsd}$) were corrected to $D_{20,w}$ by two methods. One method uses the relationship

$$D_{20,w} = D_{obsd}\left(\frac{T_s\eta_{obsd}}{T_{obsd}\eta_s}\right) \quad (4)$$

where $T_s$ is the standard temperature (293.16 K), $T_{obsd}$ the temperature of the measurement, $\eta_s$ the viscosity of water at $T_s$ and assuming the solvent viscosity ($\eta_{obsd}$) to be the viscosity of the solvent at the average sucrose concentration in which $D_{obsd}$ was measured. Calculated $D_{20,w}$ values for the standard proteins agreed with the literature values within experimental error. The other method utilizes standard proteins of known diffusion constants to determine the correction factor for temperature and viscosity. For each experiment, $D_{obsd}$ vs. $D_{20,w}$ for the standard proteins was plotted and $D_{obsd}$ for the BDH-PC(8:0) complexes corrected to $D_{20,w}$ using the relationship so obtained. The following $D_{20,w}$ values were used (all values are $\times 10^7$ in units of cm²/s): equine cytochrome *c* (13.0), human hemoglobin (6.9), BSA (5.94), and equine γ-globulins (4.8).

*Circular Dichroism.* The CD spectra were obtained with a Cary 60 spectropolarimeter equipped with a Model 6002 CD attachment. A thermostated cell holder was used with a circulating water bath to control temperatures to within ±0.2 °C. Cells of path length 0.5 and 1.0 mm were used to record spectra between ca. 205 and 240 nm under a variety of experimental conditions. The instrument was operated with a time constant of 3 s and sensitivities 2 and 4 mdeg/in. Several scans of sample and appropriate blanks were taken and averages are reported. A mean residue molecular weight of 110 was used to obtain the reduced mean-residue ellipticity, $[\theta]$, in units of deg·cm²/dmol.

## Results

*Activation of Apo-BDH with Lecithin.* The maximum specific activity which could be obtained upon activation of apo-BDH with MPL varied between 90 and 100 μmol of $NAD^+$ reduced $min^{-1}$ $mg^{-1}$ at 37 °C; this is equivalent to a specific activity of between 36 and 40 at 25 °C. Since natural lecithins are not soluble in aqueous solution and exist as macromolecular arrays (liposomes), hydrodynamic studies of the enzyme activated by them would reflect the properties of such lipid vesicles.

However, activation of apo-BDH can be achieved with the soluble lecithin PC(8:0), thus permitting characterization of the hydrodynamic properties of the active enzyme. PC(8:0), at 200 μM, activated apo-BDH (4.8 μg/mL) to the same extent as PC(18:1 cisΔ9), giving a maximum specific activity of 32 μmol of $NAD^+$ reduced $min^{-1}$ $mg^{-1}$ at 25 °C in the presence of both 1.27% ethanol and 0.4 mg/mL BSA (data not shown). At concentrations of PC(8:0) greater than 200 μM, inhibition of BDH activity occurred due to PC(8:0) micelle formation (Gazzotti et al., 1975; Tausk et al., 1974). When the concentrations, in the standard assay medium, of either potassium phosphate (pH 7.35) or ethanol are varied, the PC(8:0) concentration which gives maximum activation of BDH is altered. For example, in the absence of both BSA and ethanol, in 10 or 100 mM buffer, the CMC of PC(8:0) is reduced so that optimum activation of BDH is obtained with 120 or 130 μM PC(8:0), respectively; in 100 mM buffer with either 1.27 or 9% ethanol (v/v) and in the absence of BSA, optimum activation of BDH is obtained with 170 or 175 μM PC(8:0), respectively. These differences seem to reflect changes in the CMC of the PC(8:0) with the conditions of the medium. For

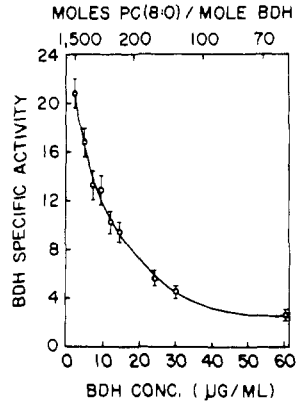


FIGURE 1: The effect of BDH concentration on the specific activity of the enzyme activated with PC(8:0). The activity obtained with different amounts of BDH, as indicated, and 125 $\mu$M PC(8:0) was measured at 25 °C, as described in the text in a medium containing 100 mM potassium phosphate (pH 7.35), 2 mM $NAD^+$, 20 mM DL-$\beta$-hydroxybutyrate, 0.5 mM EDTA and 0.3 mM DTT. BDH specific activity is expressed as $\mu$mol of $NAD^+$ reduced $min^{-1}$ (mg of BDH)$^{-1}$. The molar ratio of PC(8:0) to BDH subunit with increasing BDH concentration was calculated (upper ordinate).

the active-enzyme sedimentation of BDH–PC(8:0), the assay medium in the main compartment of the cell contained 125 $\mu$M PC(8:0) and 100 mM potassium phosphate, pH 7.35. Under these conditions, the specific activity of BDH at 4.8 $\mu$g of protein/mL is 16 $\mu$mol of $NAD^+$ reduced $min^{-1}$ $mg^{-1}$ at 25 °C. This salt concentration minimizes nonspecific electrostatic interactions and also provides a sufficient density difference between the assay medium and the enzyme sample, which is overlayed, so that a sharp boundary forms. In this assay medium, 125 $\mu$M PC(8:0) is below the apparent CMC of this lipid.

The specific activity of BDH, activated with PC(8:0), is dependent on the BDH concentration (Figure 1). The observed decrease in BDH activity with increasing protein concentration is related to the self-association of the protein (see below) and is not due to limiting amounts of PC(8:0) for activation of the enzyme. The free concentration of PC(8:0) in the presence of either 40, 120, or 390 $\mu$g of BDH/mL was measured by equilibrium dialysis. At 40 $\mu$g/mL, the concentration of PC(8:0) on both sides of the dialysis membrane was the same (125 $\mu$M) within experimental error. At 390 $\mu$g/mL, the concentration of PC(8:0) in the protein-free chamber was diminished but to only about 94 $\mu$M. The free concentration of PC(8:0), in the presence of from 2 to 160 $\mu$g of BDH/mL was also measured using rapid filtration (Paulus, 1969). Although the concentration of PC(8:0) in the effluent was diminished to approximately 100 $\mu$M by nonspecific absorption of between 10 and 15 nmol of PC(8:0) to each membrane filter, the presence of BDH (from 2 to 160 $\mu$g/mL) did not alter the free concentration of PC(8:0). Therefore, when variable amounts of BDH are activated with PC(8:0), although the molar ratio of PC(8:0) to BDH decreases rapidly with increasing protein concentration (Figure 1), the concentration of free PC(8:0) is not significantly altered, within experimental error, by the presence of from 2 to 60 $\mu$g of BDH/mL. Since the amount of PC(8:0) bound to BDH is dependent only on the dissociation constant for the interaction and the free PC(8:0) concentration, we conclude that the decrease in BDH activity with increasing protein concentration is not due to an insufficiency of PC(8:0).

*Sedimentation Velocity of Apo-BDH and of BDH–PC(8:0).* The sedimentation coefficient of apo-BDH was measured using conventional sedimentation velocity, and several typical scans

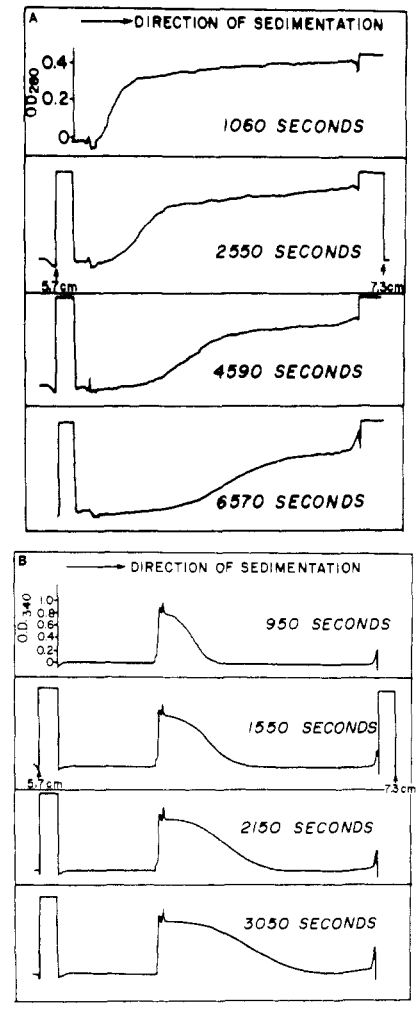


FIGURE 2: (A) Scanner data from conventional protein sedimentation of apo-BDH, 340 $\mu$g/mL, in 0.4 M LiBr, 1 mM Hepes, 2 mM DTT, 0.5 mM EDTA (pH 7.0) at 52 000 rpm, 20 °C, in the Model E ultracentrifuge. Protein was monitored by absorbance, at 280 nM, and scans were taken at 250-s intervals. Representative scans, taken at the times indicated, are shown. (B) Scanner data from active-enzyme sedimentation of BDH–PC(8:0) in 100 mM potassium phosphate (pH 7.35), 2 mM DTT, 0.5 mM EDTA, 2 mM $NAD^+$, 20 mM DL-$\beta$-hydroxybutyrate, and 125 $\mu$M PC(8:0). The enzyme was layered at an initial concentration of 14.4 $\mu$g/mL in the same buffer but with only 10 mM potassium phosphate (pH 7.35) and without either $NAD^+$ or DL-$\beta$-hydroxybutyrate. The overlayed solution, containing the enzyme, has a lower density so that a sharp initial boundary is formed, when the enzyme solution is layered over the assay solution in the main compartment of the sedimentation cell. NADH production was monitored by absorbance, at 340 nm, and scans were taken at 250-s intervals. Representative scans, taken at the times indicated, are shown.

are presented in Figure 2A. For these studies, 0.4 M LiBr and 2 mM DTT were present, since earlier work has demonstrated the stability of apo-BDH under these conditions (Bock and Fleischer, 1975). There is a single principal skewed boundary (i.e., asymmetric about its midpoint) which does not resolve as the protein sediments. The absorption profile also shows the presence of a heterogeneous high-molecular-weight material sedimenting several-fold faster than the principal peak. This material comprises some 5 to 15% of the protein absorbance and exhibits apparent sedimentation coefficients in the range of 20 to 40 S. Interestingly, the proportion of protein sedimenting in this size range did not vary appreciably as the protein concentration varied from 60 to 900 $\mu$g/ml. The high-molecular-weight material does not, therefore, appear to be in rapid reversible equilibrium with the remainder of the

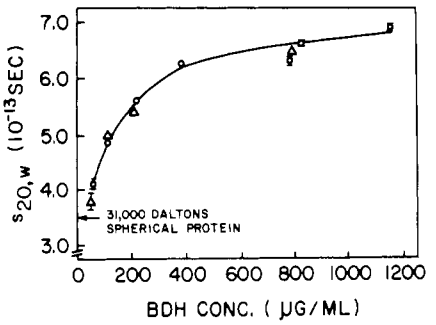


FIGURE 3: The effect of protein concentration on the sedimentation coefficient of apo-BDH, at 20 °C, in the medium described in Figure 2A (○) and with the addition of 125 μM PC(8:0) (△).

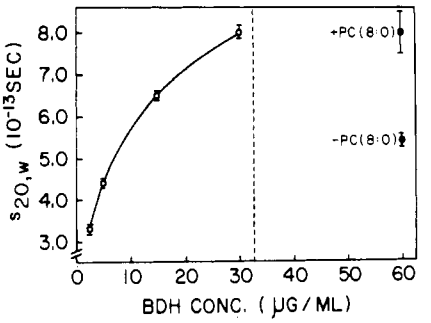


FIGURE 4: The effect of protein concentration on the sedimentation coefficient of BDH-PC(8:0) at 20 °C. The data points on the left side (○) were obtained using the active-enzyme sedimentation method, described in Figure 2B, but with the enzyme being overlayed at the concentrations indicated. The two data points on the right (●) were obtained by the conventional protein-sedimentation technique, described in Figure 2A, but in the medium for active-enzyme sedimentation (cf. Figure 2B) except without $NAD^+$ or DL-β-hydroxybutyrate.

protein. Attempts to remove this material by sedimentation prior to sedimentation velocity studies (cf. Materials and Methods) were not successful. The irreversibly aggregated material may be denatured apo-BDH, since the enzyme preparation is homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bock and Fleischer, 1975).

The BDH-PC(8:0) complex is most active at low protein concentrations (Figure 1) and, therefore, to determine the hydrodynamic properties of the active enzyme, studies must be carried out at a minimum protein concentration, in the range of 0–10 μg/mL. The sedimentation behavior of the active BDH-PC(8:0) complex was investigated using the active-enzyme sedimentation technique (Cohen and Mire, 1971; Kemper and Everse, 1973). The sedimentation of active enzyme was followed by monitoring at 340 nm the formation of NADH as the enzyme band passes through an assay solution which also contains the PC(8:0). Typical scans from one such experiment are shown in Figure 2B. The approximate method was used for calculating $s_{20}$ from the midpoint of the reaction boundary (cf. Materials and Methods); this method has been shown by Wampler (1972), Kemper and Everse (1973), and Holleman (1973) to give essentially the same calculated sedimentation coefficient for a single sedimenting boundary as does the rigorous treatment of the data (Cohen and Mire, 1971), in which $s_{20}$ is computed from the radial position of the difference distribution of successive scans. In all of the active-enzyme sedimentation experiments, substrate did not become limiting, since the amount converted to product by the sedimenting enzyme band was small.

From the principal boundary observed in conventional sedimentation, $s_{20,w}$ was measured at several initial protein concentrations (Figure 3). The apoenzyme undergoes concentration-dependent association and, under the conditions used, PC(8:0) does not significantly alter the sedimentation behavior. However, it must be emphasized that under these conditions, i.e., high protein concentration (cf. Figure 1) and 0.4 M LiBr (Nielsen and Fleischer, 1973), the enzyme is not activated by PC(8:0). Conventional sedimentation velocity was not used to study the active species, since the lowest concentration of protein which could be monitored with the instrumentation available was approximately 60 μg/mL; at this concentration apo-BDH can only be minimally activated by PC(8:0).

When the sedimentation of apo-BDH in 0.4 M LiBr was studied using the sucrose density gradient method (Martin and Ames, 1961), similar results (not shown) were obtained to those with the Model E analytical ultracentrifuge; i.e., the apparent sedimentation coefficient increased as the concentration of protein, which was layered on the top of the gradient, was increased from 0.1 to 2.4 mg/ML. However, a direct correlation of $s_{20,w}$ with protein concentration is not possible, since the protein dilutes at least tenfold during the sedimentation time (ca. 20 h).

The apparent $s_{20,w}$ of the active BDH-PC(8:0) complex (measured by the active-enzyme sedimentation method) increases markedly as the initial layered concentration is increased from 2 to 30 μg/mL (Figure 4). The single skewed boundary observed at all protein concentrations (Figure 2B), together with the shape of the plot of $s_{20,w}$ vs. concentration (Figure 4), suggests the presence of at least three species in reversible equilibrium (Cox, 1968, 1971). The right side of the graph (Figure 4) shows two measurements of the sedimentation coefficient in the same medium, using the conventional protein sedimentation technique at its lower limit (60 μg/mL). The addition of 125 μM PC(8:0) increases the apparent $s_{20,w}$ of the apo-BDH, although at 60 μg/mL the enzyme is only partially activated by PC(8:0).

The activity of the BDH-PC(8:0) complex was correlated with the apparent sedimentation coefficient, and the most active species was found to have the smallest apparent $s_{20,w}$ (3.3), which is close to that expected for a spherical protein of molecular weight 31 000 (i.e., the subunit molecular weight) (cf. Table I). However, the sedimentation coefficient depends upon both the shape and size of the protein; consequently, a second hydrodynamic parameter is necessary for the determination of the molecular weight of the active BDH-PC(8:0) complex. Thus, the diffusion coefficient was measured independently but under essentially the same conditions as those used in the active enzyme sedimentation.

*The Diffusion Coefficient and Molecular Weight of BDH.* The diffusion coefficient of the active BDH-PC(8:0) complex was measured using the diffusion technique as described under Materials and Methods. Typical experimental data for one of the protein standards (equine globulins) and for BDH-PC(8:0) at 4.8 μg of BDH/mL are shown in Figures 5A and 5B, respectively. $D_{obsd}$ was computed as described and the simulated diffusion profiles for this calculated value are also shown. The computed curve fits the experimental data to a good approximation. The values of $D_{obsd}$ for the standard proteins are given in the legend to Figure 5. The plot of $D_{obsd}$ vs. $D_{20,w}$ was linear (not shown) and gives a correction factor for the temperature and viscosity of 0.87; i.e., $D_{20,w} = 0.87D_{obsd}$.

$D_{obsd}$ was found to be independent of the diffusion time, since the concentrations did not change measurably at the ends of the tube during the time of the experiments. The $D_{20,w}$ values for the BDH-PC(8:0) complexes were determined from the $D_{obsd}$ on the high protein concentration side of the initial boundary to minimize errors due to the dissociation of the

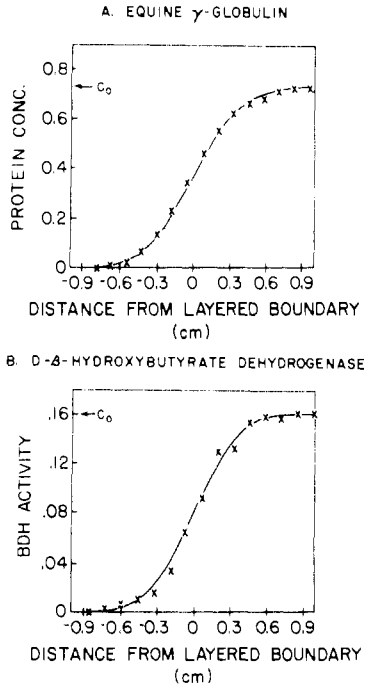


FIGURE 5: The free diffusion of protein from a layered boundary. Each sample was prepared in 2.5 mL of solution, saturated with nitrogen gas, and containing 100 mM sodium phosphate (pH 7.35), 5 mM DTT, 0.5 mM EDTA, 125 $\mu$M PC(8:0), and 5% (w/v) ultrapure sucrose. An equal volume of the same solution, but devoid of both sample and sucrose, was layered over the sample to form a sharp initial boundary. After diffusion for ca. 24 h at 25 °C, fractions were collected through the top of the tube, by displacement of the sample with 55% ultrapure sucrose. The concentration of sample in each fraction was then measured. Fraction number has been converted to distance in centimeters, and the initial layered boundary position (0 cm) was set as the position where the sample concentration was half of the initial concentration ($C_0$). The top of the tube is to the left and, for clarity, only the middle portion of each profile is shown; the concentration remains constant (zero or $C_0$) for an additional centimeter in both directions. The solid line is the simulated diffusion profile that best fits the experimental data (x). (A) Equine γ-globulins at 0.5 mg/mL after diffusion for 26.8 h; protein concentration ($OD_{750}$/fraction) in the collected fractions was measured by the modified Lowry method ($D_{obsd}$ = 5.40 ± 0.40 × $10^{-7}$ $cm^2/s$). The $D_{obsd}$ for the other standard proteins (not shown) were 6.89 ± 0.58 (BSA), 8.16 ± 0.55 (human hemoglobin), and 14.2 ± 1.0 (cytochrome *c*). (B) BDH-PC(8:0) complex at 4.8 $\mu$g of BDH/mL after diffusion for 23.1 h; BDH concentration in the collected fractions was determined by measuring enzymatic activity ($\Delta OD_{340}$ per 4 min per 100 $\mu$L of fraction), using the normal assay method with MPL activation ($D_{obsd}$ = 5.36 ± 0.45 × $10^{-7}$ $cm^2/s$).

protein as it dilutes. When the protein was layered in either the sucrose-containing buffer ("heavy") or in the upper "light" solution (no sucrose), $D_{obsd}$ was consistently lower on the high sucrose density side of the initial boundary. The difference, although within experimental error, can be accounted for by the higher average viscosity of this medium. The $D_{obsd}$ values for the BDH-PC(8:0) complexes in each solution (i.e., "light" or "heavy") were corrected to $D_{20,w}$ using the correction factor for temperature and viscosity measured in the same solution. The values of $D_{20,w}$ for BDH-PC(8:0) at different initial layered concentrations are given in Table I.

For each protein concentration, the apparent molecular weight ($M_{s/D}$) was calculated from the following equation

$$M_{s/D} = \frac{s_{20,w}RT}{D_{20,w}(1 - \phi_2'\rho)} \quad (5)$$

where $R$ is the gas constant (8.314 × $10^7$ erg $mol^{-1}$ $K^{-1}$), $T$ the standard temperature (293.16 K), $\phi_2'$ the effective partial specific volume of the BDH-PC(8:0) complex, and $\rho$ the density of water. A value of 0.735 mL/g was used for $\phi_2'$.

TABLE I: Variation of the Enzymatic Activity, Sedimentation and Diffusion Coefficients,[a] and Calculated Molecular Weight[b] of BDH-PC(8:0) with Enzyme Concentration.

| BDH ($\mu$g/mL) | sp act.[a] ($\mu$mol of $NAD^+$ red. $min^{-1}$ $mg^{-1}$) | $s_{20,w}$ × $10^{13}$ (s) | $D_{20,w}$ × $10^7$ ($cm^2/s$) | $M_{s/D}$ (daltons) |
|---|---|---|---|---|
| 2.4 | 20.9 | 3.3 | 4.9 | 61 600 |
| 4.8 | 16.8 | 4.4 | 4.6 | 87 500 |
| 7.2 | 13.8 | 5.1 | 4.4 | 106 000 |
| 10.0 | 11.5 | 5.7 | 4.2 | 124 000 |

[a] The enzymatic activity and diffusion coefficient were measured at 25 °C as described in Figures 1 and 5, respectively. The sedimentation coefficient was measured at 20 °C as described in Figure 2B. [b] The molecular weight was calculated using eq 5; the standard deviation for the value at each concentration of BDH is approximately 15%.

TABLE II: A Summary of the Hydrodynamic Parameters of the BDH-PC(8:0) Complex.[a]

| | |
|---|---|
| partial sp vol ($\bar{\nu}$) | 0.735 $cm^3/g$ |
| sedimentat. coeff. ($s_{20,w}$)[a] | 3.3 × $10^{-13}$ s |
| diffusion coeff. ($D_{20,w}$)[a] | 4.9 × $10^{-7}$ $cm^2/s$ |
| mol wt ($M_{s/D}$)[b] | 61 600 daltons |
| frictional coeff ($f$)[c] | 8.3 × $10^{-8}$ g·s |
| Stokes radius ($R_0$)[d] | 28 Å |
| frictional ratio ($f/f_0$)[e] | 1.56 |
| axial ratio, prolate ($a/b$)[f] | 9 |
| axial ratio, oblate ($a/b$)[f] | 11 |

[a] The sedimentation and diffustion coefficients were measured at a BDH concentration of 2.4 $\mu$g/mL as described in the text. The other physicochemical parameters were calculated from these values using standard relationships (Tanford, 1961). [b] Calculated from $s_{20,w}$ and $D_{20,w}$ according to eq 5. [c] $f = kT/D_{20,w}$. [d] $R_0 = [(3M/4\pi N)(\bar{\nu} + \delta\nu^\circ)]^{1/3}$, where $\delta$ = 0.2 g of solvent/g of protein as suggested by Tanford (1961). [e] $f/f_0 = (kT/6\pi\eta D_{20,w}R_0)$. [f] The axial ratios were determined graphically (Tanford, 1961).

The values of $s_{20,w}$, $D_{20,w}$, $M_{s/D}$, and the specific activity of BDH-PC(8:0) at various protein concentrations are tabulated in Table I. Other hydrodynamic parameters, calculated from $s_{20,w}$ and $D_{20,w}$ using classical equations (Tanford, 1961), are shown in Table II for the BDH-PC(8:0) complex at 2.4 $\mu$g/mL. The physical meaning of some of these parameters is not clear, since it is not possible to extrapolate the apparent $s_{20,w}$ and $D_{20,w}$ to zero concentration without knowing the type of self-associating system present. However, at 2.4 $\mu$g/mL, the lowest concentration for which $s_{20,w}$ and $D_{20,w}$ could be measured, the apparent molecular weight of the BDH-PC(8:0) complex is 61 600 which corresponds to a dimer. The specific enzymatic activity at 25 °C for this concentration of the BDH-PC(8:0) complex is 21 $\mu$mol of $NAD^+$ reduced $min^{-1}$ $mg^{-1}$, whereas the optimum specific activity of the BDH-MPL complex is 28 $\mu$mol of $NAD^+$ reduced $min^{-1}$ $mg^{-1}$ under the same conditions.

The molecular weight of apo-BDH, reduced and alkylated according to the method of Crestfield et al. (1963), was estimated by sedimentation equilibrium in the presence of 6 M GdmCl at 20 °C with 500 $\mu$g of apo-BDH/mL. Two independent experiments yielded an $M_1$ of 28 700, although the solutions were not monodisperse. Thus, it appears that at least a fraction of the BDH population is aggregated at this protein concentration, even in the presence of a strong denaturant such as GdmCl.

*CD of BDH.* The CD spectrum of apo-BDH below 250 nm, numerically averaged from a number of scans and obtained

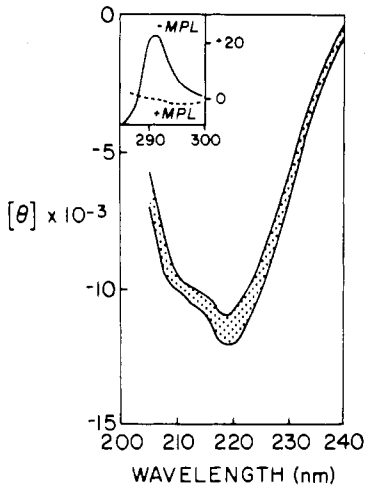


FIGURE 6: The average CD spectrum of apo-BDH at 25 °C. The reduced mean residue ellipticity, $[\theta]$, is in units of deg·cm$^2$/dmol. The spectrum represents an average of six independent spectra involving different buffer conditions and different enzyme concentrations and preparations; each of the six spectra and the respective baselines were scanned several times and averaged. The upper and lower limits indicate the mean of the six spectra and the standard deviation of the mean. BDH was diluted to concentrations of 0.13, 0.17, and 0.26 mg/mL with 1 mM Hepes (pH 7.0), 2 mM DTT, and 80, 100, or 500 mM NaCl; no significant differences were noted in the CD spectra under any of these conditions. The inset shows the near-UV CD spectra of BDH (1.85 mg/mL) in 0.2 M LiBr, 0.5 mM Hepes, 2.5 mM DTT, 0.5 mM EDTA, and 10 mM Tris-Cl (pH 8.1) in the presence and absence of MPL (73 μg of phosphorus/mg of BDH).

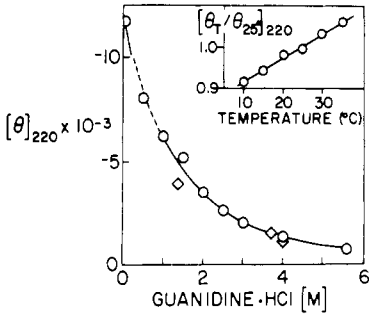


FIGURE 7: The effect of GdmCl on the reduced mean-residue ellipticity at 220 nm, $[\theta]_{220}$, of BDH at 25 °C. All solutions contained 0.25 mg of BDH/mL, 27 mM LiBr, 0.34 mM DTT, and 68 μM Hepes (pH 7.0); the sample without GdmCl also contained 150 mM NaCl: Apo-BDH (○); BDH-MPL complex (100 μg of phosphorus/mg of BDH) (◇). The dashed line between 0.3 and 0.8 M GdmCl indicates a region of limited BDH solubility; e.g., at 0.5 M GdmCl, an opalescent solution appears after the addition of GdmCl, and with time, the protein precipitates. The inset shows the variation in $[\theta]_{220}$ of apo-BDH (0.25 mg/mL, 0.2 M NaCl, 2 mM DTT, 10 mM Hepes, pH 7.0) between 10 and 35 °C. The values are normalized to the ellipticity at 25 °C.

under a wide variety of conditions, is shown in FIGURE 6/ Within experimental error, the spectra obtained at various solvent conditions and protein concentrations from 0.13 to 0.26 mg/mL were not significantly different. The spectrum is characterized by a negative extremum at 210 nm and a shoulder at 209 nm. The use of a reducing agent (DTT) to maintain the enzymatic activity prohibited meaningful CD spectra below about 205 nm.

The spectrum in Figure 6 was analyzed by comparison with reference spectra of proteins of known conformation (Chen et al., 1974), using constrained linear least-squares analysis[2] (Holladay and Puett, 1977). The following estimates of ordered secondary structures were obtained: α helicity, 29%; β structure, 6%.

The CD spectrum of BDH at protein concentrations from 0.13 to 0.26 mg/mL was also obtained with added MPL (30–170 μg of P/mg of protein), and the resulting spectrum (not shown) was quite similar to that of apo-BDH, at least between 215 and 240 nm. Below 215 nm, the light absorption and opalescence of the BDH–MPL solution (as indicated by a very noisy signal and a relatively high dynode voltage) prohibit any quantitative estimation of the conformation of the enzyme activated with MPL. The existing instrumentation is not sufficiently sensitive to permit CD measurements at low concentrations of protein (up to 10 μg/mL), so that the conformation of BDH activated with PC(8:0) could not be obtained.

The variation of $[\theta]$ with the GdmCl concentration was also determined under equilibrium conditions. Rather than the cooperative-like changes noted with many globular proteins (Pace, 1975), $[\theta]$ of apo-BDH decreased continuously with increasing denaturant concentration between 0 and 5.6 M GdmCl (Figure 7). The addition of MPL to solutions of BDH at the various GdmCl concentrations had no noticeable effect on the ellipticity as demonstrated in Figure 7. The effect of temperature on the ellipticity at 220 nm was also investigated. As shown in the inset to Figure 7, $[\theta]_{220}$ changes in a linear fashion with temperature between 10 and 35 °C.

The near-UV CD spectrum of concentrated apo-BDH (1.85 mg/mL) was also obtained (inset in Figure 6). Between about 250 and 285 nm there was very little ellipticity, presumably due to band cancellation. However, a positive CD band was observed at 291.5 nm which is assigned to the tryptophanyl $^1L_b$ electronic transition (Strickland, 1974). Interestingly, the addition of MPL (73 μg of P/mg of BDH) to the apo-BDH solution considerably reduced the magnitude of the positive extremum.

## Discussion

BDH is one of the best studied of the lipid-requiring enzymes. The enzyme has been purified to homogeneity (Bock and Fleischer, 1974, 1975) and has an absolute requirement for lecithin for activity (Fleischer et al., 1974; Gazzotti et al., 1975). The active form of the enzyme is the BDH–PL complex. In this study, we have used PC(8:0) to form an active BDH–PC(8:0) complex and have studied the hydrodynamic properties of BDH–PC(8:0) under conditions equivalent to those used in the enzymatic assay.

We have shown by conventional sedimentation velocity studies that the apoenzyme undergoes concentration-dependent self-association and, in solution, is a heterogeneous population of species. At 60 μg/mL, apo-BDH has an $s_{20,w}$ of approximately 4.0, which is close to that expected for a spherical monomer of 31 000 daltons (Figure 3). Since, in the absence of added lecithin, apo-BDH is inactive even at low protein concentrations (60 μg/mL or lower), we conclude that the dissociation of apo-BDH is not, of itself, sufficient for activation of the enzyme.

A hydrodynamic characterization of BDH activated with natural lecithins, which form liposomes in solutions, is obviously not meaningful. We have used the techniques of active enzyme sedimentation and free diffusion from an initial layered boundary to obtain $s_{20,w}$ and $D_{20,w}$, respectively, of the active BDH–PC(8:0) complex at protein concentrations equivalent to assay conditions. At 2.4 μg of BDH/mL in the presence of 125 μM PC(8:0), the enzyme has a specific activity at 25 °C of 20.9 (75% of the activity obtained with MPL activation in

[2] The least-squares analysis was constrained so that the sum of the percentages of α helicity, β structure, and random coil was set equal to 100%.

the same assay medium); at this protein concentration, $s_{20,w}$ and $D_{20,w}$ give a calculated molecular weight of 61 600 which corresponds to a dimer. With increasing protein concentration, the enzyme self-associates and the specific activity decreases. This decrease in specific activity is not due to a limiting amount of PC(8:0), since the concentration of unbound PC(8:0) does not change appreciably in this concentration range of BDH. The self-association of the BDH–PC(8:0) complex with increasing BDH concentration may result, at least in part, from the failure of the short acyl chains of PC(8:0) to cover certain hydrophobic regions of the protein, and these domains would then be available to participate in the protein–protein association.

It must be emphasized that the hydrodynamic parameters of the active enzyme (Tables I and II) pertain only to the species capable of being activated by PC(8:0), i.e., the molecular species that are active or are in rapid reversible equilibrium with the active form(s). The methods used exclude contributions from altered BDH molecules that are not activatible by lipid but which would contribute to the apparent $s_{20,w}$ and $D_{20,w}$ measured in conventional hydrodynamic studies where total protein is monitored.

We conclude from the sedimentation and diffusion coefficients of BDH–PC(8:0) at 2.4 μg of BDH/mL that the smallest species with the highest activity must be either the monomer or dimer. An active monomer in equilibrium with higher molecular weight species could exhibit sedimentation and diffusion characteristics similar to those observed, i.e., an apparent dimer. Reliable data could not be obtained at BDH concentrations lower than 2.4 μg/mL, thus preventing a direct clarification of this problem. We have shown previously by equilibrium binding measurements that only one NADH molecule binds to two subunits of active BDH (Gazzotti et al., 1974). The hydrodynamic properties and the NADH-binding characteristics taken together indicate that the dimer is the active form of BDH.

Our hydrodynamic studies contrast with those of Levy et al. (1976) who measured the sedimentation coefficient of a partially purified preparation (specific activity of 3.1 μmol of $NAD^+$ reduced $min^{-1}$ $mg^{-1}$) of apo-BDH from rat liver mitochondria. Using the sucrose gradient sedimentation velocity method, they obtained, for the apo-BDH protein, an $s_{20,w}$ of 5.0, which did not vary as the initial layered protein concentration was increased from 0.9 to 10.7 mg/mL. The lipid-activated enzyme was not studied. They concluded from this study, together with gel-exclusion chromatography, that apo-BDH, solubilized from rat liver mitochondria, is one molecular species, a dimer. We have used the sucrose density gradient sedimentation method (data not presented) with the purified bovine heart mitochondrial apo-BDH and obtained results similar to those we have arrived at with the analytical ultracentrifuge, i.e., a significant variation of $s_{20,w}$ with protein concentration. The differences between our results and those of Levy et al. cannot, therefore, be due to the different methods used but could arise from the different source and purity of the enzyme or from the different isolation methods. Cholate was used to solubilize the rat liver enzyme (Levy et al., 1976), and it is possible that sufficient detergent remained in their enzyme preparation to mask hydrophobic sites on apo-BDH which would normally participate in the self-association.

The far-UV CD spectrum of apo-BDH indicates the presence of appreciable α helicity (ca. 30%) but only limited β structure; with this percentage of α helicity, any estimate of β structure is subject to considerable error (Holladay and Puett, 1977). Under the conditions used, MPL up to 170 μg of phosphorus/mg of BDH had little effect on the CD spectrum between 215 and 240 nm; however, the problem of light scattering prohibits any quantitative conclusions. MPL diminishes the 291.5-nm CD band of apo-BDH, which suggests that lecithin binding alters the microenvironment of one or more tryptophanyl residues in the enzyme. Due to instrumental limitations, the measurement of the CD spectrum of the BDH–PC(8:0) complex at low protein concentrations where it is active was not possible.

The apparent noncooperative changes in the mean residue ellipticity at 220 nm with GdmCl indicate that apo-BDH has considerable conformational flexibility (i.e., equienergetic states) during equilibrium unfolding. Obviously, this may be related to the associated state of apo-BDH in the absence of denaturants. The temperature dependence of $\theta_{220}$ indicates that no cooperative-like thermal transitions involving major conformational changes occur over the temperature range investigated.

The effect of MPL on the CD spectrum of BDH contrasts with the effect of lecithin on the CD spectrum of high-density lipoprotein (Jackson et al., 1973). The α-helical content of the apolipoprotein, estimated to be approximately 49% in the absence of phospholipid, increases significantly to 65% after rebinding lecithin, which suggests a lecithin-induced transformation in the secondary structure of the protein. We observe no major changes in the CD spectrum between 215 and 240 nm when MPL is added to BDH; this indicates little conformational change involving secondary structure of apo-BDH with lecithin. These results are consistent with a cofactor role for lecithin, previously indicated by the half-maximal activation of apo-BDH by only a few equivalents of lecithin (e.g., two to four molecules per apo-BDH subunit give half-maximal activation, Gazzotti et al., 1975).

These studies represent the first comprehensive physicochemical characterization of the active form of a lipid-requiring enzyme. The sedimentation, diffusion, and circular dichroic properties of BDH, purified to homogeneity from the mitochondrial inner membrane, have been measured. We have used the active-enzyme sedimentation method of Cohen and Mire (1971) to study the active form of the enzyme, using the soluble lecithin, PC(8:0), to form the active BDH–PC(8:0) complex. Both the apoenzyme and BDH–PC(8:0) exhibit concentration-dependent self-association, even in the microgram concentration range. By correlating hydrodynamic, enzymatic, and NADH-binding data, we have shown that the active form of the BDH–PC(8:0) complex is a dimer.

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