with some of the experiments described above.

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Use of Phospholipase D To Alter the Surface Charge of Membranes and Its Effect on the Enzymatic Activity of D-β-Hydroxybutyrate Dehydrogenase†

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ABSTRACT: The effect of an electrostatic potential on the enzymatic activity of D-β-hydroxybutyrate dehydrogenase was examined. Phospholipase D was used to increase the surface charge and concomitantly the electrostatic potential of submitochondrial membranes. The apparent K_m for the negatively charged substrates of D-β-hydroxybutyrate dehydrogenase increased as the membranes were reacted with phospholipase D. There was a 10-fold increase in the apparent K_m for NADH when the content of acidic phospholipids was increased by 24%. The addition of monovalent or divalent cations, which reduced the electrostatic potential, largely reversed the apparent K_m changes. At the same ionic strength, divalent cations had a substantially larger effect than monovalent cations. Similar results were obtained when the purified apoenzyme was reconstituted in unilamellar vesicles containing different ratios of phosphatidylcholine and acidic phospholipids. When the apoenzme was reconstituted into phosphatidylcholine vesicles containing increasing amounts of phosphatidylethanolamine, the apparent K_m also increased but to a smaller extent, and increasing the ionic strength did not reverse this effect. The results show that the apparent K_m of D-β-hydroxybutyrate dehydrogenase can be significantly altered by an electrostatic potential as well as other properties of the phospholipid polar head group.

Electrostatic potentials have been shown to affect the catalytic activity of enzymes in a variety of systems (Douzou & Maurel, 1977). One approach to studying these effects has been to immobilize trypsin and chymotrypsin to an insoluble polyanionic carrier (Goldstein et al., 1964; Goldstein, 1972). The polyanionic environment was found to promote a strong local negative electrostatic potential which acted on the chemical properties of the medium. In particular, the local concentration of ionic species were found to be different from those of the bulk solution. Another study employing lysozyme in the polyanionic environment of the cell wall of *Micrococcus luteus* has shown similar results (Maurel & Douzou, 1976). Relatively few studies have been carried out on the effect of an electrostatic potential on the properties of membrane-bound enzymes. Recently, Wojtczak & Nalecz (1979) and Nalecz et al. (1980) used detergent methods and fatty acids to increase the electrostatic potential of a biological membrane and observed the effect on several membrane-bound enzymes.

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Significant electrostatic potentials exist at the surface of most biological membranes [for a review, see McLaughlin (1977)]. An important consequence of the bilayer organization of membrane phospholipids is that the concentration of phospholipid polar head groups at the surface is quite high. For a membrane composed of 20% acidic phospholipids, the concentration of negative charges at the membrane surface is approximately 1 M. This polyanionic environment gives rise to a strong electrostatic potential. The electrostatic potential attracts ions of the opposite charge to the membrane and repels ions of the same charge from the membrane.

The electrostatic potential that exists at a charged surface and the ion distribution at that surface are described by the Gouy–Chapman theory. This theory has been tested with electrostatic potentials that exist on phospholipid bilayers (McLaughlin, 1977). The electrostatic potential was found to be sensitive to surface charge density and the bulk salt concentration. An increase in the charge density caused the electrostatic potential to increase, while an increase in the salt concentration caused the electrostatic potential to decrease by neutralizing the surface charge.

In this study, the effect of an electrostatic potential on the catalytic activity of D-β-hydroxybutyrate dehydrogenase (BDH)[1] was examined. BDH is an integral membrane enzyme found in mitochondria which catalyzes a reaction involved in the generation and utilization of ketone bodies [for a review, see Fleischer et al. (1974a)]. It catalyzes the following reaction: D-β-hydroxybutyrate + NAD^+ ↔ acetoacetate + NADH + H^+. BDH has been purified to homogeneity (Boch & Fleischer, 1974), and it is one of the best characterized enzymes that shows a specific phosholipid requirement (Sekuzu et al., 1961; Gazzotti et al., 1974). In reconstitution experiments, it was shown that the polar head groups of the phospholipids must contain choline to reactivate the purified apoenzyme (Isaacson et al., 1979; Grover et al., 1975). All of the substrates of the forward and reverse reactions are negatively charged at pH 7.5. Previously published observations suggested to us that this enzyme is sensitive to an electrostatic potential. The kinetics of this enzyme have been shown to be sensitive to the bulk salt concentration. For example, the addition of salt lowered the apparent K_m for D-β-hydroxybutyrate (Nielson et al., 1973). An explanation for this observation is that the salt neutralized the electrostatic potential, and this caused an increase in the surface concentration of negatively charged substrates. The apparent K_m, which reflects the surface concentration of the substrate, therefore, decreased with salt addition.

For determination of whether BDH was sensitive to an electrostatic potential, the charge density in submitochondrial membranes was altered with phospholipase D. Phospholipase D catalyzes the hydrolysis of primarily zwitterionic phospholipids to phosphatidic acid, an acidic phospholipid. The results obtained with phospholipase D were compared with a reconstituted system, and these results show that the activity of BDH can be dramatically altered by an electrostatic potential as well as by other properties of the phospholipid polar head groups.

Materials and Methods

Submitochondrial Membrane Preparation. Mitochondria were isolated from beef heart by using a modified procedure described by Blair (1967). The outlined procedure was followed through the low-speed centrifugation step. The supernatant from the low-speed spin was centrifuged at 20000*g* for 10 min, and the mitochondrial pellet was resuspended with a 15-mL Dounce homogenizer to a protein concentration of 10 mg/mL in 1 mM Tris, pH 7.4, and 0.25 M sucrose. The mitochondria were converted to submitochondrial membranes as described by Fleischer et al. (1974b), suspended in 1 mM Tris, pH 7.4, and 0.25 M sucrose, and stored at 0 °C at a protein concentration of 10 mg/mL.

D-β-Hydroxybutyrate Dehydrogenase Assays. The forward and reverse reactions catalyzed by BDH were studied in reaction mixtures that contained 2 mM dithioerythritol, 0.5 mg/mL bovine serum albumin, and either 5 mM sodium phosphate, 15 mM Tris, or 15 mM imidazole, pH 7.4. The substrate concentrations are given in the text. When BDH in submitochondrial membranes was assayed, 2.5 μg/mL antimycin A was added. The reaction mixture was preincubated for 10 min at 37 °C before starting the reaction by the addition of either DL-β-hydroxybutyrate or acetoacetate. The appearance or disappearance of NADH was monitored by the absorbance at 340 nm and converted to units of activity by using an extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1}. For all K_m determinations, sodium chloride was used to maintain the ionic strength[2] constant as the substrate concentration was varied.

BDH Reconstitution. Beef heart phosphatidylcholine and egg phosphatidic acid were purchased from Sigma Chemical Co. Phosphatidylinositol was purchased from the Serdary Research Laboratories. *Escherichia coli* phosphatidylethanolamine was purchased from Sigma Chemical Co. and Avanti Polar Lipids, Inc. Reconstitution of BDH into phosphatidylethanolamine-containing vesicles from either source gave similar results. For preparation of unilamellar vesicles, the phospholipids were dissolved in chloroform, and the chloroform was evaporated to dryness under nitrogen. The phospholipids were dissolved in 10 mM Tris and 1 mM ethylenediaminetetraacetic acid, pH 8.1, and sonicated to obtained unilamellar vesicles as described by Barrow & Lentz (1980). BDH was purified to homogeneity according to the method of Boch & Fleischer (1974).

A lipid–protein complex was formed by incubating 25 μg of the enzyme at 27 °C with phospholipids in 200 μL of 75 mM NaCl, 2.5 mg/mL bovine serum albumin (fatty acid free), 25 mM Tris, pH 7.4, and 2 mM dithioerythritol. After preincubation for 15 min, an aliquot of the complex was assayed as described above.

Phospholipase D Isolation. Other degradation products along with phosphatidic acid were observed when submitochondrial membranes were reacted with phospholipase D purchased from Sigma Chemical Co. Phospholipase D was therefore purified away from other lipases contained in crude preparations of phospholipase D.

Phospholipase D was purified from Savoy cabbage to an acetone powder as described by Yang (1969). The enzyme was then further purified by using the procedure of Allgyer & Wells (1979), except the steps involving the ether wash and Sephadex G-200 fractionation were omitted. All steps were performed at 4 °C. The partially purified enzyme was stored at −20 °C in a solution of 50 mM *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid, pH 6.5, and 50% ethylene glycol. Prior to reaction with submitochondrial membranes,

[1] Abbreviations used: AcOAc, acetoacetate; BDH, D-β-hydroxybutyrate dehydrogenase; βHB, D-β-hydroxybutyrate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Tris, tris(hydroxymethyl)aminomethane.

[2] Ionic strength = $^1/_2\sum_i C_i Z_i^2$ where C_i is the molar concentration of an ion and Z_i is the charge on the ion.

the enzyme was put through a Sephadex G-25 column equilibrated with 2 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, pH 7.0, and 0.3 M inositol to remove the ethylene glycol. During the isolation of phospholipase D, the enzyme was assayed in a 200-μL mixture of 200 mM acetate pH 5.6, 50 mM $CaCl_2$, 500 μM ^{3}H-labeled egg phosphatidylcholine (1000 dpm/nmol labeled in the choline methyl groups), and 50 μL of ether. The reaction was carried out for 10 min at 30 °C, and it was terminated with the addition of 20 μL of 5 N HCl and 1 mL of chloroform. The unreacted egg phosphatidylchloline was extracted into the chloroform. An aliquot of the aqueous phase containing the free [^{3}H]choline was counted. One unit of phospholipase D is defined as the amount necessary to hydrolyze 1 μmol of phosphatidylcholine per h. Phospholipase D comprised 10–20% of the total protein in the purified fraction used in this study.

Phospholipase D Reaction with Submitochondrial Membranes. The reaction mixture was composed of 0.5 mg/mL bovine serum albumin, 1 mM dithioerythritol, 1 mM NAD, 50 mM acetate, pH 5.6, 50 mM $CaCl_2$, 2.5 mg/mL submitochondrial membrane protein, and 125 mM sucrose in a volume of 1.6 mL. The reaction mixture was started with the addition of 0.4 mL of phospholipase D. The reaction mixture was incubated for 15 min at 37 °C, and it was terminated by the addition of 5.0 mL of a solution containing 50 mM Tris, pH 7.5, and 150 mM ethylenediaminetetraacetic acid. The sample was centrifuged at 100000*g* for 60 min. The pellets were resuspended in a solution of 1 mM Tris, pH 7.4, 0.25 M sucrose, and 5 mM dithioerythritol.

Lipid Extraction and Analysis. Membrane samples were adjusted to 0.1 M HCl and the lipids extracted by the method of Bligh & Dyer (1959) as described by Ames (1968). The phospholipid compositions were determined by two-dimensional thin-layer chromatography on Redi-coat 2D (250 μm, Supelco, Inc.) with $CHCl_3$–CH_3OH–NH_4OH (65:25:5) as the solvent system for the first dimension and $CHCl_3$–acetone–CH_3OH–acetic acid–water (3:4:1:1:0.5) as the solvent system for the second dimension. The plate was exposed to iodine vapor in order to visualize the phospholipids. The phospholipids were scraped and eluted with 5 mL of $CHCl_3$–CH_3OH–acetic acid–water (5:5:1:1) and 2 mL of methanol. Phospholipid phosphate was determined by the ashing procedure of Ames (1966).

Results

The apparent K_m values for the negatively charged substrates of BDH in submitochondrial membranes changed subtantially when the ionic strength was increased. Approximately a 3-fold decrease in the apparent K_m for NADH was observed when 50 mM NaCl was added to the standard assay mixture. The maximal velocity also decreased 25% with salt addition, similar to the observations of Nielson et al. (1973). For determination of whether the salt effects were due to an electrostatic potential, submitochondrial membranes were reacted with phospholipase D to change the surface charge. Phospholipase D was purified from Savoy cabbage to eliminate lipases which are present in the crude preparations that are commercially available (see Materials and Methods). The phospholipid composition of submitochondrial membranes that were reacted with phospholipase D is shown in Table I. By addition of different amounts of phospholipase D, the amount of phosphatidic acid produced was varied. Phosphatidylcholine and phosphatidylethanolamine were the primary phospholipids hydrolyzed. When these membranes were assayed for BDH activity, the apparent velocity fell dramatically with increasing

Table I: Phospholipid Composition of Submitochondrial Membranes That Were Reacted with Phospholipase D[a]

phospholipase D (units/mL)	phospholipid composition (%)			
	PC	PE	PA	other[b]
0	42	41	0	17
7	34	34	16	16
10	31	30	24	15
20	20	19	43	19

[a] Reaction with phospholipase D was performed as described under Material and Methods. [b] This category consists of several minor phospholipids including cardiolipin, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and sphingomyelin.

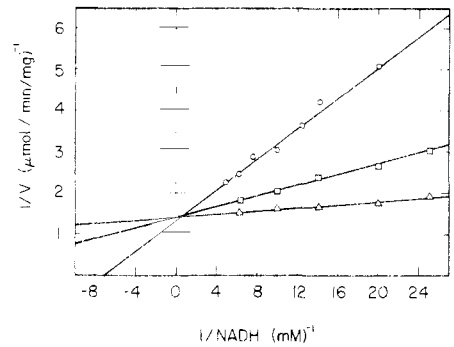

FIGURE 1: Double-reciprocal plot of NADH vs. the specific activity of BDH in submitochondrial membranes that were reacted with zero (△), 7 units/mL (□), or 10 units/mL (○) phospholipase D. The concentration of AcOAc was 4.0 mM, and the assays were performed in the standard assay mixture plus 16 mM NaCl.

phosphatidic acid content (data not shown). When up to 24% phosphatidic acid was produced, the decrease in the velocity reflected an increase in the apparent K_m for the charged substrates for BDH. Representative data for the activity of BDH assayed at different concentrations of NADH are shown in Figure 1. The apparent K_m increased as the phospholipid polar head groups of the submitochondrial membranes were hydrolyzed with phospholipase D while the maximal velocity did not change. Data from an extensive analysis of the kinetics of BDH for the substrates of the forward and reverse reactions, assayed at an identical ionic strength, are shown in Table II. With the accumulation of 24% phosphatidic acid, approximately a 10-fold increase in the apparent K_m for NADH in the reverse reaction and NAD in the forward reaction was observed. For βHB and AcOAc, approximately a 3-fold maximal increase in the apparent K_m was observed. The error associated with the K_m measurements did not exceed 25%. More extensive hydrolysis of these membranes did not yield any further increase in the apparent K_m, although there was a decrease in the maximal velocity.

The activity of BDH in phospholipase D reacted membranes was sensitive to the addition of salt, as illustrated in Figure 2. For a phospholipase D reacted sample that contained 24% phosphatidic acid, the K_m's for the substrates AcOAc and NADH were 1.3 mM and 0.24 mM, respectively, under the assay conditions employed. In this experiment, the AcOAc concentration was set at a near saturating concentration of 4.0 mM, and the NADH concentration was set at the subsaturating concentration of 0.16 mM. The assays were then performed with the addition of different concentrations of NaCl. The addition of NaCl increased the apparent velocity approximately 3-fold at 60 mM ionic strength (Figure 2A). Because the addition of NaCl also affected the velocity of control membranes, the velocity of the reacted sample is expressed as the percent of a control sample (Figure 2C). The addition of divalent cations also caused an approximately 3-fold

Table II: Apparent K_m for the Forward and Reverse Reactions of D-β-Hydroxybutyrate Dehydrogenase in Submitochondrial Membranes with Different Amounts of Phosphatidic Acid[a]

substrate	PA (%)	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
NADH	0	0.014	0.71
	16	0.053	0.71
	24	0.14	0.70
	43	0.12	0.22
NAD	0	0.19	2.2
	16	1.0	2.2
	24	2.4	1.5
	43	2.2	0.43
AcOAc	0	0.50	0.75
	24	1.3	0.65
	43	1.4	0.25
βHB	0	1.8	2.3
	24	6.5	1.7
	43	6.0	0.53

[a] When the K_m values were determined for NADH, AcOAc, NAD, or βHB, the assay mixture contained 4.0 mM AcOAc, 0.2 mM NADH, 10 mM βHB, or 4.0 mM NAD, respectively. K_m values were determined from primary double-reciprocal plots. The assays for the K_m of NADH and AcAc contained an additional 16 mM NaCl.

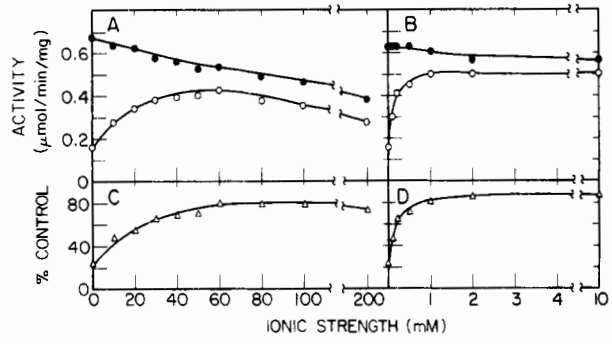

FIGURE 2: Effect of ionic strength on the apparent velocity of control (●) membranes and membranes that were reacted with 10 units/mL phospholipase D (○). Either NaCl (A) or $CaCl_2$ (B) was added to the standard assay mixture plus 10 mM NaCl containing 4.0 mM AcOAc and 0.16 mM NADH. In panels C and D, the velocity of the samples is expressed as a percent of the control velocity vs. the increase in ionic stength due to the addition of either NaCl or $CaCl_2$.

increase in the apparent velocity at 1 mM ionic strength (Figure 2B and 2D). Divalent cations, however, were over 2 orders of magnitude more effective at increasing the apparent velocity of BDH in the reacted sample than monovalent cations, as shown in Figure 3. Similar titration profiles were obtained with $MgCl_2$, $BaCl_2$, or $CaCl_2$. Monovalent and divalent cations had a similar effect on the velocity of BDH measured with the other substrates as well (data not shown). This increase in the apparent velocity when the ionic strength increased was due to changes in the apparent K_m. Representative data for NADH are shown in Figure 4. Small changes in the maximal velocity and a dramatic decrease in the apparent K_m were observed with an increase in the ionic strength. The apparent K_m for the substrates of the forward and reverse reactions of BDH assayed with different concentrations of sodium or calcium chloride are shown in Table III. For a phospholipase D reacted sample that contained 24% phosphatidic acid, increasing the ionic strength largely reversed the changes in K_m for charged substrates in both the forward and reverse reactions. Divalent cations were more effective at lowering the apparent K_m at lower ionic strengths than were monovalent cations. Addition of 0.34 mM $CaCl_2$ resulted in approximately a 5-fold and a 2-fold decrease in the K_m for the

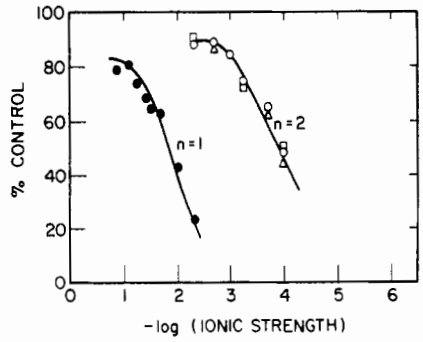

FIGURE 3: Effect of NaCl (●), $CaCl_2$ (○), $BaCl_2$ (△), or $MgCl_2$ (□) on the velocity of BDH in submitochondrial membranes that were reacted with 10 units/mL phospholipase D. The data are expressed as a percent of the control velocity at each ionic strength for monovalent (n = 1) or divalent (n = 2) cations. All assays were performed as described in Figure 2.

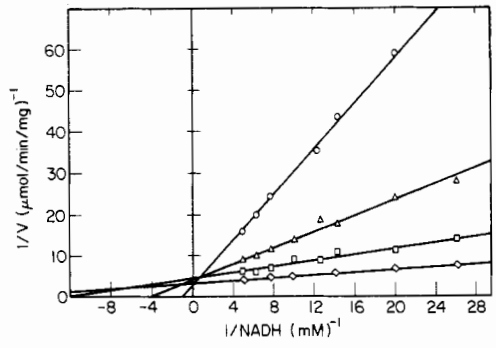

FIGURE 4: Double-reciprocal plot of NADH vs. specific activity of BDH in submitochondrial membranes that were reacted with 10 units/mL phospholipase D. The assays were carried out in the standard assay mixture containing 4.0 mM AcOAc, with either no addition (○), 10 mM NaCl (△), 50 mM NaCl (□), or 0.34 mM $CaCl_2$ (◇).

Table III: Apparent K_m for the Forward and the Reverse Reactions of BDH in Submitochondrial Membranes Assayed with Different Concentrations of Sodium Chloride or Calcium Chloride[a]

substrate	phospholipase D reacted	salt	K_m (mM)
NADH	−	no addition	0.030
		0.34 mM $CaCl_2$	0.022
	+	no addition	0.24
		40 mM NaCl	0.081
		0.34 mM $CaCl_2$	0.046
NAD	−	no addition	0.14
		0.34 mM $CaCl_2$	0.14
	+	no addition	1.9
		0.34 mM $CaCl_2$	0.33
AcOAc	−	no addition	0.50
		0.34 mM $CaCl_2$	0.61
	+	no addition	1.3
		0.34 mM $CaCl_2$	0.77
βHB	−	no addition	1.5
		0.34 mM $CaCl_2$	1.6
	+	no addition	6.5
		0.34 mM $CaCl_2$	2.1

[a] Mitochondrial membranes were reacted with phospholipase D to produce approximately 25% phosphatidic acid as described in the text. When the K_m values were determined for NADH, AcOAc, NAD, or βHB, the assay mixture contained 4.0 mM AcOAc, 0.2 mM NAD, 10 mM βHB, or 4.0 mM NAD, respectively. K_m values were determined from primary double-reciprocal plots. The assays for the K_m of NADH and AcOAc also contained 10 mM NaCl.

substrates NADH and AcOAc, respectively, while in the other direction there was a 6-fold and a 3-fold decrease for NAD and βHB, respectively.

The electrostatic potential at the active site of BDH has an opposite effect on the concentration of cations and anions. When the electrostatic potential produces a 10-fold decrease in the concentration of negatively charged substrated (as observed in Table II), a 10-fold increase in the proton concentration, 1 pH unit, would also be expected to occur. K_m measurements for NAD and NADH were made for BDH in submitochondrial membranes at pH 6.4 and 7.4 which is the normal pH of the assay mixture. For either substrate, the K_m values were found to be essentially identical (data not shown). Therefore, a change in the pH that would result from changes in the electrostatic potential produced in this study did not alter the K_m of BDH.

For substantiation of the results on the effect of an electrostatic potential obtained by the use of phospholipase D, additional experiments were carried out on the purified apoenzyme reconstituted into phospholipid vesicles. The surface charge was varied by reconstituting BDH into unilamellar vesicles containing phosphatidylcholine and different amounts of either phosphatidylinositol or phosphatidic acid. As the concentration of negatively charged phospholipids and the surface charge increased, the K_m for NADH and AcOAc increased (Table IV). For example, the addition of 25% acidic phospholipids resulted in approximately a 4-fold increase in the apparent K_m for NADH and a 2-fold increase in the apparent K_m for AcOAc. The addition of 0.34 mM $MgCl_2$ to the reaction mixture lowered the apparent K_m to values approaching that of the control. Reconstitution of the apoenzyme into unilamellar vesicles containing different amounts of the zwitterionic phospholipid, phosphatidylethanolamine, also increased the apparent K_m for NADH, but to a smaller extent. The addition of $MgCl_2$ to these vesicles had no effect, indicating that the change in the apparent K_m was due to other parameters besides the electrostatic potential. The apparent K_m for AcOAc was not significantly altered when the concentration of phosphatidylethanolamine was increased. DeKruijff et al. (1980) have reported that phosphatidylethanolamine as well as some other phospholipids can form nonbilayer phases. Phosphatidylethanolamine from *E. coli* which was used in this study forms a bilayer (Gally et al., 1980), and all the phospholipid mixtures contained at least 50% phosphatidylcholine.

Table IV: Apparent K_m of Purified BDH Reconstituted into Unilamellar Vesicles[a]

substrate	phospholipid composition (%)		K_m (μM)		lipid/protein[b] (mol/mol)
			$-MgCl_2$	$+MgCl_2$	
NADH	PI	PC			
	0	100	8	10	620
	25	75	33	21	160
	50	50	52	24	80
	PA	PC			
	0	100	8	10	620
	25	75	39	25	160
	50	50	75	24	80
	PE	PC			
	0	100	8	10	620
	25	75	15	15	620
	50	50	21	23	620
AcOAc	PI	PC			
	0	100	200	240	620
	25	75	330	180	160
	50	50	540	230	80
	PA	PC			
	0	100	200	240	620
	25	75	460	200	160
	50	50	690	280	80
	PE	PC			
	0	100	200	240	620
	25	75	250	270	620
	50	50	190	230	620

[a] When the K_m values were determined for NADH or AcOAc, the assay mixture contained either 4.0 mM AcOAc or 0.16 mM NADH, respectively. K_m values were determined from primary double-reciprocal plots. BDH was reconstituted as described under Materials and Methods and assayed in the standard assay mixture containing an additional 10 mM NaCl plus 5 mM LiBr. K_m values were determined in the presence and absence of 0.34 mM $MgCl_2$. [b] Reconstitution mixtures were made that contained 25 μg of BDH and different concentrations of phospholipids. The protein to lipid ratio that gave the maximum activity was used to determine the K_m. Similar maximal velocities were observed for BDH in all of the reconstitutions.

Discussion

Two different methods were used in this study to determine the effect an electrostatic potential would have on the activity of BDH. The first involved changing the phospholipid composition of submitochondrial membrane with phospholipase D, and the second involved reconstitution of the purified apoenzyme with different phospholipid vesicles. Of these two methods, the use of phospholipase D appears to be the preferred means to alter the surface charge because it is not subject to several potential problems inherent in the reconstitution studies. In reconstitution studies, one must be concerned with the stability of the apoenzyme, the efficiency of the reconstitution, and the integrity of the lipid vesicles. Some phospholipids, for example, may not form unilamellar vesicles and may form nonbilayer structures (DeKruijff et al., 1980). Also different phospholipid vesicles may not allow proper exposure of the phosphatidylcholine head group to BDH or may not allow the proper insertion of BDH into the bilayer. The variations in these properties between different phospholipids may be the reason why different protein to lipid ratios had to be used to obtain maximal activities (Table IV). The use of phospholipase D, on the other hand, allowed the study of BDH in submitochondrial membranes under conditions where there was no change in the total activity of the enzyme (i.e., the maximal velocity remained constant until the membranes contained more than 24% phosphatidic acid). This method also seems preferable to the use of fatty acids or detergents to manipulate the surface charge (Wojtczak & Nalecz, 1979; Nalecz et al., 1980) since these agents can have multiple effects on the bilayer as well as directly affecting the enzymes.

The results reported in this study show that the apparent K_m's for the charged substrates of BDH were sensitive to parameters such as the surface charge and bulk salt concentration that modulate an electrostatic potential. When the charge density at the surface of the membrane was increased by using phospholipase D, the apparent K_m for NADH increased by 10-fold at 24% phosphatidic acid, for example. Increasing the ionic strength largely, but not entirely, lowered the apparent K_m values to the values for a nonreacted sample. At the same ionic strength, divalent cations had a substantially larger effect than monovalent cations on the electrostatic potential.

Despite the possible problems in the reconstitution experiments, the data were generally consistent with the phospholipase D results. As the surface charge increased, so did the

apparent K_m for the charged substrates, and these changes were largely reversed by the addition of salt. Reconstitution of BDH into vesicles which contained phosphatidylethanolamine and phosphatidylcholine gave an interesting result. As the phoshatidylethanolamine increased, the K_m for NADH also increased. However, this increase was not reversed by increasing the ionic strength. This observation may reflect the fact that BDH requires phosphatidylcholine for enzymatic activity (Sekuzu et al., 1961) and utilizes phosphatidylcholine as a necessary cofactor for the binding of NADH (Gazzotti et al., 1974). This also may explain why the changes observed with phospholipase D were not completely reversible by increasing the ionic strength. One membrane property cannot be changed without perturbing others. That is, as the concentration of phosphatidylethanolamine or phosphatidic acid was increased, another surface property changed that was important to the K_m of BDH, perhaps the accessibility of the choline polar head groups or the reduced phosphatidylcholine concentration itself.

The reach of an electrostatic potential at the surface of a membrane probably extends no more than 10 Å. The active site of the enzyme must be very close to the surface of the membrane in order to be influenced by an electrostatic potential. Therefore, different enzymes would be affected by an electrostatic potential to different extents. The reach of an electrostatic potential can be increased by increasing the charge density. In this study, as the charge density increased, so did the electrostatic effect on the kinetics of BDH. Another result observed in this study was that divalent cations were more effective at eliminating the electrostatic potential than monovalent cations, as predicted by the Gouy–Chapman theory (McLaughlin, 1977). One ramification of this result might be that physiological changes in divalent cations might regulate metabolic processes by modulating an electrostatic potential. Although large changes in the content of zwitterionic phosholipids can occur in cells, the content of acidic phospholipids appears to be closely regulated (Hubbard & Brody, 1975; Becker & Lester, 1977; Glaser et al., 1974). In a recent study carried out by Esko & Raetz (1980) of a mutant mammalian cell line deficient in the synthesis of phosphatidylinositol, for example, compensatory increases in the composition of phosphatidylglycerol were observed. The total acidic lipid composition was found to be maintained at levels comparable to those of the wild type. One explanation for these observations is that many membrane surface phenomena are regulated by an electrostatic potential, and it is, therefore, important to keep the charge density constant.

In conclusion, the results reported in this study show that the apparent K_m of an enzyme such as BDH can be significantly altered by the membrane charge density as well as other properties of the phospholipid polar head groups. The effect of the zwitterionic polar head-group composition on the activity of BDH in a biological membrane is currently being investigated. Further studies such as manipulating the phospholipid composition during cell growth (Glaser et al., 1974; Hale et al. 1977) are necessary to evaluate how these properties alter enzyme kinetics under physiological conditions.

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