Regulation by Lipids of Cofactor Binding to a Peripheral Membrane Enzyme: Binding of Thiamin Pyrophosphate to Pyruvate Oxidase†

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ABSTRACT: Pyruvate oxidase is a peripheral membrane flavoenzyme isolated from *Escherichia coli*. Lipids have been shown to influence dramatically the kinetics of the enzymatic reaction; the V_{max} is enhanced by about 25-fold, and the K_m for both the substrate, pyruvate, and the cofactor, thiamin pyrophosphate, are altered in the presence of lipids. In addition, the Hill coefficient for thiamin pyrophosphate determined using steady-state kinetics is influenced by lipids. In this paper the interaction between thiamin pyrophosphate and pyruvate oxidase has been studied both by equilibrium dialysis and by measuring the ligand-induced quenching of the protein fluorescence. It has been shown that the enzyme possesses one thiamin pyrophosphate binding site per subunit, and that a divalent cation such as Mg^{2+} is required for binding. The dependence of the initial rate of the enzymatic reaction on thiamin pyrophosphate concentration is an excellent reflection of the cofactor binding. It has been demonstrated that both the average dissociation constant and the shape of the isotherm describing cofactor binding are influenced very strongly by lipids. The observed effects are different for the different lipids and detergents used in this study. The results clearly illustrate one manner in which lipids can affect the behavior of a membrane enzyme, by modulating the interaction between the enzyme and those ligands involved in catalysis.

Pyruvate oxidase is a peripheral membrane enzyme which is released from *Escherichia coli* upon sonication and has been purified to homogeneity (O'Brien et al., 1976). The enzyme catalyzes the oxidative decarboxylation of pyruvate to yield acetate plus CO_2. Pyruvate oxidase has been shown previously (O'Brien et al., 1976; Williams and Hager, 1966; Raj et al., 1977) to consist of four identical subunits (mol wt 60 000) each containing a tightly bound FAD which remains associated with the enzyme. A second cofactor, thiamin pyrophosphate, is lost during the enzyme purification and must be added to elicit catalytic activity. This redox enzyme couples with the *E. coli* electron transport chain (Cunningham and Hager, 1975); the evidence indicates that pyruvate oxidase may be located on the inner surface of the *E. coli* cytoplasmic membrane (Shaw, Gennis, and Walsh, manuscript in preparation).

In the presence of lipids, pyruvate oxidase kinetics are dramatically altered. The V_{max} is increased by about 25-fold, and the dependence of the reaction velocity on both the concentration of pyruvate and thiamin pyrophosphate is strongly influenced by lipids (Cunningham and Hager, 1971; Blake and Gennis, manuscript in preparation). For example, in the presence of phosphatidylglycerol, the K_m for thiamin pyrophosphate is decreased from 6 to 3 μM, and the Hill coefficient increases from 1 to 1.4. Only in the presence of those lipids, such as phospholipids. which activate the oxidase at concentrations greater than their critical micelle concentration (cmc),[1] are non-Michaelis–Menten kinetics observed (Blake and Gennis, manuscript in preparation).

The purposes of this paper are to characterize the interaction between pyruvate oxidase and thiamin pyrophosphate, to demonstrate the relation between the results obtained by steady-state kinetics with the cofactor binding isotherm, and to document the effect that various lipids bound to pyruvate oxidase have on the cofactor binding isotherm.

Materials and Methods

Materials. The following reagents were used without further purification: thiamin pyrophosphate, sodium pyruvate, 2,6-dichloroindophenol (DCIP), phosphatidylglycerol prepared from egg yolk lecithin, Triton X-100 (Sigma); thiamin hydrochloride (Nutritional Biochemicals); P_2O_5 (Baker); 85% H_3PO_4, sodium ferricyanide (Fisher); [*thiazole*-2-^{14}C]thiamin hydrochloride (Amersham/Searle). Other materials used were dialysis membranes (Visking tubing, Union Carbide) and Selectron RC52 dialysis membranes (Schleicher and Schuell). All other reagents were analytical grade. Glass-distilled deionized water was used for all solutions. Prior to use phosphatidylglycerol (1 mg/mL) was sonicated under a stream of nitrogen in an ultrasonic bath at room temperature for 1 h. The phosphatidylglycerol was determined to be at least 95% pure by thin-layer chromatography.

[^{14}C]*Thiamin Pyrophosphate.* [*thiazole*-2-^{14}C]Thiamin hydrochloride (50 μCi) with a specific activity of 15.5 μCi/μmol was diluted with 35 mg of unlabeled thiamin hydrochloride. The diluted [^{14}C]thiamin hydrochloride was phosphorylated and partially purified according to the procedure of Tanaka (Matsukawa et al., 1970) and was further purified on an Amberlite CG-50 column as described by Moe and Hammes (1974). The final product was shown to be pure thiamin pyrophosphate by thin-layer chromatography in the solvent system 1-propanol–0.2 M potassium phosphate buffer (pH 4.9)–water (60:20:20, v/v). An extinction coefficient of 8550 M^{-1} cm^{-1} (at 267 nm in 0.02 M sodium phosphate buffer, pH 7.0) was used to determine a specific activity of 7.97 $\times$ 10^5 cpm/μmol for the final [^{14}C]thiamin pyrophosphate product (Wittorf and Gubler, 1970).

Pyruvate Oxidase. Pyruvate oxidase was purified as de-

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[1] Abbreviations used: FAD, flavin adenine dinucleotide; cmc, critical micelle concentration; DCIP, 2,6-dichloroindophenol; EDTA, (ethylenedinitrilo)tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; NADH, reduced nicotinamide adenine dinucleotide.

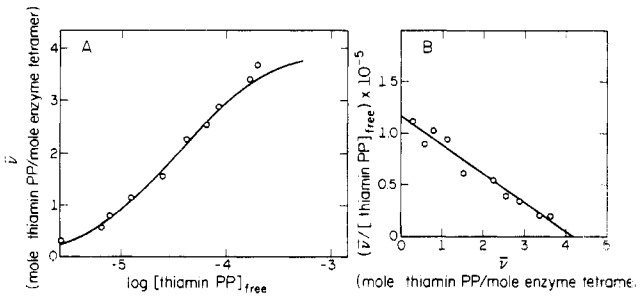

FIGURE 1: (A) Thiamin pyrophosphate binding at 4 °C to active pyruvate oxidase. Binding data were obtained by equilibrium dialysis at 4 °C, 0.1 M sodium phosphate, 10 mM $MgCl_2$, 1.2 mg/mL enzyme, pH 5.7. At the end of the experiment, there was an average recovery of 58 ± 3% of the initial enzymatic activity, and the data were adjusted to take this average loss of active enzyme into account. The solid line is a calculated binding curve assuming 4 independent binding sites with K_d of 28 μM. (B) Scatchard plot of the binding data from part A. The straight line was calculated by a least-squares linear regression analysis of the data and is consistent with 4.2 independent binding sites with a K_d of 28 μM.

scribed previously (O'Brien et al., 1976). The enzyme was judged to be greater than 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970). The three preparations of enzyme which were use for these studies had specific activities between 4900 and 5200 decarboxylase units/mg. A decarboxylase unit has been previously defined as the evolution of 1 μM of CO_2 in 30 min under standard assay conditions (Williams and Hager, 1966).

Kinetic assays to determine the reaction velocity of pyruvate oxidase were performed using a modification of the procedure described previously with either DCIP of sodium ferricyanide as the electron acceptor (O'Brien et al., 1976). When sodium ferricyanide was employed, the final concentration of dye was 8.5 mM, and the catalytic activity was measured by following the decrease in the absorbance at 450 nm. Purified pyruvate oxidase has an absolute requirement for thiamin pyrophosphate in the assays. The assay buffer contained 100 μM thiamin pyrophosphate, 10 mM $MgCl_2$, 200 mM sodium pyruvate, 0.1 M sodium phosphate, pH 5.7, in addition to the lipid activators when specified. When indicated, the thiamin pyrophosphate concentration was varied.

Equilibrium Dialysis. Equilibrium dialysis experiments were done in plexiglass dialysis blocks which contained six 200-μL dialysis chambers. Binding experiments were performed at 4, 20, and 28 °C. In the low temperature experiments Visking tubing (Union Carbide) was used and the blocks were rotated at 10 rpm for 16 h. Large pore RC52 membranes were used for the 20 and 28 °C experiments. Equilibration was reached within 5 h at these temperatures. Before being used for dialysis experiments both membranes were repeatedly rinsed with distilled-deionized water.

After equilibration had been reached [^{14}C]thiamin pyrophosphate binding was determined by measuring the radioactivity in 50-μL samples taken from the dialysis chambers. Samples were counted in 10 mL of Bray's scintillation fluid (Bray, 1960) using a Nuclear Chicago Isocap 300 scintillation counter. Samples were also assayed for enzymatic activity and protein in order to determine the recovery of pyruvate oxidase specific activity over the course of the experiment.

The binding data were graphed in a Bjerrum plot (Weber, 1975) in order to obtain a value for the maximum binding. This value was then used to normalize the binding data to a percent saturation scale. The dissociation constant, K_d, is the thiamin pyrophosphate concentration at 50% saturation.

Fluorescence Measurements. When thiamin pyrophosphate binds to pyruvate oxidase, the intrinsic fluorescence from the protein is quenched by 16%, and this was used as an additional technique for monitoring cofactor binding. All fluorescence measurements were made with a Perkin-Elmer MPF-III fluorimeter equipped with a circulating constant-temperature bath. The temperature of the sample was measured using a YSI Model 42SC telethermometer. To avoid large inner filter corrections due to thiamin pyrophosphate absorption, all fluorescence titrations were done with excitation at 300 nm. Fluorescence intensity was followed at 333 nm using an excitation band-pass of 6 nm and an emission band-pass of 10 nm. The Raman scattering intensity was negligible.

At the end of a titration the fluorescence intensity values were corrected for small volume changes (always less than 5%) and for thiamin pyrophosphate inner filter effects. Since thiamin does not bind to pyruvate oxidase, as judged by steady-state kinetics, this allowed a thiamin quenching curve to be used as an empirical inner filter correction for the thiamin pyrophosphate titration data.

For each titration a value for the binding constant and the final fluorescence intensity at saturating thiamin pyrophosphate were obtained from double-reciprocal plots of $F_0/(F_0 - F)$ vs. $[TPP]^{-1}$, where F_0 is the fluorescence intensity in the absence of the ligand and F is the value at the given ligand concentration. This minimum fluorescence intensity was then used to calculate the percent saturation corresponding to each thiamin pyrophosphate addition.

Results

The binding of thiamin pyrophosphate to pyruvate oxidase at 4 °C in pH 5.7 phosphate buffer is shown in Figure 1. The Bjerrum plot in Figure 1A compares the binding data with the expected binding isotherm assuming four independent and equivalent binding sites per active enzyme tetramer with a dissociation constant of 28 μM. Figure 1B shows the same data in the form of a Scatchard plot (Scatchard, 1949). The best straight line by a least-squares analysis yields a value of 4.2 sites with a dissociation constant of 28 μM. In this set of experiments, the data have been adjusted to reflect the 40% loss of specific activity during the course of the experiment.

In the presence of a saturating concentration of thiamin pyrophosphate, the intrinsic fluorescence emission of pyruvate oxidase is quenched by 16%. Due to the selection of the excitation wavelength at 300 nm, the emission spectrum should be nearly entirely due to tryptophan; the emission peak is at 333 nm and this indicates an average chromophore environment which is hydrophobic (Burstein et al., 1973).

The extent of fluorescence quenching was measured at 20 °C as a function of thiamin pyrophosphate concentration. The results are given in Figure 2. Also shown on the same scale are the results for both equilibrium dialysis experiments and measurements of the steady-state catalytic reaction velocity as a function of cofactor concentration. All three parameters saturate at high concentrations of thiamin pyrophosphate, and all are plotted on a percent saturation scale. These data clearly establish that both fluorescence quenching and steady-state kinetics accurately monitor cofactor binding to the enzyme. Figure 2 also demonstrates other features of thiamin pyrophosphate binding. In the absence of Mg^{2+} the cofactor no longer binds to the enzyme. The data show that, even at concentrations of thiamin pyrophosphate greater than 100 μM, there is no fluorescence quenching of the enzyme if Mg^{2+} is omitted from the buffer. In this experiment, EDTA has been added to remove traces of endogenous divalent cation. Neither

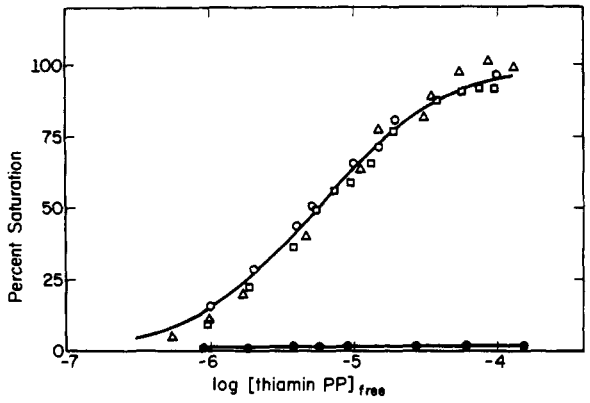

FIGURE 2: Thiamin pyrophosphate binding at 20 °C. Data were obtained at 20 °C under the following conditions: steady-state reaction velocity measured using the ferricyanide reductase assay (○), 30 μg/mL enzyme under normal assay conditions in the absence of any lipid activator; fluorescence quenching (□), 0.1 M sodium phosphate, 10 mM $MgCl_2$, 62 μg/mL enzyme, pH 5.7; fluorescence of a sample plus EDTA (●), 0.1 M sodium phosphate, 10 mM EDTA, 62 μg/mL enzyme, pH 5.7; equilibrium dialysis (△), 0.1 M sodium phosphate, 10 mM $MgCl_2$, 1.7 mg/mL enzyme, pH 5.7. The average percent recovery of enzymatic activity was 55% for the equilibrium dialysis experiment and 89% for the fluorescence experiments. The upper solid line is a calculated binding curve assuming independent binding sites with a single K_d of 5.8 μM.

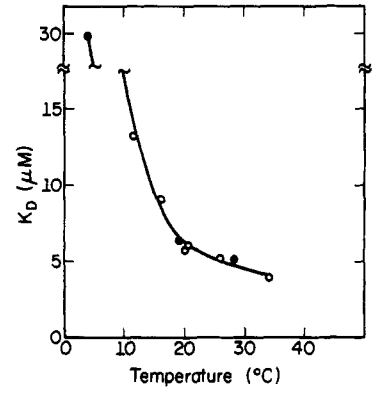

FIGURE 3: The temperature dependence of thiamin pyrophosphate binding. Thiamin pyrophosphate titrations were carried out at a number of temperatures under the conditions described in the legend of Figure 2: equilibrium dialysis (●); fluorescence quenching (○). The values for K_d were obtained as described under Materials and Methods.

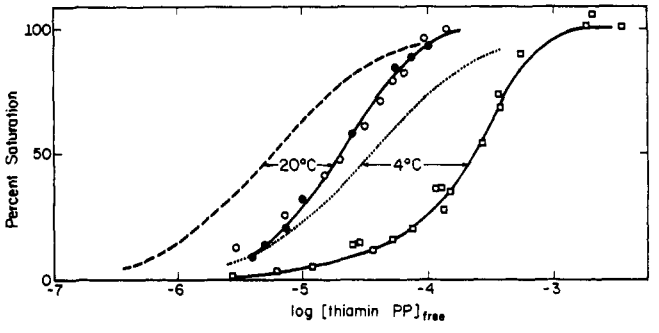

FIGURE 4: The effect of Triton X-100 micelles on thiamin pyrophosphate binding. Binding data were obtained under the following conditions: reaction velocity at 20 °C (●), 30 μg/mL enzyme in a normal assay where 10 mg/mL Triton X-100 is used as the activator; equilibrium dialysis at 4 °C (□), 0.1 M sodium phosphate, 10 mM $MgCl_2$, 1.2 mg/mL and 6.0 mg/mL enzyme for thiamin pyrophosphate concentrations below and above 100 μM, respectively, 10 mg/mL Triton X-100, pH 5.7; equilibrium dialysis at 20 °C (○), same as described in the legend of Figure 2 with the addition of 10 mg/mL Triton X-100. The average percent recovery of enzymatic activity was 90 and 95% for the equilibrium dialysis experiments at 4 and 20 °C, respectively. The two curves from Figures 1 and 2 are reproduced and correspond to thiamin pyrophosphate binding to unactivated enzyme at 4 (···) and 20 °C (- - -).

Mg^{2+} alone nor EDTA has any influence on the enzyme fluorescence. Inorganic pyrophosphate, which is a competitive inhibitor of pyruvate oxidase (K_I, 3 mM), also has no influence on the protein fluorescence, when measured in the presence or absence of the divalent cation. Other metals, such as Mn^{2+}, can substitute for Mg^{2+} with full enzyme activity, and the same fluorescence quenching is observed upon cofactor binding. Equilibrium dialysis experiments performed in the absence of divalent metal ions (not shown) confirm the conclusions based on fluorescence quenching. Virtually no binding of cofactor is evident when Mg^{2+} is omitted from the buffers.

The curve in Figure 2 is the theoretical isotherm expected for independent, equivalent binding sites with a K_d of 6 μM. Thus, the binding of thiamin pyrophosphate to the enzyme is stronger at higher temperatures. Both equilibrium dialysis and fluorescence quenching have been used to determine the K_d as a function of temperature. This is shown in Figure 3. There is a steep temperature dependence of the dissociation constant, especially at low temperatures. The data do not fit a linear Van't Hoff plot. At no temperature is there any evidence of cooperativity in the binding isotherm. These data certainly indicate that thiamin pyrophosphate binding to pyruvate oxidase is entropically driven. This kind of thermal effect on cofactor–enzyme interaction has been noted for other thiamin pyrophosphate dependent enzymes (Moe and Hammes, 1974; Heinrich et al., 1972). Fluorescence quenching was also used to measure the thiamin pyrophosphate K_d as a function of pH. No substantial change was observed between pH 5.0 and 6.5, although the enzyme is virtually inactive at pH 5.0 and has a pH optimum at pH 5.7.

Table I as well as Figures 4 and 5 show the effect of activating amphiphiles on thiamin pyrophosphate binding to the enzyme. Each of the amphiphiles activates the enzyme when present in the assay buffer, resulting in an increased specific activity of about 25-fold. In each case it is shown that the effects of these lipids on the constants derived from steady-state kinetics, the K_m and n_H of the cofactor, are direct results of changes in the binding isotherm of the thiamin pyrophosphate induced by the lipids.

The ionic detergent sodium dodecyl sulfate ($NaDodSO_4$) has no significant effect on the interaction between the cofactor and the enzyme. The results obtained by both the equilibrium binding methods and the kinetics studies are in good agreement. The isotherm determined in the presence of 20 μM $NaDodSO_4$ (not shown) is the same as that shown in Figure 2, demonstrating that $NaDodSO_4$ does not substantially influence the binding of the cofactor to the enzyme. In the presence of the $NaDodSO_4$, the maximum fluorescence quenching observed is reduced to about 12% from a value of 16% observed in the absence of the detergent.

The most dramatic effects were observed with the neutral detergent, Triton X-100, as illustrated in Figure 4. At 20 °C,

TABLE I: Thiamin Pyrophosphate Binding Parameters at 20 °C.

Activator	K_d (μM)	n_H[a]
None	6	1
$NaDodSO_4$	7	1
Triton X-100	21	1.6
Phosphatidylglycerol	3	1.4

[a] n_H is the Hill coefficient.

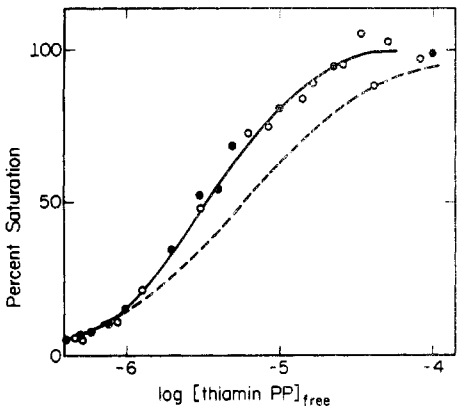

FIGURE 5: The effect of phosphatidylglycerol on thiamin pyrophosphate binding. Binding data were obtained under the following conditions at 20 °C: reaction velocity (○), 30 μg/mL enzyme in a normal assay where 0.1 mg/mL phosphatidylglycerol was present; equilibrium dialysis (●), same as described in the legend of Figure 2 with the addition of 1 mg/mL phosphatidylglycerol. The percent recovery of enzymatic activity was a function of the thiamin pyrophosphate concentration. It varied from 55% at very low thiamin phosphate concentrations to 95% at saturating thiamin pyrophosphate. The dashed line (- - - -) was taken from Figure 2 and corresponds to thiamin pyrophosphate binding to unactivated enzyme at 20 °C.

Triton X-100 increases the K_m of thiamin pyrophosphate and markedly increases the cooperativity between subunits. Equilibrium dialysis studies show that the dependence of the reaction velocity on thiamin pyrophosphate concentration is in excellent agreement with the binding isotherm measured in the presence of Triton X-100. The isotherm from Figure 2, which is determined under identical conditions except for the absence of the detergent, is shown in Figure 4 for comparison. Also shown are the binding isotherms of thiamin pyrophosphate at 4 °C both with and without Triton X-100. The effect of the detergent is again to reduce the average affinity of the enzyme for the cofactor. The cooperativity is again very strikingly increased in the presence of the detergent. One notable effect of the Triton X-100 is on the stability of the enzyme. In the absence of any lipids and in the presence of NaDodSO$_4$, the specific activity at the end of the dialysis experiments was about 55% of the initial value. However, in the presence of Triton X-100, the recovery was between 90 and 100%. This is reflected in the fact that the isotherms shown in Figure 4 saturate at about 4 sites per enzyme tetramer based on the total protein present (data not shown).

The influence of phosphatidylglycerol is shown in Figure 5. The isotherm from Figure 2, determined in the absence of phospholipid, is reproduced for comparison. The phospholipid clearly increases both the affinity of the enzyme for the cofactor and the cooperativity between subunits. Presumably, this closely approximates the in vivo situation. The data obtained using both kinetics and equilibrium dialysis are in excellent agreement. As observed with Triton X-100, the recovery of enzymatic activity was enhanced by the presence of the phospholipid. However, in this case, the recovery of activity was greater in the presence of the high concentrations of thiamin pyrophosphate. Hence, the specific activity of the enzyme was not the same in each dialysis sample. This was not observed in any of the other dialysis experiments. The equilibrium dialysis data in Figure 5 were normalized per unit of active enzyme to take this variation in specific activity into account. Fluorescence quenching experiments were not performed in the presence of either phosphatidylglycerol or Triton X-100 due to technical problems.

Discussion

The influence of lipids and detergents on the steady-state kinetics of pyruvate oxidase has been thoroughly examined (Blake and Gennis, manuscript in preparation). The enzyme is activated by a wide variety of amphiphiles, including anionic and cationic detergents at concentrations well below their critical micelle concentrations, neutral detergents such as Triton X-100 at concentrations where detergent micelles are present, and phospholipids such as phosphatidylglycerol and phosphatidylcholine in the form of bilayer vesicles. In all cases, the V_{max} is increased by about 25-fold and the K_m for pyruvate is decreased by nearly an order of magnitude. In contrast, only in the presence of those activating amphiphiles that are either micellar or exist as bilayers is there any substantial effect observed on the kinetic parameters relating to the cofactor thiamin pyrophosphate. It is clear that there is an interaction mediated by the protein between the activating lipids and the ligands involved in catalysis. It has been shown that in the simultaneous presence of both pyruvate and thiamin pyrophosphate the affinity of the enzyme for charged detergents (Schrock and Gennis, 1977) and for phospholipids (Schrock and Gennis, manuscript in preparation) is dramatically enhanced. Both the bound ligands and the redox state of the flavin appear to play important roles in modulating the lipid binding properties of the enzyme, and a complete analysis of these interrelationships is not yet feasible. It is the purpose of this work to characterize the binding of the cofactor, thiamin pyrophosphate, with pyruvate oxidase and to compare the results with the kinetics parameters previously examined. For all the binding experiments reported in this work, the bound flavin was in the oxidized state. By accumulating information concerning the protein–lipid interactions under equilibrium conditions, it will eventually be possible to analyze completely this complex situation in thermodynamic terms. Thiamin pyrophosphate was selected for this initial study because the magnitude of the K_m indicated that the direct measurement of cofactor binding by equilibrium dialysis would be feasible. It was also discovered that the quenching of the oxidase intrinsic fluorescence could be used as an indirect monitor of cofactor binding, and both methods were used. The fluorescence technique allows the experiments to be performed at much lower protein concentrations than the equilibrium dialysis, but the binding results are the same, demonstrating that the binding isotherm is not dependent on protein concentration.

The results show that there are four cofactor binding sites per tetramer of pyruvate oxidase. The data shown in Figure 1 have been adjusted to reflect the substantial loss of activity during the course of the experiment. There is no reason to believe a priori that cofactor binding and specific activity will necessarily be strongly correlated; however, under the conditions described for Figure 1, the corrected data consistently yield the same stoichiometry. The fact that the binding isotherm saturates at four sites per tetramer based on total protein under conditions where the activity is fully stabilized by Triton X-100 tends to confirm this conclusion. It is fortunate that the shape and location of the isotherms are so similar by the different techniques employed despite the variation in final specific activity.

The data in Figure 2 also demonstrate that a divalent cation such as Mg^{2+} is absolutely required for cofactor binding. It is probably a metal-cofactor complex which is binding to the enzyme. The thermal dependence of cofactor binding (Figure 3) is somewhat unusual, but may be a general feature of thiamin pyrophosphate binding to enzymes. For example, a

similar effect was reported with the *E. coli* pyruvate dehydrogenase complex (Moe and Hammes, 1974). Clearly, the formation of the cofactor-enzyme complex is driven by a highly favorable entropy change upon binding.

An important conclusion of this work is that the K_m and n_H values for thiamin pyrophosphate obtained by steady-state kinetics are identical with the K_d and n_H values obtained by studying the cofactor binding to the enzyme under equilibrium conditions in the absence of pyruvate and any electron acceptor. Furthermore, this is true both in the presence and absence of lipid activators. Thus, it can be stated that the enzymatic reaction appears cooperative with respect to thiamin pyrophosphate in the presence of a phospholipid because the phospholipid has altered the thiamin pyrophosphate binding isotherm, rather than changing some catalytic rate constant. It is apparent from this paper that, at least under the conditions employed for equilibrium dialysis, the enzyme can interact with Triton X-100 and also with phosphatidylglycerol in such a manner as to alter the thiamin pyrophosphate binding isotherm. It is probably a critical feature that the experiments with phosphatidylglycerol were done by equilibrium dialysis in which the protein concentration is relatively high, because it is likely that at lower protein concentrations there would be little lipid-protein complex. The addition of pyruvate to the buffer would probably be required to enhance the phospholipid affinity of pyruvate oxidase to the point that the enzyme would be bound to the lipid at the low protein concentrations employed in the assay. The unusual pattern of recovery of enzyme activity in the dialysis experiment with phosphatidylglycerol makes it seem likely that the enzyme is binding to and stabilized by the phospholipid at high concentrations of thiamin pyrophosphate, but may not be binding at lower concentrations of the cofactor.

These data demonstrate one manner in which phospholipids can modulate the behavior of a membrane enzyme, by altering the thermodynamic binding parameters describing the interactions with the ligands involved in catalysis. It is possible that the K_m change observed with pyruvate when lipids are bound to pyruvate oxidase is also a result of an altered binding isotherm. However, this has not yet been shown.

One clearcut case has been described in the literature where lipid activation of a membrane enzyme is correlated with altered cofactor binding. This is β-hydroxybutyrate dehydrogenase from liver, which is activated specifically by phosphatidylcholine (Gazzotti et al., 1975; Grover et al., 1975; Houslay et al., 1975). The changes in the binding isotherm of NADH in the presence of the phospholipid are much less subtle than those reported in this work; no binding of NADH to β-hydroxybutyrate dehydrogenase could be detected in the absence of the lipid activator (Gazzotti et al., 1974).

It should be noted that β-hydroxybutyrate dehydrogenase is an intrinsic membrane enzyme and is not likely to exist in vivo apart from the membrane. This may not be so for pyruvate oxidase. Pyruvate oxidase is loosely bound to the membrane and it requires some care to avoid dissociating the enzyme from *E. coli* membrane vesicle preparations. It appears from work in vitro that this enzyme might exist in equilibrium between a highly active membrane-bound form and a water-soluble form with a low specific activity. The presence of ligands involved in catalysis and the redox state of the flavin are factors which determine this equilibrium constant. Furthermore, it is clear from this work that the effect is reciprocal. The interactions between the cofactor, thiamin pyrophosphate, and also probably the substrate, pyruvate, are strongly modulated by the lipid-protein interactions.

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