Biochimica et Biophysica Acta, 614 (1980) 215 —220

BBA Report

BBA 61375

SPECIFIC LIGAND ENHANCEMENT OF THE AFFINITY OF *E. COLI* PYRUVATE OXIDASE FOR DIPALMITOYL PHOSPHATIDYLCHOLINE

HERBERT L. SCHROCK* and ROBERT B. GENNIS

Departments of Chemistry and Biochemistry, University of Illinois, Urbana, IL 61801 (U.S.A.)

(Received April 4th, 1980)

Key words: Ligand enhancement; Pyruvate oxidase; Dipalmitoyl phosphatidylcholine; Enzyme affinity; (E. coli)

Summary

Pyruvate oxidase (pyruvate: ferricytochrome b_1 oxidoreductase, EC 1.2.2.2) is a peripheral membrane flavoenzyme isolated from *Escherichia coli*. The enzyme catalyzes the oxidative decarboxylation of pyruvate to acetate plus CO_2, and is coupled to the *E. coli* electron transport chain. In vitro, pyruvate oxidase activity is measured spectrophotometrically using ferricyanide as an electron acceptor. In the presence of dipalmitoyl phosphatidylcholine or a number of other phospholipids or detergents, the enzymatic specific activity is enhanced about 25-fold. In this paper the interaction between pyruvate oxidase and dipalmitoyl phosphatidylcholine is examined. It is demonstrated that the presence of the ligands involved in catalysis has a substantial influence on the affinity between pyruvate oxidase and dipalmitoyl phosphatidylcholine. In the absence of the substrate (pyruvate) and cofactor (thiamin pyrophosphate) there is no detectable complex formation. However, when both ligands are present, a condition which results in the reduction of the flavoprotein, the interaction between the protein and phospholipid is greatly enhanced. It is clearly shown that the protein-lipid interaction is dramatically modulated by the ligands bound at the catalytic active site on the enzyme and/or by the oxidation-reduction state of the flavin.

Pyruvate oxidase (pyruvate: ferricytochrome b_1 oxidoreductase, EC 1.2.2.2) is a peripheral membrane enzyme isolated from *Escherichia coli*.

*Present address: Cornell University, Section of Biochemistry and Molecular Biology, Ithaca, 14850, U.S.A.

The oxidase has been shown to be a tetramer, composed of four indentical subunits [1—3]. The enzyme catalyzes the oxidative decarboxylation of pyruvic acid to acetic acid and CO_2. Two cofactors are required for this activity, thiamin pyrophosphate, which must be added to the purified enzyme, and FAD which remains bound to the oxidase throughout the purification. 1 molecule of each of these cofactors is bound to each subunit [1,2,4]. In vivo, pyruvate oxidase is bound to the inner surface of the *E. coli* cytoplasmic membrane and is coupled to the electron transport chain [5,6]. Enzymatic activity is measured in vitro using a ferricyanide reductase assay.

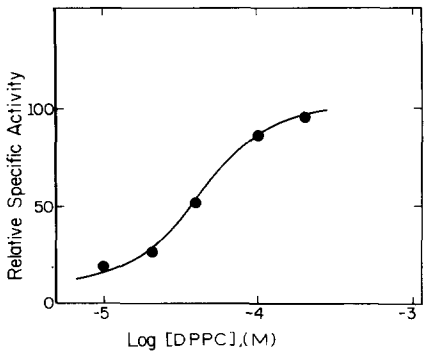
Like many membrane enzymes [7,8], pyruvate oxidase requires the addition of lipids to the in vitro assay in order to attain maximal specific activity. Pyruvate oxidase activity is stimulated about 25-fold by a wide variety of amphipathic activators, including detergents and phospholipids [9—11]. A previous study with detergent activators demonstrated the existence of hydrophobic lipid binding sites on the protein [12]. In addition, it was shown that the affinity between the enzyme and the charged detergents was enhanced when both the substrate (pyruvate) and cofactor (thiamin pyrophosphate) were present. This paper demonstrates that these catalytic ligands also have a dramatic effect in enhancing the interaction between pyruvate oxidase and dipalmitoyl phosphatidylcholine vesicles. Clearly, there is a reciprocal coupling between the catalytic active site and the lipid binding site on pyruvate oxidase.

Pyruvate oxidase. Details of the enzyme purification and characterization have been described elsewhere [1]. Enzymatic activity was measured using a ferricyanide reductase assay [13]. Activation of the enzyme by dipalmitoyl phosphatidylcholine was performed as recently reported [11].

Reagents. Sodium pyruvate, thiamin pyrophosphate and synthetic dipalmitoyl phosphatidylcholine were obtained from Sigma. Ultrapure sucrose (density gradient grade) and SDS were purchased from Schwartz/Mann. Fluorescamine was obtained from Pierce. (1-^{14}C)-labeled dipalmitoyl phosphatidylcholine was obtained from Applied Science Laboratory. All other chemicals were reagent grade and deionized, glass-distilled water was used for all solutions.

Density gradient centrifugation. Details of the sucrose density gradient centrifugation experiments have been given elsewhere [14]. Two modifications were made in the procedures. Instead of a linear gradient, a step gradient was prepared by layering 2 ml 40% sucrose, 2 ml 25% sucrose and 1 ml 5% sucrose. The stock solutions contained specified levels of different ligands as described in the text. Samples were applied after the gradient was prepared and the centrifugation started immediately. Protein was determined using a fluorescamine assay [14]. SDS (200 mM) was included in the borate buffer to eliminate any masking effects of phospholipid on protein quantitation.

The activation of pyruvate oxidase activity by dipalmitoyl phosphatidylcholine is shown in Fig. 1. This phospholipid will activate the enzyme to approximately the same extent as will numerous other phospholipids [11],



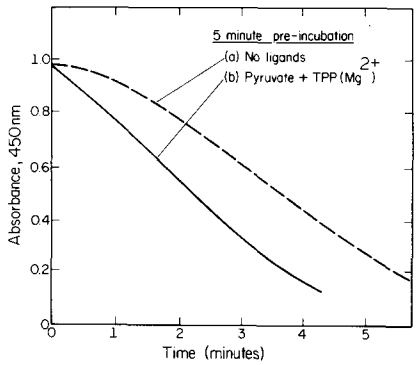


Fig. 1. Activation of pyruvate oxidase at 24°C by dipalmitoyl phosphatidylcholine (DPPC). The ferricyanide reductase assay was performed as previously reported [13].

Fig. 2. Ferricyanide reductase assays of pyruvate oxidase after pre-incubation of the enzyme with the lipid activator, dipalmitoyl phosphatidylcholine, in the presence and absence of the catalytic ligands. The reduction of ferricyanide is monitored by the loss of absorbance at 450 nm. Curve (a): The enzyme (13 μg) and lipid (150 μg) are allowed to pre-incubate for 5 min in 0.8 ml 0.1 M phosphate buffer pH 5.7 containing 10 mM $MgCl_2$. The reaction is initiated by the addition of pyruvate, thiamin pyrophosphate (TPP), and ferricyanide at time zero. Curve (b): Same as curve (a) except that the pyruvate and thiamin pyrophosphate are present with the enzyme during the 5-min pre-incubation period, and the reaction is initiated by the addition of ferricyanide. In all cases, the final concentrations are as follows in a total volume of 1.0 ml: 200 mM pyruvate, 100 μM thiamin pyrophosphate, 8.5 mM sodium ferricyanide. All experiments were performed at room temperature.

corresponding to a 25-fold increase in the V using the ferricyanide reductase assay. The half-maximally activating concentration of dipalmitoyl phosphatidylcholine is about 40 μM, and, while other phospholipids activate at lower concentrations, the dipalmitoyl phosphatidylcholine activation is typical of phospholipid vesicle activation of pyruvate oxidase. Since these measurements were made at 24°C, it is clear that pyruvate oxidase will interact with dipalmitoyl phosphatidylcholine at temperatures well below the gel-liquid crystalline phase transition temperature (41°C). It has previously been reported that the 'fluidity' of the phospholipid bilayer is not a critical parameter in the ability of phospholipids to activate pyruvate oxidase [11].

In Fig. 2, the requirements for maximal stimulation of pyruvate oxidase by dipalmitoyl phosphatidylcholine in the ferricyanide reductase assay are emphasized. Previous work has shown that in order to obtain full activation, the enzyme must be allowed to pre-incubate with the phospholipid for a period of several minutes prior to the initiation of the reaction. Fig. 2 compares the results when the pre-incubation is performed in the presence and absence of the catalytic ligands. When saturating levels of both pyruvate and thiamin pyrophosphate are present during the pre-incubation, and the reaction is initiated by the addition of ferricyanide, the resulting reaction proceeds rapidly and is linear in time. However, if the protein and lipid are incubated in the absence of the two catalytic ligands, and the reaction is initiated by the simultaneous addition of pyruvate, thiamin pyrophosphate, and ferricyanide, the results are quite different. The reaction begins slowly and accelerates to its maximum rate over a period of about 2 min. The increase in the rate of reaction presumably reflects the formation of the highly active protein-lipid complex which does not form unless the ligands involved in catalysis are present.

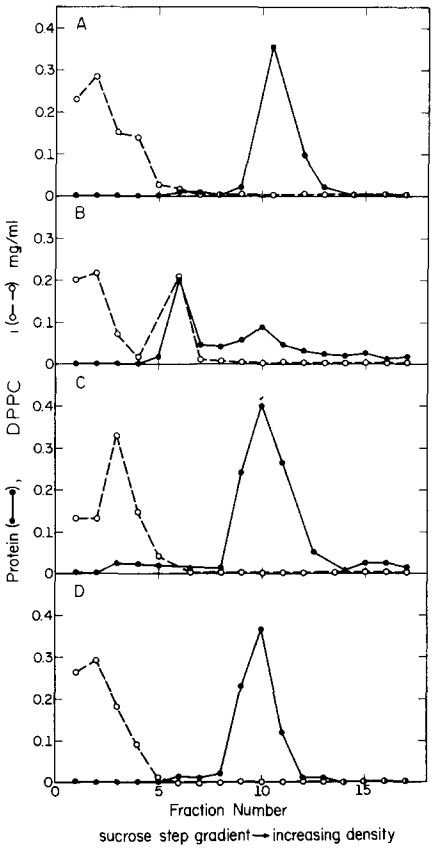


Fig. 3. Sucrose step-gradient centrifugation of pyruvate oxidase in the presence and absence of the catalytic ligands. The samples contained 0.3 mg of dipalmitoyl phosphatidylcholine (DPPC) and 0.3 mg of pyruvate oxidase in 0.3 ml. The step gradient is described in the text. Panel A: no catalytic ligands present. Panel B: 100 μM thiamin pyrophosphate, 100 mM sodium pyruvate and 10 mM $MgCl_2$. Panel C: 100 μM thiamin pyrophosphate and 10 mM $MgCl_2$. Panel D: 100 mM sodium pyruvate. All solutions contain 0.1 M sodium phosphate buffer, pH 5.7, in addition to the ligands. Samples were centrifuged at 40 000 rev./min (192 000 × g) for 24 h at 4°C as described previously [14].

The intimate relationship between the presence of the catalytic ligands and phospholipid binding is evident in Fig. 3. Sucrose density gradient centrifugation experiments clearly demonstrate significant dipalmitoyl phosphatidylcholine interaction with pyruvate oxidase only when pyruvate and thiamin pyrophosphate are both present. Panel A shows that in the absence of these ligands no observable interaction occurs between the enzyme and dipalmitoyl phosphatidylcholine vesicles. The lipid and protein elute at the same position as when either are sedimented above. However, when both ligands are present in saturating concentrations, a complex that contains both active pyruvate oxidase and dipalmitoyl phosphatidylcholine is formed, as seen in panel B. The presence of either thiamin pyrophosphate or pyruvate alone is insufficient to induce this complex formation (panels C and D).

Sodium 3-fluoropyruvate, a competitive inhibitor of pyruvate oxidase (K_i, 3mM), will not substitute for pyruvate in enhancing protein-lipid complex formation. In the presence of 100 mM fluoropyruvate and 10 μM thiamin

pyrophosphate, the sedimentation profile (not shown) is identical to that shown in panel A (Fig. 3), indicating no protein-lipid complex formation.

The interactions between pyruvate oxidase, dimyristoyl phosphatidylcholine and egg phosphatidylcholine were also examined using sucrose density gradient centrifugation. The results (not shown) were qualitatively similar to those shown for dipalmitoyl phosphatidylcholine. The simultaneous presence of pyruvate and thiamin pyrophosphate resulted in an enhanced protein-lipid interaction. Similar results were obtained using egg phosphatidylcholine, and separating the protein-lipid complex using gel filtration chromatography.

Pyruvate oxidase is stimulated by charged detergents below their critical micelle concentrations, as well as by phospholipid vesicles. Specific ligand effects on SDS and cetyltrimethyl ammonium bromide binding to pyruvate oxidase has been previously reported [12].

This paper reports data that indicate a similar specific ligand effect on the interaction of pyruvate oxidase and dipalmitoyl phosphatidylcholine vesicles. When the substrate, pyruvate, and the cofactor, thiamin pyrophosphate, are present the phospholipid-enzyme interaction is greatly enhanced. If either ligand is absent, no interaction can be detected.

Dipalmitoyl phosphatidylcholine was selected for this study because its activation profile (Fig. 1) indicated relatively weak interaction with pyruvate oxidase under steady state assay conditions [11]. Hence, the effect of the ligands on the protein-lipid interaction is seen dramatically as an all-or-none phenomenon (e.g., compare panels A and B, Fig. 3). Phospholipids which apparently interact with the enzyme more strongly, such as phosphatidylglycerol [11], do not show such dramatic differences in complex formation with pyruvate oxidase when examined using the sedimentation procedure in the presence and absence of pyruvate and thiamin pyrophosphate. However, it is presumed that in these cases the catalytic ligands are also modulating the nature of the protein-lipid interaction. Experiments to quantitate these effects have not yet succeeded. At concentrations of protein and lipid such as those used in the sedimentation experiments (Fig. 3), the protein-lipid complex is actually a co-aggregate.

Clearly, events at the catalytic active site on the protein affect the lipid binding properties of pyruvate oxidase. In the absence of pyruvate and thiamin pyrophosphate, the enzyme does not manifest the solution properties usually associated with membrane proteins. Such properties become manifest when the enzyme is in the presence of saturating concentrations of the substrate plus cofactor. The protein self-aggregates, becomes surface labile, and shows enhanced interaction with activating amphiphiles such as detergents [14] and, as shown in this work, phospholipid vesicles. In the presence of these ligands, the flavin moiety on pyruvate oxidase becomes reduced and the nature of the bound ligands is uncertain. The fact the 3-fluoropyruvate will not substitute for pyruvate in causing the enhanced interaction with dipalmitoyl phosphatidylcholine indicates that the reduction of the flavin is a critical requirement for causing the protein conformational change which is responsible for the altered solution properties of pyruvate oxidase. This protein conformational change has recently been investigated by using mild proteolysis [13,14].

Presumably, once the protein-lipid complex has been formed, the turnover of the enzyme occurs sufficiently fast so that the complex does not dissociate.

The apparent higher affinity of the reduced form of pyruvate oxidase for membranes is intriguing in view of possible regulatory mechanisms. It is conceivable that the enzyme will bind to the *E. coli* membrane only when the substrate level is sufficient to reduce the flavin. Once bound, pyruvate oxidase would function normally, coupled to the electron transport chain. It should be stressed that there is no evidence that such a mechanism is actually operating in the *E. coli* cell.

What is clear from these studies is that the lipid binding properties of this protein are modulated by events at the catalytic site. The same kinds of allosteric properties and subtleties observed in studies of protein subunit interactions, protein-nucleic acid interactions and protein-small molecule interactions are clearly manifested in the protein-lipid interactions of pyruvate oxidase. The physiological relevance in this case will have to await further experimentation, but these results call attention to a new dimension of the protein-lipid interaction which may turn out to be of general importance.

We thank Robert Blake II, L.P. Hager, Thomas. A. O'Brien and Patricia Russell for many helpful discussions. This work was supported by grants from the National Institutes of Health (HL-16101) and the American Heart Association. H.L.S. was supported in part as a Trainee of the Cellular and Molecular Biology Training Program at the University of Illinois by P.H.S. GM-07283. R.G.B. is grateful for support by P.H.S. Career Development Award K04 HL-00040.

References

1 O'Brien, T.A., Schrock, H.L., Russell, P., Blake, R., II and Gennis, R.B. (1976) Biochim. Biophys. Acta 452, 13—29
2 Williams, F.R. and Hager, L.P. (1961) J. Biol. Chem. 236, PC 36-PC 37
3 Raj, T., Russell, P., Flygare, W.H. and Gennis, R.B. (1977) Biochim. Biophys. Acta 481, 42—49
4 O'Brien, T.A. and Gennis, R.B. (1977) Biochemistry 18, 804—809
5 Cunningham, C.C. and Hager, L.P. (1975) J. Biol. Chem. 250, 7139—7146
6 Shaw-Goldstein, L.A., Gennis, R.B. and Walsh, C. (1978) Biochemistry 17, 5605—5613
7 Coleman, R. (1973) Biochim. Biophys. Acta 300, 1—30
8 Gennis, R.B. and Jonas, A. (1977) Annu. Rev. Biophys. Bioeng. 6, 195—238
9 Cunningham, C.C. and Hager, L.P. (1971a) J. Biol. Chem. 246, 1575—1582
10 Cunningham, C.C. and Hager, L.P. (1971b) J. Biol. Chem. 246, 1583—1589
11 Blake, R., II, Hager, L.P. and Gennis, R.B. (1978) J. Biol. Chem. 253, 1963—1971
12 Schrock, H.L. and Gennis, R.B. (1977) J. Biol. Chem. 252, 5990—5995
13 Russell, P., Hager, L.P. and Gennis, R.B. (1977) J. Biol. Chem. 252, 7877—7882
14 Russell, P., Schrock, H.L. and Gennis, R.B. (1977) J. Biol. Chem. 252, 7883—7887