Identification, Localization, and Function of the Thiamin Pyrophosphate and Flavin Adenine Dinucleotide Dependent Pyruvate Oxidase in Isolated Membrane Vesicles of *Escherichia coli* B†

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ABSTRACT: The enzymatic activity responsible for pyruvate-dependent O_2 uptake in membrane vesicles of *E. coli* B is identified as the thiamin pyrophosphate requiring flavoprotein pyruvate oxidase, a peripheral membrane enzyme which oxidatively decarboxylates pyruvate to acetate and CO_2. Enzyme activity was inhibited by antibody to authentic pyruvate oxidase (from *E. coli* W191-6) after solubilization with Triton X-100, but was insensitive to antibody added exogenously either to freshly prepared isolated cytoplasmic membrane vesicles or to spheroplasts. This latency suggests an essentially exclusive location of the membrane-associated pyruvate oxidase at the inner face of the cytoplasmic membranes. This membrane location was confirmed using analogues of thiamin pyrophosphate, thiamin thiazalone pyrophosphate, and thiamin thiothiazolone pyrophosphate. These coenzyme analogues are membrane impermeant. While they show K_i values of 10^{-7} and 2×10^{-8} M for solubilized *E. coli* B pyruvate oxidase, they cause no more than 20% inhibition of vesicle-associated enzyme at high exogenous concentrations. The metabolic function of pyruvate oxidase is not the provision of activated acetyl groups (the role of the pyruvate dehydrogenase multienzyme complex) but appears rather to provide substrate-derived electrons to the membrane respiratory chain for the generation of the electrochemical potential, so that $\Delta\mu_{H^+}$ can be used to drive solute active transport (shown here for amino acid transport). Exogenous addition of solubilized pyruvate oxidase to isolated membrane vesicles results in weak binding and apparent saturation, as judged by a 25-fold increase in pyruvate-stimulated O_2 uptake. However, there is only a 1.6-fold stimulation in the rate of active transport of amino acids, indicating a much lower coupling efficiency of exogenous enzyme. For example, we calculate that for endogenous (cytoplasmic side) pyruvate oxidase some 55 electron pairs pass down the membrane electron-transport chain per molecule of proline actively transported; with exogenous pyruvate oxidase, 510 electron pairs are required per molecule of proline actively accumulated.

In previous studies on solute active transport in isolated cytoplasmic membrane vesicles from *E. coli* B, we noted a membrane-associated pyruvate-oxidizing, O_2-reducing activity which was coupled as an energy source to solute uptake (Kaczorowski et al., 1975a). We searched for such an enzyme to explain why L-chloroalanine, metabolizable only to pyruvate by a membraneous alanine racemase (Kaczorowski et al., 1975b; Wang and Walsh, 1978), could stimulate solute concentrative uptake. As an α-keto acid, pyruvate is most logically oxidized by oxidative decarboxylation to CO_2 and a two-carbon acetate fragment. Enzymes which oxidatively decarboxylate α-keto acids require both thiamin pyrophosphate and a redox active coenzyme, such as FAD. Two known examples are the multienzyme pyruvate dehydrogenase complex and the TPP[1]- and FAD-dependent pyruvate oxidase first isolated as a crystalline enzyme by Hager and associates from *E. coli* sonicates (Koike et al., 1960; Hager, 1957; Williams and Hager, 1966). The multienzyme complex produces the activated acyl fragment acetyl-CoA, while pyruvate oxidase produces unactivated acetate, and its cellular physiological function has been obscure. However, recent studies by both Hager and Gennis and their colleagues have characterized the dramatic lipid activation (15- to 25-fold) of the soluble pyruvate oxidase (Cunningham and Hager, 1971a,b; Blake et al., 1978; Russell et al., 1977).

In this paper, we show that the membrane vesicle pyruvate-oxidizing activity is due to the TPP- and FAD-requiring pyruvate oxidase, a peripheral membrane enzyme. We report studies on its location on the cytoplasmic side of the membrane both in isolated vesicles and in spheroplasts by antibody and TPP analogue susceptibility studies. We suggest its physiological function is to provide substrate-derived low-potential electrons to build an electrochemical membrane potential. We also examine its ability to rebind to membrane vesicles from the outside and to show differential coupling to the electron transport chain and to solute active transport.

Materials and Methods

Preparation of Membrane Vesicles from E. coli B. *E. coli* B (wild type) was grown on a minimal salts media with either 0.5% glucose, 1% DL-alanine plus 2% glycerol or 1% pyruvate plus 2% glycerol as described by Kaczorowski et al. (1975a). The carbon source did not affect the amount of pyruvate oxidase activity in vesicles. Vesicles were prepared by osmotic shock of lysozyme-EDTA treated cells according to the method of Kaback (1971) with modifications as described in

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[1] Abbreviations used: PMS, phenazine methosulfate; $TPMP^+Br^-$, triphenylmethylphosphonium bromide; Me_2SO, dimethyl sulfoxide; TPP, thiamin pyrophosphate; TTPP, thiamin thiazolone pyrophosphate; TTTPP, thiamin thiothiazolone pyrophosphate; DCIP, dichlorophenolindophenol; IPTG, isopropyl thiogalactoside; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

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Kaczorowski et al. (1975a). They were stored at 8 mg/mL in 0.1 M potassium phosphate at pH 6.2 or 7.5 as indicated.

Transport Assays. Radioactive amino acid uptake in the presence of various energy sources was assayed by established procedures (Kaback, 1971). A final volume of 50 μL contained 0.25 mg of membrane vesicles, 50 mM potassium phosphate (pH 7.5), 10 mM $MgSO_4$, either 40 mM potassium pyruvate or 20 mM of the other appropriate energy source or 0.1 mM PMS plus 20 mM ascorbate, and a radioactive solute. The pyruvate-stimulated chemical membrane potential was assayed by using either [^{3}H]triphenylmethylphosphonium bromide ($TPMP^+Br^-$) or $^{86}RbCl$ in the presence of valinomycin, as described (Ramos and Kaback, 1976). Since valinomycin increases the permeability of membranes to both K^+ and Rb^+, vesicles were washed by centrifugation three times with 0.1 M sodium phosphate (pH 6.2) to remove K^+. After dilution to their original volume in a plastic vial, valinomycin was added to the vesicles at a final concentration of 2 nmol/mg of protein. Stock solutions of valinomycin in dimethyl sulfoxide were freshly made for each use in plastic vials because valinomycin is absorbed on glass. The amount of Me_2SO in the incubation mixtures had no effect on transport. Due to the short half-life of ^{86}Rb (18.6 days), corrections for the specific activity of ^{86}Rb were made just prior to the experiments. Samples were analyzed by liquid scintillation counting with approximately 85% efficiency for ^{14}C and 30% for ^3H. The specific activities and final concentrations of radioactively labeled substrates were employed at the following saturating concentrations: L-proline (230 mCi/mmol), 1.3×10^{-5} M; L-phenylalanine (495 mCi/mmol), 9.6×10^{-6} M; $^{86}RbCl$ (18.15 mCi/mmol), 2×10^{-3} M; and [^{3}H]TPMPBr (36 mCi/mmol), 3.9×10^{-4} M.

Measurement of Dehydrogenase or Oxidase Activities. Substrate-dependent rates of oxygen consumption could be measured in membrane vesicle suspensions using a Clark type oxygen electrode (YSI Model 53). An alternate assay involved coupling the dehydrogenase activity to the dye dichlorophenolindophenol (DCIP) and monitoring the reduction spectrophotometrically at 600 nm. Assay conditions are described in Kaczorowski et al. (1975a,b).

Isolation of [^{14}C]Acetate from [^{14}C]Pyruvate. Acetate was separated from pyruvate after incubation with vesicles by a modification of a procedure by Lockridge et al. (1972). Vesicles (0.8 mg), $MgSO_4$ (10 mM), and [3-^{14}C]pyruvate (10.5 mCi/mmol) (2.3×10^{-3} M) were incubated for 45 min at room temperature. Phospholipase A (50 μg) was added to disrupt the integrity of the vesicles, and the mixture was incubated for an additional 15 min. The mixture was diluted with 1 mL of 0.1 M potassium phosphate (pH 7.5) and centrifuged. The supernatant was saved, and the pellet was washed twice with 1 mL of the same buffer. The supernatants were combined and added to a 12-mL Dowex 1-X8Cl^- column. The column was washed with 50 mL of water, and acetate and pyruvate were separated using a step elution, 4 and 50 mM HCl; 2-mL fractions were collected. The elution profiles were compared with those of a mixture of authentic pyruvate and acetate.

Isolation of $^{14}CO_2$ from [^{14}C]Pyruvate. The release of radioactive CO_2 was assayed by the method of Cromartie and Walsh (1976). In a test tube with a magnetic stirring bar a total volume of 50 μL containing 0.4 mg of vesicles, 50 mM potassium phosphate (pH 6.2), and 10 mM $MgSO_4$ was incubated for 5 min. The solution was put under an oxygen atmosphere, [U-^{14}C]pyruvate (6.45 mCi/mmol, 2.5 mM) was added, and the test tube was immediately sealed with a stopper containing a sanded glass rod dipped in a 1% solution hyamin hydroxide in methanol. The reaction was quenched by the addition of 50 μL of 2 N HCl and stirred for an extra 15 min. The hyamin hydroxide was washed off from the rod into a scintillation vial with 2 mL of water. Cocktail D (10 mL) was added, and the rod was allowed to remain in the vial for an additional 15 min before counting.

Purification of Soluble Pyruvate Oxidase. E. coli B was grown under the same conditions as for vesicle preparation until late-log or stationary phase in a 25-L fermentor and frozen in the pellet form until use. Pyruvate oxidase was prepared following the general lines describes by O'Brien et al. (1976), through the DEAE-Sephadex A50 column. Aliquots of the concentrated active fractions from the column were transferred to 30-mL Corex tubes with a magnetic spin bar and submerged in an 80 °C water bath with stirring until the solution reached 70 °C. The tubes were rapidly cooled at 4 °C. The pink precipitate was removed by centrifugation. The pellet was washed with 1 mL of 0.1 M KP_i (pH 5.7) and 50% glycerol, and the two supernatants were combined. The pH was adjusted to 5.7 and cold glycerol was added to a final concentration of 50%. The enzyme was stable for ca. 6 months and was ~20–30% pure as judged by the A_{438}/A_{280} ratio (O'Brien et al., 1976) and $NaDodSO_4$-polyacrylamide gels (Weber and Osborn, 1969).

Determination of Soluble Pyruvate Oxidase Activity. Soluble pyruvate oxidase activity was assayed by either following DCIP or $Fe(CN)_6^{3-}$ reduction spectrophotometrically at 600 or 450 nm, respectively. Final incubation mixtures contained 0.1 M KP_i (pH 6.0), 10% glycerol, 0.06–0.09 mg of pyruvate oxidase, 10 mM $MgSO_4$, 10 μM TPP, 60 μM $NaDodSO_4$, and 40 mM potassium pyruvate in 1-mL volume. The mixture was incubated at room temperature for 20 min, to fully activate the protein. Assays were initiated by the addition of either 0.15 mM PMS plus 82.4 μM DCIP or 5 mM $K_3Fe(CN)_6^{3-}$.

Preparation of Spheroplasts. E. coli B were grown as described for vesicle preparation with the addition of 0.5 mM isopropyl thiogalactoside (IPTG) to induce β-galactosidase activity. Spheroplasts were made by following the method for vesicle preparation through centrifugation of the EDTA-lysozyme treated cells (Kaback, 1971). The formation of spheroplasts was followed by the decrease in absorbance at 600 nm of an aliquot of cells suspended in glass-distilled water and occurred within 2 min of the addition of lysozyme. After centrifugation, spheroplasts were resuspended in 0.1 M KP_i (pH 6.2), 20% sucrose, 20 mM $MgSO_4$ buffer. RNase and DNase were added (50 mg each) to remove any nucleic acids and prevent the spheroplast solution from becoming too viscous. The spheroplasts were repelleted, diluted in the above buffer, and used immediately. Spheroplasts stored at 4 °C were stable for 2–3 h before substantial lysis would occur. Pyruvate oxidase activity in spheroplasts was assayed by PMS–DCIP reduction as in the procedure for vesicles. The amount of lysis of spheroplasts was assayed by β-galactosidase activity; β-galactosidase is located in the cytoplasmic side of the cells. The increase in absorbance at 430 nm was monitored, indicating the hydrolysis of *o*-nitrophenyl galactopyranoside (50 mM) to *o*-nitrophenol and galactose (Neu and Heppel, 1965).

Anti-Pyruvate Oxidase Preparation and Characterization. Antibody to pure pyruvate oxidase from *E. coli* W-191-6 was elicited in an adult New Zealand white rabbit. An emulsion was prepared using 1 mL of enzyme solution (2 mg/mL) and 1 mL of complete Freund's adjuvant. The rabbit was injected in the rear foot pads and in the interscapular region. After the primary immunization, two booster injections (2 mg) were administered at approximately 3-month intervals. Blood was collected 14 days postinjection via the marginal ear vein. Serum obtained after centrifugation was stored frozen.

The IgG fraction was obtained using the following procedure at 4 °C. To the serum was added 0.05 volume of 5% sodium dextran sulfate and 0.09 volume of 11.1% $CaCl_2$. After stirring for 30 min the solution was stored overnight at 4 °C, and the supernatant was collected after centrifugation. IgG was precipated by bringing the solution to 40% saturation with ammonium sulfate, and the pellet was redissolved in about half the original serum volume with 0.02 M KP_i (pH 7.7). This was dialyzed against the same buffer. After clarifying the solution by centrifugation, the solution was passed through a DEAE-cellulose column (DE-52) (25 × 120 mm) which had been equilibrated with the same buffer. The IgG fraction does not bind to this column under the ionic conditions employed and elutes in the initial peak of protein. Anti-pyruvate oxidase activity was measured using the inhibition of the ferricyanide reductase activity of the enzyme. The concentration of IgG was determined using the extinction coefficient at 278 mm of 1.50 mL/mg·cm.

The IgG preparation was examined by both the Ouchterlony double-diffusion test and by immunoelectrophoresis. The Ouchterlony assay revealed a line of identity using pure pyruvate oxidase antigen. Immunoelectrophoresis was performed using a crude lysate from *E. coli* obtained from an early step in the preparation of the enzyme. The only arc of precipitation which appeared corresponded to the pyruvate oxidase within the mixture. The antibody preparation was not challenged with solubilized *E. coli* membrane proteins. Although the IgG preparation is likely monospecific, this cannot be stated with certainty.

Materials. Pyruvic acid was distilled under vacuum and stored tightly sealed under nitrogen. The acid was freshly diluted and neutralized with an equivalent of potassium hydroxide before use. The thiamin analogues, TTPP and TTTPP, were generous gifts of Professors G. Lienhard and L. J. Reed. The compounds were stored, desiccated at −20 °C. Before use, the purity of the compounds was checked by thin-layer chromatography as described by Gutowski and Lienhard (1977). Stock solutions of the analogues were prepared with 10 mM KP_i buffer (pH 7.0). [^{3}H]TPMP$^+$ was a generous gift of H. R. Kaback. Radioactive material was purchased from New England Nuclear or Amersham Searle. Hyamin hydroxide was from New England Nuclear. All other chemicals were from commercial sources and of reagent-grade quality. The New Zealand rabbit was obtained from Morgan Rabbitry, Collum, Ill. Dextran sulfate was obtained from Sigma, and the complete Freund's adjuvant was purchased from Difco.

Results

Properties of Pyruvate-Oxidizing Activity in E. coli B Membrane Vesicles. The pyruvate-oxidizing activity of isolated *E. coli B* cytoplasmic membrane vesicles could be assayed by oxygen consumption (a measure of electron flow through the membrane respiratory chain) or by reduction of the dye dichlorophenolindophenol (DCIP, which accepts electrons from the purified enzyme, vide infra). For O_2 uptake, V_{max} rates averaged 3 nmol min^{-1} (mg of vesicle protein)$^{-1}$, while DCIP reduction averaged 6.8 nmol min^{-1} mg^{-1}. The O_2 uptake (but not DCIP reduction) was specifically inhibited by 3 mM cyanide, as expected for respiratory chain inhibition. The addition of 10 μM thiamin pyrophosphate typically stimulated rates (both O_2 and DCIP) up to twofold. Addition of sodium dodecyl sulfate (60 μM) disrupted membrane integrity but did not increase V_{max} rates [in contrast to the effects reported by Williams and Hager (1966) for soluble, lipid-free pyruvate oxidase]. If the *E. coli* B cells were grown not on the typical alanine-glycerol medium but on glucose or pyruvate-glycerol, there was no alteration in membrane-associated pyruvate-oxidizing activity. On the other hand, membranes from *E. coli* ML 308-225 grown on DL-alanine-glycerol had small amounts of activity, but when grown on succinate no pyruvate-oxidizing activity was detectable in the subsequently isolated membrane vesicles. Membrane vesicles from *E. coli* W191-6, the strain used for isolating pyruvate oxidase (Hager, 1957), showed rates of pyruvate oxidation equivalent to *E. coli* B membranes, but the W191-6 vesicles were smaller, quite fragile to mechanical manipulation, and not useful for solute active transport. All experiments reported here are for *E. coli* B.

Product Identification and Kinetic Constants from Vesicular Oxidation of [^{14}C]Pyruvate. Given an identity of the membrane enzyme to the isolated soluble TPP- and FAD-independent pyruvate oxidase (Gennis and Hager, 1976), the vesicles should process pyruvate to acetate and CO_2. When [3-^{14}C]pyruvate was incubated with vesicles for 45 min as noted under Materials and Methods, 190 nmol of [^{14}C]acetate was separated from pyruvate by Dowex column chromatography and was the only detectable radioactive product. In a complementary experiment with [U-^{14}C]pyruvate, the time-dependent production of radioactive carbon dioxide, trapped in hyamine hydroxide solution, was observed.[2] These results show that pyruvate is oxidized to acetate and CO_2 by the membrane vesicles.

The K_m for pyruvate in vesicular oxidation varied dramatically with pH: it was 80 mM at pH 7.5 but 3.2 mM at pH 6.2, as judged by the O_2-uptake assay. On addition of 60 μM $NaDodSO_4$ to vesicles at pH 7.5, the K_m dropped from 80 to 16 mM, an observation consistent with the data of Williams and Hager (1966) and Cunningham and Hager (1971a,b) on pure *E. coli* W191-6 pyruvate oxidase: K_m (pH 6.0) = 80 mM; K_m (pH 6.0 and $NaDodSO_4$) = 8 mM.

Soluble Pyruvate Oxidase from E. coli B. Pyruvate oxidase was solubilized by sonication and purified from intact *E. coli* B following the procedure of O'Brien et al. (1976) up to the point of 20–30% purity as judged by the $A_{280/450}$ ratio, $NaDodSO_4$-gel electrophoresis, and specific activity compared to the *E. coli* W191-6 enzyme. The soluble *E. coli* B enzyme shows the typical lipid activation behavior expected and, after maximal activation in a 20-min preincubation with 60 μM $NaDodSO_4$, 10 μM TPP, 10 mM $MgSO_4$, and 40 mM pyruvate, showed a K_m for pyruvate of 14 mM. The K_m for TPP was determined to be 10 μM in contrast to the 1 μM value for the W191-6 enzyme. The *soluble* enzyme does *not use* O_2 as cosubstrate, consistent with results for the W191-6 enzyme and validating the idea that the O_2 consumption assay in membrane vesicles measures electron flow down a functional respiratory chain.

Effect of Antibody to E. coli W191-6 Pyruvate Oxidase on E. coli B Pyruvate Oxidase. Antibody to homogeneous W191-6 pyruvate oxidase affects the activity of 30% pure *E. coli* B pyruvate oxidase as shown in Figure 1. At low concentrations of antibody (<0.05 mg of IgG protein/mg of enzyme protein), there is a stimulation up to ~160% initial activity, but at higher ratios the expected decrease in activity occurs, with 50% inactivation at 0.2 mg of antibody/mg of enzyme protein and residual 10% activity at 4.0 mg/mg of enzyme. The initial stimulation is probably due to nonspecific protein activation

[2] Only about 25% of the $^{14}CO_2$ expected from the [^{14}C]acetate production was trapped in the hyamine hydroxide solution. This could be due to incomplete trapping of the CO_2 as we have suggested in another context with this assay (Cromartie and Walsh, 1976), and we have not actually tested to see that the [U-^{14}C]pyruvate, in fact, had one-third of the radioactivity at carbon-1.

TABLE I: Effect of Antibody on Fresh and Frozen Vesicles.

freshly prepared vesicles[a]		once frozen vesicles[a]			
mg of IgG / mg of protein	nmol of DCIP min^{-1} mg^{-1}	mg of IgG / mg of protein	nmol of O_2 min^{-1} mg^{-1}	mg of IgG / mg of protein	nmol of DCIP min^{-1} mg^{-1}
0	14.6 (0%)	0	2.6 (0.0%)	0	7.4 (0.0%)
0.29	14.6 (0%)	0.23[b]	2.5 (3.6%)	0.93	5.98 (19.2%)
0.88	14.6 (0%)	0.24[c]	2.0 (20.0%)		

[a] Fresh vesicles were assayed immediately after the preparation before storage in liquid nitrogen. Frozen vesicles have been stored in liquid nitrogen and rapidly thawed before assaying. [b] Pyruvate was added to the reaction mixture before antibody addition. [c] Antibody and vesicles were preincubated for 3 min before the addition of pyruvate.

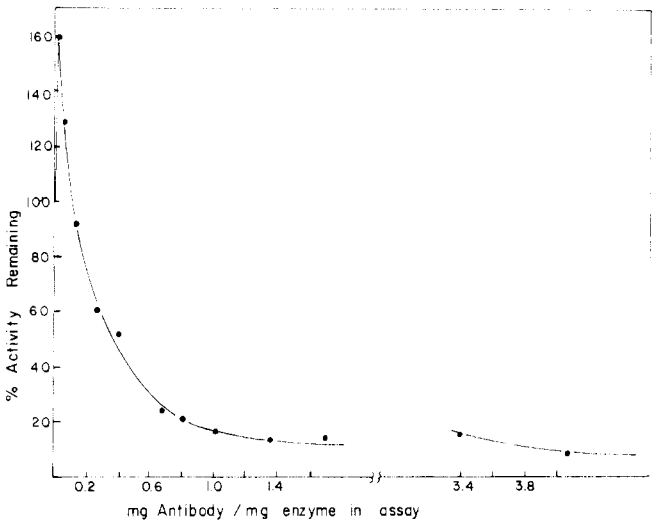
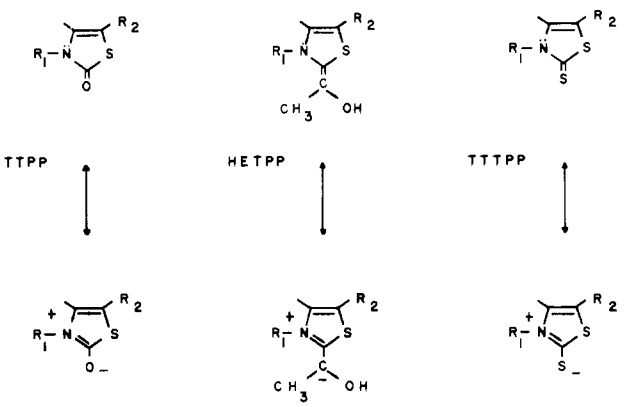


FIGURE 1: Effect of antibody to homogeneous *E. coli* W191-6 pyruvate oxidase on partially purified *E. coli* B pyruvate oxidase. $NaDodSO_4$-activated enzyme was assayed by DCIP reduction after a 20-min incubation with antibody as described under Materials and Methods.



of the enzyme, since the W191-6 enzyme is activated by bovine serum albumin (Williams and Hager, 1966). Preimmune γ-globulin, in fact, activated this soluble *E. coli* B enzyme twofold at 0.42 mg of globulin/mg of enzyme.

The time dependence of antibody-mediated enzyme inactivation was probed at 20 and at 4 °C. Maximal activity loss occurred within 20 min at room temperature and within 90 min at 4 °C, in contrast to the 6–12 h required for inactivation of *E. coli* membrane-derived D-lactate dehydrogenase by anti-D-LDH at 37 or 0 °C (Short et al., 1975a,b).

These results show that the antibody to soluble *E. coli* W-191-6 pyruvate oxidase may be usable to probe the membrane location of the enzyme in vesicles as noted later.

Effect of Thiamin Thiazolone Pyrophosphate on Soluble E. coli B Pyruvate Oxidase. Gutowski and Lienhard (1976) have recently demonstrated that the TPP analogue thiamin thiazolone pyrophosphate (TTPP) (possibly an analogue of the reaction intermediate, the hydroxyethyl-TPP anion (HETPP)) is a potent inhibitor of the *E. coli* pyruvate dehydrogenase complex with an estimated $K_D = \sim 5 \times 10^{-10}$ M for dissociation of the E–TTPP complex. The thiothiazolone analogue, TTTPP, showed similar inhibition and K_D values. Since TPP has a K_D of $\sim 10^{-5}$ M, the thiazolone pyrophosphate and the thiothiazolone pyrophosphate bind ~20 000-fold more tightly than normal TPP coenzyme. The sensitivity of soluble *E. coli* B pyruvate oxidase to TTPP amd TTTPP was tested using the dye reduction assay. Because of the $NaDodSO_4$-activation characteristics of soluble enzyme, assays were performed after a 20-min preincubation with 30 μM $NaDodSO_4$, 40 mM pyruvate, and 10 μM TPP present (required to get full activity in subsequent catalytic assays), as well as the indicated concentration of thiazolone. There is 50% inhibition at 7.2 μM TTPP (data not shown). The thiothiazolone pyrophosphate caused 50% inhibition under identical conditions at an 0.8 μM analogue concentration. K_i values of 10^{-7} M for TTPP and 2×10^{-8} M for TTTPP were subsequently determined. Thus, while both coenzyme analogues are effective inhibitors, there is no dramatically selective binding to pyruvate oxidase. [Although HETPP has been shown to bind to, and undergo catalysis with, pyruvate oxidase, the K_D was not reported (Hager and Krampitz, 1963).]

Location of E. coli B Pyruvate Oxidase in the Cytoplasmic Membrane. Both the antibody and the TPP coenzyme analogues could then be used to probe the sidedness distribution of the membrane-associated form of pyruvate oxidase in the isolated cytoplasmic membrane vesicles which remain competent for solute active transport. Table I shows the degree of antibody inhibition of pyruvate oxidase activity in vesicles both freshly prepared and after a freeze–thaw cycle. There is no inhibition in fresh vesicles, and only 20% inhibition (by either O_2 reduction or DCIP reduction assay) in vesicles after freezing and thawing. The amount of antibody used was five to tenfold higher than the amount needed to inhibit enzyme completely on Triton X-100 solubilization (vide infra). Even after a 16-h incubation (4 °C) of vesicles with 0.29 mg of antibody/mg of vesicle protein, the vesicular pyruvate oxidase was still inhibited only 20%.

These results raised the possibility that the antibody did not have direct access to the membrane-bound enzyme, i.e., the enzyme may be located in the interior or cytoplasmic side of the membrane; such an inner location is postulated for D-lactate dehydrogenase in *E. coli* ML308-225 vesicles (Short et al., 1975a,b). To test this idea, we attempted to disrupt the membrane vesicles, which had been stored frozen at pH 7.5, and see if antibody was then fully inhibitory. However, neither brief sonication with or without glycerol nor incubation with phospholipase A, with $NaDodSO_4$, Triton X-100, or Brij 36-T released much pyruvate oxidase acitivity, nor did the mem-

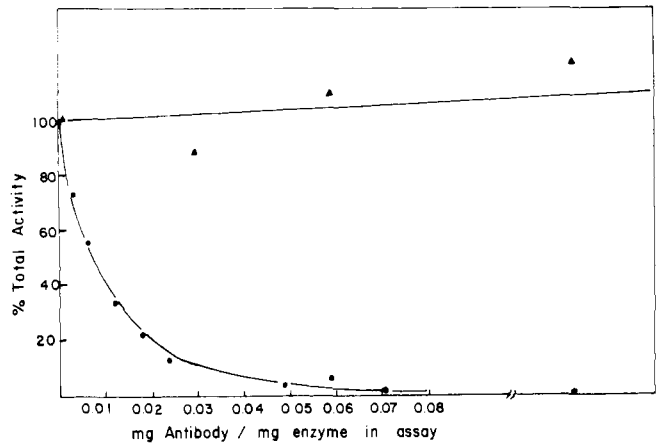


FIGURE 2: Effect of antibody to *E. coli* W191-6 pyruvate oxidase on *E. coli* B membrane vesicle's pyruvate oxidase activity. Vesicles were incubated for 10 min under normal assay conditions plus the indicated concentrations of antibody either in the presence (●) or absence (▲) of 5% Triton X-100. Vesicles were completely solubilized, as determined by lack of absorbance at 600 nm.

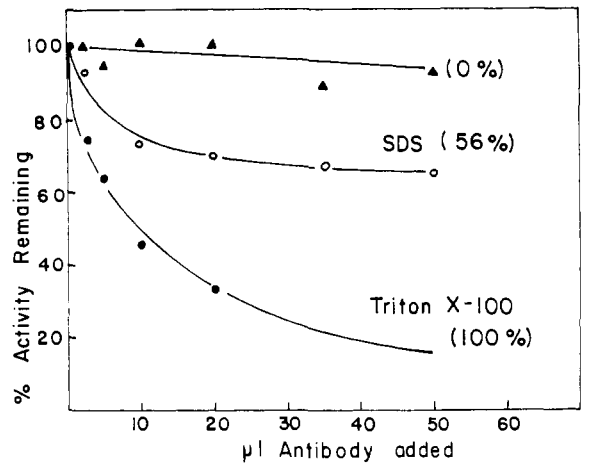


FIGURE 3: Effect of antibody on pyruvate oxidase activity in spheroplasts. *E. coli* B spheroplasts were prepared as described under Materials and Methods. Pyruvate oxidase activity was assayed for by dye reduction as described in untreated (▲), $NaDodSO_4$-treated (1 mM) (○), and Triton X-100 (0.01%) (●) treated spheroplasts after a 5 min. incubation with antibody. The numbers in parentheses indicate the percent of cell lysis, as detected by β-galactosidase activity as described under Materials and Methods.

branes themselves retain detectable activity after such treatments. The source of this apparent enzyme lability turned out to be the pH. The membranes were stored at pH 7.5 to maximize solute active transport; the soluble enzyme is isolated at pH 5.7 and, as we then determined, has a narrow pH optimum range of 5.8 to 6.4 when solubilized and activated. Repetition of the membrane-disruption experiments with 5% Triton X-100 at pH 6.2 in the presence of varying amounts of antibody yielded the results shown in Figure 2. There was 50% inactivation at 6 μg of antibody/mg of vesicle protein *only when Triton* X-100 *is present.* If the amount of antibody causing 50% inhibition on Triton solubilization of vesicles is identical to that inhibiting 50% of soluble pyruvate oxidase (with Triton X-100 present), it can be calculated that pyruvate oxidase represents about 0.2% of the vesicular protein in these preparations. The same antibody inhibition results were obtained if O_2 consumption was used as an assay. No stimulation of Triton-solubilized enzyme was seen at low antibody concentrations.

The antibody results suggest two conclusions at this point: the membrane-associated enzyme is, indeed, pyruvate oxidase and it would appear to be located in the membrane inaccessible to exogenous antibody. A potential ambiguity remains with the antibody that the membrane-bound enzyme may not be the same antigen as the solubilized enzyme. It might be that the antibody does not detect the total amount of pyruvate oxidase on the external surface of the vesicle because the lipid interface causes some protection.

Thus, the thiamin thiazolone pyrophosphate (TTPP) and the thiamin thiothiazolone pyrophosphate (TTTPP) analogues could be used as complementary probes, since they are not likely to be able to cross the membrane due to the negatively charged pyrophosphate group. We have determined separately that neither radioactive thiamin or thiamin pyrophosphate is transported into the membrane vesicles.[3] The degree of inhibition of pyruvate-stimulated DCIP reduction in vesicles should be equivalent to the amount of pyruvate oxidase located on the outside surface of the vesicle membrane. When 40 μM TTTPP (sufficient to inhibit 98% of purified, solubilized pyruvate oxidase activity) was added to the vesicles, 18% of the vesicular pyruvate oxidase was inhibited after a 10-min preincubation. This value is essentially the same amount of inhibition determined in the antibody studies and confirms that about 20% of the enzyme is on the surface of the isolated cytoplasmic membrane vesicles (previously frozen and then thawed).

Location of Pyruvate Oxidase in Spheroplasts. Some questions (Weiner, 1974; Futai, 1974) have been raised that loosely attached, peripheral membrane enzymes (such as pyruvate oxidase) might undergo redistribution during the hypotonic lysis involved in functional cytoplasmic membrane vesicle isolation protocols (Kaback, 1971, 1974). Such movement would tend to randomize the location of enzymes (rather than move them directionally from outside to inside), but we felt the question could be put to rest by using the antibody in spheroplasts, since spheroplast formation precedes any lysis step.

Spheroplasts were prepared as described under Materials and Methods and assayed for pyruvate-stimulated DCIP reduction in the presence of antibody with or without detergent (0.10% Triton X-100 or 1 mM $NaDodSO_4$). Concurrent assays of β-galactosidase with *o*-nitrophenyl galactoside were carried out to determine that no spheroplast lysis was occurring (no β-galactosidase activity detected in absence of detergent). Figure 3 shows that, indeed, there is negligible inhibition of enzyme (pyruvate oxidase) in fresh spheroplasts until $NaDodSO_4$ or Triton X-100 was added. Both $NaDodSO_4$ and Triton X-100 treated spheroplasts showed inhibition corresponding to the degree of spheroplast lysis.

Coupling of Membranous Pyruvate Oxidase to Active Transport. We have previously noted preliminarily that pyruvate oxidation in *E. coli B* membrane vesicles is coupled to the active transport of radioactive proline (Kaczorowski et al., 1975a). This result is general for concentrative uptake of amino acids (Figure 4 shows data for [^{14}C]phenylalanine active transport) and the disaccharide lactose. The pyruvate-coupled rate of solute influx is about one-eighth that of the best artificial electron donor ascorbate–phenazine methosulfate. Pyruvate is about one-third to one-half as effective as D-lactate (oxidized by D-LDH) in data not shown. However, the slower

[3] J. S. Hong and C. Walsh, unpublished observations. To test permeability directly would require highly radioactive TTPP and TTTPP, compounds not available. The argument is by analogy to results with [^{35}S]thiamin and TPP which do not cross the cytoplasmic membrane of these vesicles.

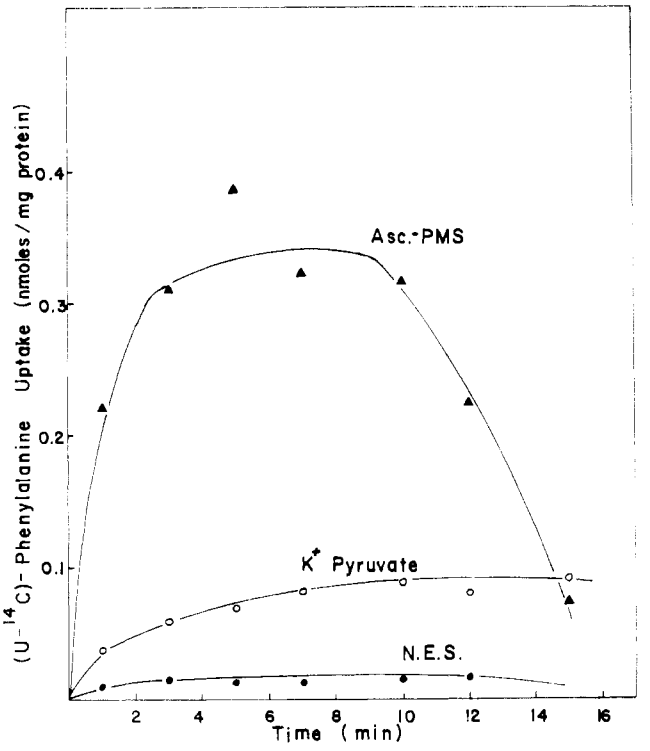


FIGURE 4: Uptake of [U-^{14}C]phenylalanine in *E. coli* B membrane vesicles at pH 7.5, stimulated by ascorbate–PMS, an artificial election donor (▲), 40 mM potassium pyruvate (○), or no energy source (NES) (●). Conditions are described in Kaback (1971).

rate of pyruvate-stimulated solute uptake is not due to specifically inefficient coupling of oxidation to transport but rather due to the small amounts of enzyme associated with the vesicles (a consequence of its peripheral membrane nature). At pH 6.0 the efficiency of D-lactate-driven transport is about 59 nmol of D-lactate oxidized per nmol of radioactive proline actively transported;[4] the efficiency of pyruvate-driven transport is 56 nmol of pyruvate oxidized per nmol proline actively transported. As expected (Ramos and Kaback, 1977a,b), pyruvate oxidation by membranous pyruvate generates a chemical potential across the vesicle membrane; we measured the electrical component both by [^{3}H]TPMP$^+$Br$^-$ distribution or ^{86}Rb$^+$ transport (in the presence of valinomycin) (data not shown) to be ca. −20 to −25 mV.

Effect of Exogenous Addition of Pyruvate Oxidase to E. coli B Membrane Vesicles. Since solubilized pyruvate oxidase is activated by detergents and lipids (Blake et al., 1978) and will bind to liposomes (Schrock and Gennis, unpublished observations), we reasoned that enzyme might bind to vesicles, on the outer face of the membrane presumably, when added to suspensions of cytoplasmic membrane vesicles. Because purified, soluble pyruvate oxidase does not use O_2 as a reducible cosubstrate, but membrane-bound enzyme *functionally* coupled to the membrane electron-transport chain does reduce O_2 (via terminal oxidase), then pyruvate-stimulated O_2 consumption signals vesicular association. An increase in rate of O_2 consumption was detected when ca. 25% pure soluble pyruvate oxidase of *E. coli* B was added back to *E. coli* B vesicles. This reconstitution behavior, as measured by the O_2-reduction assay, showed saturation kinetics (data not shown). The "K_m" value for added enzyme is 0.098 mg of soluble pyruvate oxidase (ca. 25% pure) per mg of vesicle protein, and the maximal rate of O_2 consumption is 62.5 nmol min^{-1} (mg of vesicle protein)$^{-1}$, a 25-fold stimulation over the rate (2.5 nmol min^{-1} mg^{-1} in these vesicles) due to the endogenous level of bound pyruvate oxidase at the start of the experiment.

As expected for functionally reconstituted pyruvate oxidase, 3 mM cyanide completely abolished oxygen consumption in these vesicle suspensions (but was without effect on soluble enzyme). An important control is antibody susceptibility. The subsequent addition of antibody to vesicle suspensions saturated with exogenous pyruvate oxidase (by O_2 assay) inhibited O_2 reduction to endogenous background levels, consistent with the expectation that exogenously bound enzyme stays on the outer face of the membranes and is accessible to antibody. This is in contrast to the endogenous membrane-associated pyruvate oxidase in vesicles which is not accessible to antibody (vide supra).

The functional reconstitution of the electron flow to oxygen suggests that exogenous pyruvate oxidase can find cytochrome *b* molecules (Deeb and Hager, 1964) on the outer surface of the membrane to receive electrons from the bound $FADH_2$. Since pyruvate oxidase is a peripheral membrane enzyme and has K_D values for many lipids in the micromolar range, we next determined in a qualitative way how tightly the enzyme adhered to the outside face of cytoplasmic membrane vesicles. Vesicles were incubated with saturating concentrations of soluble oxidase for 10 min, diluted with a tenfold excess of 0.1 M KP_i (pH 6.2), centrifuged, and rediluted to the original volume. At this point, addition of pyruvate to the vesicles showed only a 1.3-fold increase in rates of O_2 consumption compared to control vesicles that had been exposed similarly to an equivalent amount of bovine serum albumin. Thus, although vesicles can become functionally coupled to exogenous soluble pyruvate oxidase, those enzyme molecules do not become tightly integrated into the membrane structure. Guanidine hydrochloride (0.6 M) used in a similar (but tight) reconstitution of vesicles with D-lactate dehydrogenase (Short et al., 1974a) was of no use. Nor did TPP, $NaDodSO_4$, pyruvate, Triton X-100, or combinations of these factors provide reconstitution stable to dilution and centrifugation. Subsequent experiments, then, simply used suspensions of vesicles to which saturating levels of exogenous soluble pyruvate oxidase had been added.

Since the rate of O_2 consumption can be accelerated 25-fold, it was anticipated that exogenous enzyme should increase the rate of solute active transport coupled to pyruvate oxidation. At low enzyme concentrations, only a 1.2-fold (120% increase) rate effect on [^{14}C]proline uptake was detected. Higher levels of enzyme inhibited active transport but that was due to the 4% glycerol used as a stabilizer with the soluble enzyme. With dialyzed (glycerol-free and moderately unstable) soluble pyruvate oxidase, the highest effect on initial rates of proline transport was 1.6-fold, which was real (see Figure 5), but disappointingly, low. When active-transport rates of [^{14}C]-serine, [^{14}C]phenylalanine, and [^{14}C]lysine were also monitored, the maximal effect was a 2.4-fold rate increase with serine uptake. The "K_m" for this stimulation by enzyme was 0.06 mg of enzyme (25% pure) per mg of vesicle protein.

At this juncture, it seems that the exogenously (and loosely) bound pyruvate oxidase rapidly turns over pyruvate and feeds electrons into the respiratory chain, but the electrons are not coupled properly to establish the full electrochemical potential gradient expected across the membrane (the proximal driving force for solute uptake). We added coenzyme Q1 and vitamin K derivatives with six or eight isoprenyl groups to the reconstituted vesicles but without effect on either O_2 reduction or

[4] Proline uptake is only one of the nine amino acid transport systems coupled to electron flow.

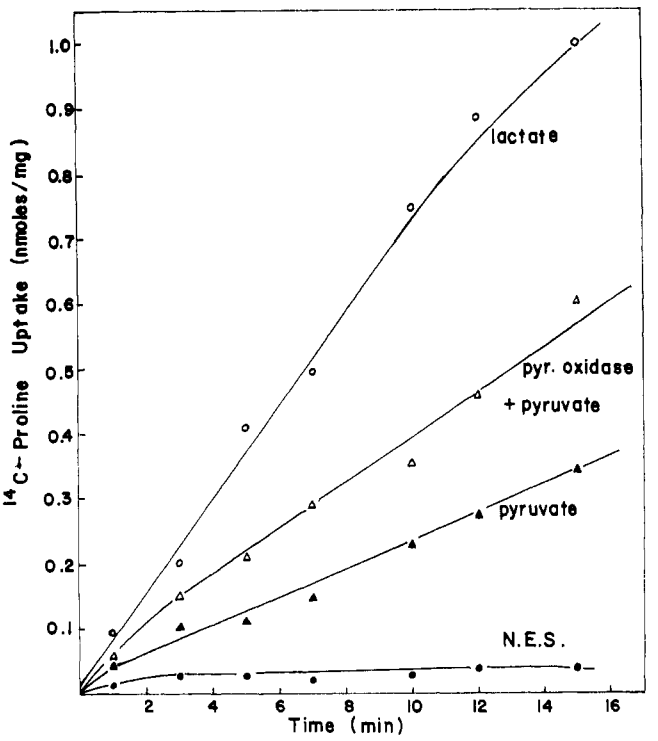


FIGURE 5: Effect of exogenously added, soluble pyruvate oxidase on [^{14}C]proline active transport in *E. coli* B vesicles (pH 6.2). D-Lactate (20 mM) (○) and pyruvate (40 mM) (△) were used as energy sources. Endogenous proline uptake in the presence of pyruvate oxidase (●) and pyruvate-stimulated proline uptake in the absence of additional enzyme (▲) are shown for reference. Soluble pyruvate oxidase was dialyzed against 0.1 M KP_i (pH 5.7), 4% glycerol for 6 h prior to the addition to vesicles, soluble enzyme was added directly to vesicles (0.11 mg of enzyme/mg of vesicle protein) and proline transport was measured under standard conditions (Kaback, 1971).

solute transport rates, in contrast to the observations of Stroobant and Kaback (1975) (*E. coli*) and Bisschop and Konings (1976) (*B. subtilis*) that NADH dehydrogenase, poorly coupled to active transport in the absence of Q or K (respectively), is dramatically coupled by addition of quinone cofactors.

When we measured the effect of exogenous pyruvate oxidase addition not on amino acid solute uptake but on the rate of $^{86}Rb^+$ uptake (in the presence of valinomycin), again a small 1.6-fold stimulation occurred and the enzyme showed a "K_m" = 0.06 mg of added enzyme protein per mg of vesicle protein.

Discussion

E. coli pyruvate oxidase is an unusual enzyme in the sense that it requires both TPP (loosely bound) and FAD (tightly bound, four per tetramer) for catalysis—the oxidative decarboxylation of pyruvate. The other well-studied case of a TPP- and FAD-dependent enzyme is glyoxalate carboligase, from *E. coli* or *P. oxalyticus* (Chung et al., 1971), but there are no redox steps with that reaction and the FAD has been shown conclusively not to undergo reduction in that latter enzyme (Cromartie and Walsh, 1976). There is a clear functional analogy between the single protein pyruvate oxidase and the multicomponent pyruvate dehydrogenase complex, but the oxidative decarboxylation mediated by the latter complex, of course, yields the thermodynamically activated acetyl-CoA rather than the unactivated acetate anion as product. Pyruvate oxidase is also notable for its interaction with a variety of lipids, phospholipids, fatty acids, and detergents via a freely reversible, stoichiometric interaction which dramatically lowers the K_m for pyruvate (up to tenfold) and raises the V_{max} (up to 25-fold). Selective proteolysis apparently cleaves one peptide bond per subunit, reducing the subunit molecular weight from 60 000 to 56 000 and thereby desensitizing the enzyme to the lipid activators and suppressing its association with model membranes[5] (e.g., liposomes) (Russell et al., 1977). These observations are consistent with categorizations of the enzyme as a peripheral membrane protein which interacts with lipid monomers or surfaces by a hydrophobic tail (Schrock and Gennis, 1977) and suggest analogies to microsomal cytochrome b_5 and the flavoprotein dehydrogenase, cytochrome b_5 reductase (Strittmatter and Rogers, 1975).

The results described in this paper allow the identification of pyruvate oxidase as the pyruvate-oxidizing enzyme activity associated with *E. coli* membrane vesicles, functional for active transport, that we detected in 1975 while studying how L-chloroalanine could serve as an oxidizable energy source for active transport. L-Chloroalanine is processed by α,β-elimination to yield HCl, NH_4^+, and pyruvate by the membrane-associated alanine racemase (Kaczorowski et al., 1975a,b), a process that does not put electrons into the respiratory chain, yet L-chloroalanine addition to vesicles powers solute accumulation. Thus, we anticipated and found that pyruvate must in turn be oxidized in a way which puts two electrons functionally into the membrane respiratory chain.

The membrane location of the pyruvate oxidase could be anticipated to be on the cytoplasmic side given the normal production of pyruvate in cellular metabolism. This is the expectation for all such membrane-bound flavoproteins, i.e., accessibility to intracellular oxidizable metabolites, and has been borne out initially by antibody-localization studies for D-lactate dehydrogenase in isolated membrane vesicles of *E. coli* ML308-225 and related strains (Short et al., 1975a,b). Our data with pyruvate oxidase fit this pattern. Of course enzyme localization studies in a population of membrane vesicles are meaningful only if the vesicle population is also homogeneous with respect to sidedness, and, although there has been debate (Hare et al., 1974), a variety of experimental probes have indicated that Kaback's procedures for isolated membrane vesicles produce "right-side-out" vesicles (Stroobant and Kaback, 1975; Short et al., 1974b; Owen and Kaback, 1978). In this work, we have used two different probes for membrane location of the enzyme, antibody against homogeneous soluble enzyme and impermeant inhibitory analogues of the coenzyme TPP, taking specific advantage of the catalytic requirement of pyruvate oxidase for the coenzyme form of vitamin B_1. Both methods suggest the enzyme is not on the outside face of the vesicle membrane. This assignment was corroborated by also analyzing enzyme accessibility in spheroplasts where no question of some preferential redistribution of the peripheral enzyme on lysis to form vesicles can be raised. The complementary use of antibody and thiamin pyrophosphate analogues rules out the worry that antibody may not detect membrane-bound pyruvate oxidase on the external membrane surface because it looks like a different antigen in that environment.

The functional role for pyruvate oxidase in cellular metabolism has been questioned, since it appears energetically wasteful to oxidatively decarboxylate pyruvate to acetate when the pyruvate dehydrogenase complex oxidatively decarboxylates it to metabolically useful, activated acetyl-CoA. However, if one focuses not on the nature of the two-carbon product but

[5] We have not yet examined the behavior of the active proteolytic fragment on exogenous addition to *E. coli* membrane vesicles.

on what happens to the electrons removed, then the electrons removed from the substrate by pyruvate dehydrogenase action end up in NADH, while those from pyruvate oxidase, in its membrane-associated state, pass into the membrane electron-transport chain, fall through a potential drop, and are two of the four electrons eventually used to take O_2 to H_2O by the membrane terminal oxidase. These electrons can do work; in particular, they are used to generate $\Delta\mu_{H^+}$, the electrochemical potential across the cytoplasmic membrane, that can be used to power solute active transport. The degree of functional coupling (of electron flow to membrane potential generation and solute concentrative uptake) of pyruvate oxidase is equal to that seen for D-lactate dehydrogenase and D-alanine dehydrogenase (Kaczorowski et al., 1975a). There is simply less of the pyruvate oxidase per mg of vesicle membrane protein.

The effects of the exogenous addition of the soluble form of pyruvate oxidase were examined in order to study its interaction with a natural membrane. Given the in vivo sidedness (inside) of endogenous membranous pyruvate oxidase, we had no special expectation of specific interactions, although similar previous experiments with D-lactate dehydrogenase (Short et al., 1974) showed functional reconstitution of electron flow to oxygen and active-transport stimulation. As in that instance, a convenient assay for the functional reassociation of pyruvate oxidase with the membrane vesicles is effected on O_2 consumption, since only the membrane-associated, *not the soluble,* form of the enzyme can pass electrons to O_2. Indeed, saturation kinetics in O_2 stimulation were observed with a 25-fold increase in the rate of O_2 reduction. However, due to the weak binding of the pyruvate oxidase to the vesicles, the stoichiometry of that binding cannot be determined by the O_2 uptake assay.

The coupling efficiency of exogenously bound pyruvate oxidase in terms of number of electron pairs per molecule of solute actively transported (e.g., [^{14}C]proline) is very low. The rate of oxygen consumption goes up 25-fold, but the rate of solute transport goes up only 1.6-fold. This difference did not occur with exogenously bound D-lactate dehydrogenase (Short et al., 1974a). We can calculate that for endogenous (cytoplasmic side) pyruvate oxidase 55 electron pairs flow down the chain per molecule of proline transported, but for exogenous pyruvate oxidase 510 electron pairs flow per molecule of solute across the membrane. This inefficiency is directly mirrored in the size of the membrane potential generated per electron pair.[6] In contrast, for exogenous and endogenous D-lactate dehydrogenase bound to membrane vesicles (Short et al., 1974a), the ratio is ca. 20 electron pairs per solute molecule transported. Whether the peripheral vs. integral membrane protein nature accounts for the difference in coupling of exogenous pyruvate oxidase vs. D-lactate dehydrogenase is unclear, but it does reiterate that we do not know what *functional* coupling of electron flow to the establishment of a transmembrane potential means, whether there are specific loops or mobile carriers in one part of the electron transport chain or selectively accessible from one face of the membrane. Despite the dramatic increase in efficiency which coenzyme Q (Stroobant and Kaback, 1975) or menaquinone (Bisschop and Konings, 1976) provide for NADH dehydrogenase, there is no such effect of the mobile redox agents for exogenous (or endogenous) pyruvate oxidase coupling. It may be instructive to do competition experiments with exogenously added dehydrogenases to membrane vesicles to see how many common or exclusive sites of interaction are titrable and how coupling efficiency varies.

[6] Exogenous enzyme addition generates another ca. −15 to −20 mV of $\Delta\psi$ over the −20 to −25 mW of $\Delta\psi$ generated by endogenous pyruvate oxidase (data not shown) as measured by $^{86}Rb^+$ steady-state accumulation levels. The efficiency drop is not at the $\Delta\psi$ increment but in the fact that 25-fold as many substrate electron pairs flow with exogenous vs. endogenous enzyme to achieve a doubling in the transmembrane $\Delta\psi$ value.

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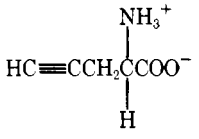
Sequence of Reactions Which Follows Enzymatic Oxidation of Propargylglycine†

Patrick Marcotte‡ and Christopher Walsh*,§

ABSTRACT: The nonenzymatic reactions which follow enzymatic oxidation of the γ-δ acetylenic amino acid propargylglycine (2-amino-4-pentynoate) have been studied. The product which accumulates in solution has been identified as 2-amino-4-hydroxy-2,4-pentadienoate γ-lactone, formed by intramolecular attack of the carboxylate anion on the electrophilic fourth carbon of 2-iminium-3,4-pentadienoate. This previously unknown substance was characterized by its reactions in acid and base and by its nuclear magnetic resonance spectrum. The lactone is preceded in the pathway by 2-amino-2-penten-4-ynoate, a transient electron-rich species which binds tightly to D-amino-acid oxidase and induces a charge-transfer complex with the electron-deficient bound flavin coenzyme. The aminediene lactone is converted by base treatment to 2-amino-4-keto-2-pentenoate, which is also a strong inhibitor of D-amino-acid oxidase and induces a charge-transfer complex.

We have recently described the properties of D-amino-acid oxidase covalently modified upon its oxidation of the acetylenic amino acid D-propargylglycine (**1**) (Marcotte and Walsh, 1978a). Since the enzyme has been demonstrated to carry out a large number of catalytic oxidations before suffering alkylation (Marcotte and Walsh, 1976), the identification of the species produced in such incubations was undertaken. Study of the interaction of D-amino-acid oxidase with D-propargylglycine has been found to be complicated by the presence of two noncovalent inhibitors, which are formed following enzymatic oxidation. Evidence as to the probable structures of these inhibitors is presented in this work, as well as the identification of the oxidation product which accumulates in solution.



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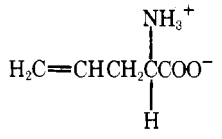

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In the following paper, the sequence of reactions following enzymatic oxidation of allylglycine (**2**) is described, studies providing a comparison of the reactivities of the olefinic and acetylenic functionalities. Two species produced in that pathway also act as strong noncovalent inhibitors of D-amino-acid oxidase and have been characterized. Although similar in some aspects to the propargylglycine pathway, the substitution of the olefin function (in allylglycine) for the acetylene induces several striking differences in the various reactions of the enzymatic oxidation products.

Experimental Section

Materials

Enzymes. D-Amino-acid oxidase from frozen hog kidneys (purchased from Pel-Freez biologicals) was initially purified as described by Brumby and Massey (1968) and then passed through DEAE[1]-Sephadex as described by Curti et al. (1973). Final purification to homogeneity was effected by chromatography on Sephadex G-100 (Pharmacia). D-Amino-acid transaminase from *Bacillus sphaericus* was purified by the procedure of Soda et al. (1974). L-Amino-acid oxidase (*Crotalus adamanteus* venom) and catalase (beef liver) were purchased from Sigma.

Reagents. DL-4-Ketonorvaline was synthesized as reported by Wiss and Fuchs (1952). Propargylglycine (2-amino-4-pentynoic acid) was synthesized and resolved by the method of Jansen et al. (1969). Hepes,[1] phenylpyruvic acid, and acetopyruvic acid were purchased from Sigma. Buffer salts and solvents were commercially available reagent-grade materials.

Chemical Synthesis of 2-Amino-4-keto-2-pentenoate. The ammonium salt of ethyl acetopyruvate was prepared by the addition of 2.5 mL of 15 N NH_4OH to 3.5 g of ethyl acetopyruvate (Marvel and Dreger, 1941) dissolved in 100 mL of tetrahydrofuran. After 30 min of stirring, the precipitate was

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl.

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