The catalytic efficiency ($k_{cat}/K_m$) of Escherichia coli flavin pyruvate oxidase can be stimulated 450-fold either by the addition of lipid activators or by limited proteolytic hydrolysis. Previous studies have shown that a functional lipid binding site is a mandatory prerequisite for the in vitro functioning of this enzyme (Grabau, C., and Cronan, J. E., Jr. (1986) Biochemistry 25, 3748-3751). The effect of activation on the transient state kinetics of partial reactions in the overall oxidative conversion of pyruvate to acetate and CO$_2$ has now been examined. The rate of decarboxylation of pyruvate to form CO$_2$ and hydroxyethylthiamin pyrophosphate for both activated and unactivated forms of the enzyme is identical within experimental error. The decarboxylation step was measured using substrate concentrations of the enzyme in the absence of an electron acceptor. The pseudo-first order rate constant for the decarboxylation step is 60-80 s$^{-1}$. The rate of oxidation of hydroxyethylthiamin pyrophosphate and concomitant enzyme-bound flavin reduction was analyzed by stopped-flow methods utilizing synthetic hydroxyethylthiamin pyrophosphate. The pseudo-first order rate for this step with unactivated enzyme was 2.85 s$^{-1}$ and increased 145-fold for lipid-activated enzyme to 413 s$^{-1}$ and 61-fold for the proteolytically activated enzyme to 173 s$^{-1}$. The analysis of a third reaction step, the reoxidation of enzyme-bound FADH$_2$, was also investigated by stopped-flow techniques utilizing ferricyanide as the electron acceptor. The rate of oxidation of enzyme-FADH$_2$ is very fast for both unactivated (1041 s$^{-1}$) and activated enzyme (645 s$^{-1}$). The data indicate that the FAD reduction step is the rate-limiting step in the overall reaction for unactivated enzyme. Alternatively, the rate-limiting step in the overall reaction with the activated enzyme shifts to one of the partial steps in the decarboxylation reaction.

Flavin pyruvate oxidase (pyruvate:ferricytochrome-b oxidoreductase, EC 1.2.2.2) catalyzes the oxidative decarboxylation of pyruvate to yield carbon dioxide, acetate, and reduced coenzyme. Under in vitro conditions ferricyanide serves as an excellent electron acceptor for the oxidation of the reduced enzyme, and can be used in a convenient spectrophotometric activity assay (1, 2). Under in vivo conditions, POX$^1$ functions as a peripheral membrane-associated flavoenzyme with ubiquinone 8 serving as electron acceptor (3-5). The active enzyme species released from the Escherichia coli cell membrane by sonication is a homotetramer with a subunit molecular weight of 60,000 composed of 572 residues of known sequence (6-13). Each subunit also contains one noncovalently tightly bound coenzyme FAD and requires addition of one TPP cofactor for catalytic activity (2, 6, 7, 14, 15).

Reduction of the flavin prosthetic group of POX plus occupation of the substrate binding site by pyruvate or a substrate analogue produces a dramatic conformational change in the enzyme (16). This conformational change exposes a high affinity lipid binding site in the carboxyl-terminal domain of the enzyme (17) and expresses the carboxyl-terminal domain of POX to hydrolysis by a variety of endo- and exoproteases.

The rate of oxidation of pyruvate by POX can be enhanced by two independent but related activation processes. Stimulation of enzyme activity occurs by binding lipid amphiphiles to the hydrophobic domain exposed by reduction of the enzyme (17-21) or by specific limited proteolytic cleavage of a 23-residue peptide (the a-peptide) from the carboxyl-terminal domain of each subunit (8, 9, 16, 22, 23). The simplest procedure for achieving activated enzyme is by incubating POX with the standard components of the assay reaction mixture minus an electron acceptor. POX is nonautooxidizable so it remains in its reduced form in the absence of an external electron acceptor. The addition of lipid amphiphiles or protease treatment at this point produces the activated enzyme. There are alternative procedures for activation of POX; for example the enzyme can be chemically reduced with dithionite, and the substrate binding site can be occupied by a substrate analogue such as fluoropyruvate (8, 9).

Spectral analysis of the activated enzyme (oxidized form) indicates that the FAD prosthetic group has moved from a hydrophobic to a hydrophilic environment (18). The conformational change which results in the spectral change of the enzyme-bound flavin prosthetic group is thought to be related to the activation process which leads to the enhancement of catalytic activity (16, 24). However, the steps in the catalytic mechanism which respond to the activation process have not been clearly identified. Rapid quench studies measuring the release of $^{14}$CO$_2$ from labeled pyruvate have suggested that the decarboxylation step is enhanced upon activation of POX by lipid amphiphiles (25, 26). Other investigations have presented data which indicate that activation appears to result from increased rates of electron flow both into and out of the flavin prosthetic group (27).

The steady state and pre-steady state kinetic studies presented in this paper were undertaken to identify those steps in the overall catalytic mechanism that are significantly changed upon activation of POX. To achieve this goal, the decarboxylation step was studied apart from the reduction...
and oxidation steps in the overall reaction. A second intermediate reaction was investigated by incubating activated and unactivated POX with hydroxethylthiamin pyrophosphate and measuring the rate of reduction of enzyme-bound FAD under stopped-flow conditions. Finally, pre-steady state means were also employed to follow the rates of oxidation of reduced enzyme-FAD with ferricyanide as the electron acceptor. The data indicate that lipid and protease activation of POX fixes or freezes a conformational form of the enzyme which oxidizes hydroxethylthiamin pyrophosphate at a greatly accelerated rate.

**MATERIALS AND METHODS**

**Purification of POX**—Oxidized enzyme free of TPP and contaminating lipids was isolated from E. coli, W-191-6, by following the thioflavin T fluorescence, sonication, heat treatment, ammonium sulfate precipitation, and DEAE-column chromatography steps developed earlier (29) with the following modifications. Immediately before ion chromatography, EDTA and phenylmethanesulfonyl fluoride were added to final concentrations of 1.0 mM and 100 μM, respectively. The pellet from the 40-70% ammonium sulfate fractionation step was resuspended in a volume of 50 mM sodium phosphate buffer, pH 6.8, at 4 °C plus 20% glycerol (v/v) and 70 mM sodium chloride and was dialyzed overnight against three changes of 5 liters each of the same buffer mixture. Unless otherwise noted all purification steps and handling of POX fractions were carried out at 4 °C. The dialyzed POX was then applied to a 5.8 × 30-cm DEAE-Sephadex A50 column previously equilibrated with the same buffer mixture and was then eluted with the same buffer mixture until little protein appeared in the eluant. The yellow oxidized form of the enzyme was eluted from the column in a step-gradient of the same buffer mixture supplemented with 110 mM sodium chloride. The eluted enzyme was concentrated using a 76-mm YM-100 ultrafiltration membrane (Amicon), and the same steps given above for dialysis and DEAE column chromatography were repeated with the exception that the size of the second column was 2.5 × 20 cm. The 10-mI final stock enzyme was stored in the dark at -18 °C in 100 mM sodium phosphate buffer, pH 5.8, plus 25% glycerol (v/v) to prevent freezing of the solution.

Protein concentrations were estimated by the Bio-Rad dye reagent microassay based on the method of Bradford (29) with bovine albumin fraction V powder from Sigma as the protein standard. The activity of the enzyme preparation was determined at pH 6 and 22 °C by the ferricyanide assay given in the following paragraph. The mean specific activity for five enzyme preparations was 168.7. Recently and Hager (30) reported a specific activity of 180 for HPLC-purified enzyme.

**Spectrophotometric Assay for Enzyme Activity Using Ferricyanide**—The millimolar extinction coefficient for ferricyanide at 450 nm and at pH 6 and 22 °C is 0.218 (25). The decrease in absorbance associated with ferrocyanide formation was monitored in 1-cm cuvettes in a Cary-Varian 219 spectrophotometer. The reaction mixture contained the following final concentrations of components added in sequence: 100 mM sodium phosphate buffer, pH 6.0; 20 mM magnesium chloride; 200 μM thiamin pyrophosphate; 200 mM sodium pyruvate; -24 μg of POX (0.1 μM tetramers); and 35 μM SDS. The reaction was started by the addition of 9 mM ferrocyanide after a 2-min incubation period which permits the formation of the lipid-activated form of the enzyme.

**Stopped-flow Analysis of the Decarboxylation Step**—Aliquots of the stock pyruvate oxidase preparation were pipetted into 50,000 molecular weight cut-off tubing and dialyzed overnight against two changes of 5 liters of 10 mM NaCl adjusted to pH 5.7. This step was necessary to remove phosphate buffer and glycerol from the stock enzyme preparation (E-FAD). The low concentration of NaCl prevented POX from precipitating out of the solution. Assays of enzyme activity indicated a 17% loss in activity in this dialysis step. The procedure used for obtaining proteolytically activated POX is the same as that given under "Materials and Methods" with the exception that the activated enzyme was dialyzed by the same procedure outlined above. The pseudo-first order reaction rates for the decarboxylation step were monitored in the stopped-flow apparatus by following the decrease in absorbance of bromocresol green at 615 nm due to the production of hydrogen ion resulting from the hydrated carbon dioxide produced in the decarboxylation step. The reaction mixture was adjusted to pH 5.8, at 4 °C plus 20% glycerol (v/v) and 70 mM sodium chloride and was dialyzed overnight against three changes of 5 liters each of the same buffer mixture. The dialyzed POX was then applied to a 5.8 × 30-cm DEAE-Sephadex A50 column previously equilibrated with the same buffer mixture and was then eluted with the same buffer mixture until little protein appeared in the eluant. The yellow oxidized form of the enzyme was eluted from the column in a step-gradient of the same buffer mixture supplemented with 110 mM sodium chloride. The eluted enzyme was concentrated using a 76-mm YM-100 ultrafiltration membrane (Amicon), and the same steps given above for dialysis and DEAE column chromatography were repeated with the exception that the size of the second column was 2.5 × 20 cm. The 10-mI final stock enzyme was stored in the dark at -18 °C in 100 mM sodium phosphate buffer, pH 5.8, plus 25% glycerol (v/v) to prevent freezing of the solution.

**Synthesis of Hydroxethylthiamin Pyrophosphate**—Synthesis of 2-(1-hydroxyethyl)thiamin pyrophosphate (HETPP) was performed by the method of Grues et al. (31). Peak fractions from the Sephadex SP-C25 anion-exchange column were pooled and evaporated to a 2-mI volume, freeze-dried, and stored in the dark at 4 °C in a dessicator. UV, NMR, and mass spectrometry were used to identify and establish the purity of the HETPP preparation. Ultraviolet spectra from 210 to 320 nm were recorded for the HETPP dissolved in 50 mM sodium phosphate buffer, pH 6.0, after a first freeze. UV, NMR, and mass spectrometric analyses were also determined using a ZAB-SE Vacuum General (Manchester, England) mass spectrometer operated at 8 kV. "HNMR analysis of HETPP was done on a General Electric 500 MHz model GN500 spectrometer.

**Carbonamide Linking of Lauric Acid to the High Affinity Lipid Binding Site of POX and Reoxidation of the Activated Enzyme**—The carbodiimide procedure developed by Hamilton et al. (17) to conveniently link a lipid activator to POX was modified as follows. The first step was to convert the oxidized enzyme to its reduced form (E-FADH), since only the reduced enzyme binds lipids at its high affinity site. Chemical reduction with dithionite was used in order to avoid the addition of TPP to the enzyme preparation. The presence of bound TPP in the enzyme preparation would interfere with the subsequent binding and utilization of HETPP in the stopped-flow experiments for the measurement of the kinetic parameters of the reduction step. Reduction of POX with dithionite has been successfully accomplished in earlier studies by Russell et al. (8, 9). The reaction mixture contained the following components: 100 mM sodium phosphate buffer, pH 5.8; 10 mM magnesium chloride; 100 mM sodium pyruvate; 6.5 mM POX; and 20 mg sodium dithionite which was slowly added with gentle swirling over a 3-min period. Dimethyl sulfoxide and an aliquot of a freshly prepared 10 mM stock solution of lauric acid in dimethyl sulfoxide was added to the above mixture to give a final concentration of 300 μM lauric acid in 8% dimethyl sulfoxide. The solution was incubated at 22 °C for 3 min with an occasional gentle swirl. Covalent linkage of the lauric acid to lysine 544 of POX was accomplished by addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to a final concentration of 5 mM. The mixture was incubated at 22 °C for 30 min. The carbodiimide-affinity-labeling reaction was terminated by the addition of 2 M ammonium acetate to a final concentration of 100 mM at pH 6.0. The reaction mixture was then dialyzed in 50,000 molecular weight cut-off tubing against 5 liters of 100 mM sodium phosphate buffer, pH 5.8, plus 20% glycerol (v/v) for 3 h. The lipid-activated enzyme in its reduced form was reoxidized back to E-FAD by incubating the enzyme preparation with 500 μM ubiquinone Q. The solution was again dialyzed under the same conditions as given above. The oxidation with ubiquinone Q and dialysis was repeated once more except that dialysis was carried out overnight. The activated enzyme was used immediately in stopped-flow experiments. Although some loss of enzyme activity was to be expected in the affinity-binding reaction, the activity of an aliquot of the lauric acid-activated enzyme, determined for the carbodiimide-affinity-labeling reaction, was 71% of the rate achieved by an aliquot of the same amount of fresh stock enzyme activated by the addition of 35 μM SDS or 300 μM lauric acid.

**Proteolytic Activation of the Reduced Form of POX**—Limited proteolytic hydrolysis in the carboxyl-terminal region of each POX subunit provides the same extent of activation as achieved by the addition of lipid activators (8, 9, 16, 22). Chemical reduction of the native oxidized stock enzyme, E-FAD, to E-FADH was carried out under the same conditions given above for carbodiimide linking of the lipid activator. An aliquot of freshly prepared α-chymotrypsin (1 mg of protease in 1 ml of 100 mM sodium phosphate buffer, pH 5.8) was added to the reduced POX in a 90:1 (w/w) ratio of POX to protease and incubated at 22 °C for 60 min. Proteolysis was stopped by the addition of phenylmethylsulfonyl fluoride at a final concentration of 80 μM. The solution was dialyzed and POX was reoxidized to E-FAD by the same procedure utilizing ubiquinone Q as described above for the carbodiimide method. After correction for dilution and volume changes, an aliquot of the proteolytically activated POX gave a rate that was 78% that of a fresh aliquot of stock enzyme assayed in the presence of 35 μM SDS.

**Stopped-flow Kinetic Analyses**—A Union Giken (Atago Bussan Co., Ltd., Tokyo, Japan) stopped-flow spectrometer was used for all of the transient state kinetic measurements. First order exponential decays were linearized by programs stored in the data base processing unit, and the Guggenheim plot was used to extract kobs s-1 and the mean, plus or minus the sample standard deviation, was calculated for each set of five determinations.
The Guggenheim plot calculates the apparent rate constant, $k_{obs}$, from the equation $y = C + D\cdot e^{-kt}$. The values of $C$ and $D$ are calculated by the least squares method from a specified portion of the reaction curve. The values of $x$ and $y$ record the time coordinates and absorbancies, respectively. The rate constant, $k_{obs}$, is obtained from the relationship $k_{obs} = k_n/\text{full scale on the x axis (in milliseconds)}$. In some cases, data collection was started with an imposed time delay in order to avoid the more noisy part of the time curve.

RESULTS

The catalytic cycle for POX can be separated into three sequential events (Fig. 1), which can be monitored independently of each other (25). The first overall reaction, which probably represents a composite of (a) a substrate binding reaction, (b) the formation of a lactylthiamin pyrophosphate intermediate, and (c) its decarboxylation to form an enzyme-bound HETPP and CO$_2$ can be measured by monitoring CO$_2$ formation. A second easily measured step is the reduction of enzyme-bound FAD resulting from oxidation of HETPP to its acyl derivative. The third step, which completes the cycle, is the oxidation of reduced enzyme (E. FADH) to E. FAD by an electron acceptor such as ferricyanide.

Decarboxylation Experiments—The transient state kinetics of the decarboxylation reaction was assayed under stopped-flow conditions by measuring hydrogen ion production following the hydration of CO$_2$ to carbonic acid. The reaction was carried out in unbuffered solutions which contained brom cresol green, and carbonic anhydrase. Brom cresol green was found to function as a sensitive probe for monitoring the pH changes associated with the formation bicarbonate and hydrogen ions. Carbonic anhydrase was included in the reaction mixtures in order to make certain that the rate-limiting step in the assay was the decarboxylation reaction and not the hydration of CO$_2$. All of the components of the reaction mixture in both flow cells were adjusted to pH 5.7. The reaction mixture in flow cell 1 contained brom cresol green, MgCl$_2$, TPP, carbonic anhydrase, and POX. The components in flow cell 2 contained brom cresol green, MgCl$_2$, pyruvate, and POX. The calculated pseudo-first order rate constant for the decarboxylation step with unactivated enzyme was 62 s$^{-1}$ (Fig. 2A). The pseudo first rate constant for decarboxylation with proteolytically activated POX was 79 s$^{-1}$ (Fig. 2B). In a single experiment with lipid-activated enzyme, the pseudo-first order rate constant was calculated to be 75 s$^{-1}$. These results indicate that, within experimental error, the rate constant of the decarboxylation step is the same for both activated and unactivated enzyme.

Control experiments were performed to prove that the hydrogen ions produced in these decarboxylation experiments came from hydrated carbon dioxide and not from some other source such as the enzyme or substrate. Fluoropyruvate is a substrate inhibitor of the pyruvate oxidase reaction and was used as one control. The substitution of equimolar amounts of fluoropyruvate for pyruvate in the reaction mixture did not promote hydrogen ion formation. In a second control experiment, pyruvate was included, but thiamin pyrophosphate was omitted from the reaction mixture. Again no hydrogen ion formation could be detected in this control. These control experiments rule out the possibility of hydrogen ion production from pyruvate or enzyme.

It is interesting to note that the absence of carbonic anhydrase from the reaction mixtures did not affect the measured $k_{obs}$ values. All of the experiments gave $k_{obs}$ values in the range of 60–80 s$^{-1}$ in the absence or in the presence of 22 μM carbonic anhydrase. This was somewhat surprising since the reported pseudo-first order rate constant for the hydration of CO$_2$ in an uncatalyzed reaction is 0.04 s$^{-1}$ (32). However, control experiments showed that the components of the POX assay significantly stimulate the hydration reaction.

Reduction Step Experiments—The initial stopped-flow reduction experiments determined the rate of reduction of POX in the presence of TPP and pyruvate but in the absence of an electron acceptor. The $k_{obs}$ values obtained in these measurements represent a composite of both the decarboxylation and reduction reactions. The reduction of POX was monitored by following the decrease in absorbance at 438 nm as the enzyme changes from the yellow oxidized form, E. FAD, to the colorless reduced form E-FADH. In five determinations, a mean $k_{obs}$ value of 3.47 s$^{-1}$ was obtained for unactivated enzyme. A tracing of the monitor scope displaying the reaction time curve and the conditions under which the experiment was run are given in Fig. 3A. In the absence of either TPP or pyruvate no reduction was observed. Also, in preliminary experiments, rates were measured at various levels of TPP over the range of 50–800 μM final concentration in the measuring chamber with no statistically significant increase in $k_{obs}$ above 400 μM TPP.

Synthetic HETPP was used in order to isolate and study
**Activation of Pyruvate Oxidase**

**Fig. 3.** Transient state kinetic measurements on the reduction of unactivated POX. Stopped-flow analysis of the reduction of unactivated POX in the oxidized state, E-FAD, to the reduced enzyme form E-FADH, was measured either by the addition of TPP plus the substrate pyruvate (A) or by the addition of the synthetic HETPP (B). The dotted lines are tracings of the pseudo-first order reaction curves obtained experimentally. The solid lines are the computer-generated fits to the reaction curves. The pseudo-first order reaction rate constants were measured by monitoring the decrease in absorbance at 438 nm associated with the conversion of the oxidized to the reduced enzyme (E-FAD → E-FADH). For the reduction of E-FAD by TPP and substrate, flow cell 1 contained in 2 ml the following final concentrations: 100 mM sodium phosphate buffer (pH 5.8), 20 mM magnesium chloride, 400 μM TPP, and 400 mM sodium pyruvate. The second flow cell contained in 2 ml: 100 mM sodium phosphate buffer, pH 5.8, and 6.5 μM POX (E-FAD). For the reduction of E-FAD by HETPP one flow cell contained in 2 ml the following final concentrations: 100 mM sodium phosphate buffer, pH 5.8, 20 mM magnesium chloride, and 800 μM HETPP. The second flow cell contained 100 mM sodium phosphate buffer, pH 5.8, and 6.5 μM POX (E-FAD). The arrows in A and B indicate the time point at which data collection started.

**Fig. 4.** Transient state kinetic measurements on the reduction of activated POX by HETPP. Stopped-flow analysis of the reduction of lipid activated POX (A) or prolytically activated POX (B) was measured by addition of synthetic HETPP. The dotted lines are tracings of the pseudo-first order reaction curves obtained experimentally. The solid lines are the computer-generated fits to the reaction curves. One flow cell contained in 2 ml the following final concentrations: 100 mM sodium phosphate buffer, pH 5.8, 20 mM magnesium chloride, and 800 μM HETPP. The other flow cell contained 100 mM sodium phosphate buffer, pH 5.8, and 6.5 μM of either lipid or proteolytically activated enzyme (E-FAD). The pseudo-first order rate constants were measured by following the decrease in absorbance at 438 nm associated with the conversion of E-FAD to E-FADH.

**Fig. 5.** Transient state kinetic measurements on the oxidation of reduced POX by ferricyanide. Stopped-flow analysis of the rate of oxidation of unactivated reduced enzyme, E-FADH (A) or lipid-activated reduced enzyme (B) by reaction with ferricyanide. The dotted lines are tracings of the pseudo-first order reaction curves obtained experimentally. The solid lines are the computer-generated fits to the reaction curves. One flow cell contained in 2 ml the following final concentrations: 100 mM sodium phosphate buffer, pH 5.8, 20 mM magnesium chloride, and 0.78 mM potassium ferricyanide. The other flow cell contained 100 mM sodium phosphate buffer, pH 5.8, and either 6.5 μM unactivated reduced enzyme (E-FADH) or reduced enzyme plus 35 μM SDS as lipid activator. The formation of oxidized enzyme was monitored at 438 nm.

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The transient state kinetics of the reduction step for activated POX were investigated with both the protease and the lipid-activated forms of the enzyme (see Fig. 4, A and B). The lipid-activated form of the enzyme used in these experiments was the lauric acid-affinity labeled species (17). The calculated pseudo-first order rate constant for the reaction of lipid-activated enzyme with HETPP is 413 s⁻¹ (Fig. 4A). This value represents a 145-fold increase over the rate for unactivated enzyme. The pseudo-first order rate constant for the reaction of protease-activated enzyme with HETPP is 173.6 s⁻¹, a 61-fold increase over the unactivated rate (Fig. 4B). Since these rates are fairly fast and difficult to measure with great accuracy, the standard deviation values are relatively large. Thus the difference between 145-fold activation for lipid-activated enzyme and 61-fold for proteolytically activated enzyme is less significant than appears by the comparison of the mean values.

**Oxidation Step Experiments**—In these experiments stock enzyme was reduced to E-FADH by the addition of 200 μM TPP and 200 μM pyruvate. Excess cofactor, substrate, and products were rapidly removed by dialysis (50,000 molecular weight cut-off tubing) against 100 mM sodium phosphate buffer, pH 5.8, plus 20% (v/v) glycerol. The rate of oxidation of E-FADH was monitored by following the increase in absorbance at 438 nm as the colorless reduced enzyme was converted to the yellow oxidized form. The pseudo-first order rate constant for unactivated enzyme for ferricyanide oxidation was 1041 s⁻¹ (Fig. 5A). The fast oxidation rates with ferricyanide push the limits of the instrumentation to an extreme and are best viewed as a good estimate of the rate.

As shown in Fig. 5B, lipid-activated E-FADH gave a pseudo-first order rate constant of 645 s⁻¹ in the oxidation reaction with ferricyanide. This value is similar to that displayed for unactivated enzyme. Very similar rates of oxidation were obtained with the proteolytically activated form of POX. The kobs with ferricyanide was 688 s⁻¹.

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### Table I

| Reaction | Unactivated | Activated |
|----------|-------------|-----------|
| 1-Decarboxylation | 62 ± 8.1 | 79 ± 9.7 |
| 2-Oxidation of HETPP | 2.85 ± 0.27 | 418 ± 168 |
| 3-Oxidation of E-FADH with ferricyanide | 1041 ± 936 | 645 ± 104 |
| kobs (V/E0) for overall enzyme catalyzed reaction | 4.5 ± 1.2 | 87 ± 12 |
**DISCUSSION**

*E. coli* pyruvate oxidase has served as a model in numerous investigations aimed at defining the mechanism of lipid and/or limited proteolytic activation of peripheral membrane proteins (8, 9, 16, 19). Phenylalanine hydroxylase and protein kinase c are two other members of the enzyme family which resembles the activation behavior of pyruvate oxidase (35-38). It has been firmly established that lipid activation, not protease activation, of pyruvate oxidase is the physiologically important process. Grabau and Cronan (12) have constructed a mutant pyruvate oxidase gene which codes for the production of a truncated form of the enzyme which lacks the final 23 carboxyl-terminal residues of the protein. These 23 carboxyl-terminal residues encompass the high affinity lipid binding site of pyruvate oxidase and comprise the α-peptide which is cut from the enzyme by chymotryptic activation. The engineered protein, like the protease-activated enzyme, is fully active in vitro in the absence of lipid. However, strains producing the truncated protein were devoid of *in vivo* pyruvate oxidase activity. The Grabau and Cronan experiment shows that membrane binding via the high affinity binding site in the carboxyl-terminal domain of the enzyme is specifically required for *in vivo* function. Thus, in physiological terms, activation performs two important steps in the functioning of pyruvate oxidase. First, activation promotes the binding of the enzyme to the plasma membrane so that it can transfer electrons to the terminal electron transport system of *E. coli*. Second, activation greatly enhances the catalytic activity of the oxidase.

The experiments reported here have focused on a kinetic analysis of individual steps in the overall pyruvate oxidase reaction. A summary of the pseudo-first order rate constants for the decarboxylation, reduction, and oxidation reactions are presented in Table I. A comparison of the rate constants in Table I indicate that activation of POX shifts the rate-limiting step in the overall reaction from the enzyme reduction step clearly is not the rate-limiting step. Instead, the overall decarboxylation reaction must become the rate-limiting determinant. Unactivated POX as well as lipid or protease-activated enzyme exhibit equivalent first order rate constants in the decarboxylation reaction, 60-80 s⁻¹.

Results for the oxidation of activated and unactivated POX with electron acceptors are in good agreement with those obtained by other investigators. With ferricyanide as electron acceptor, Blake (25) had shown that the oxidation rate was much faster than the decarboxylation rate with a value for *kₐ* of 1500 s⁻¹ which agrees very well with the estimate for *kₐ* of 1041 s⁻¹ obtained from stopped-flow studies presented in this paper. Thus, in contrast to the large enhancement of the reduction step upon activation of the enzyme, the turnover for the oxidation step with ferricyanide as electron acceptor is not significantly changed and remains relatively fast at 645 s⁻¹. These results do not agree with the suggestion from an earlier study (27) that activation results from an increased flow of electrons out of bound FAD, i.e. *kₐ* is much faster with ferricyanide for activated enzyme.

In conclusion, the single notable change in activated versus unactivated enzyme rates is in the reduction step. A change in orientation of the isoalloxazine ring of enzyme-bound FAD gives a quite different picture. In particular the oxidation of HETPP with concomitant formation of E-FADH is enhanced over 100-fold for lipid-activated enzyme. Under these conditions the enzyme reduction step clearly is not the rate-limiting reaction. Instead, the overall decarboxylation reaction must become the rate-limiting determinant. Unactivated POX as well as lipid or protease-activated enzyme exhibit equivalent first order rate constants in the decarboxylation reaction, 60-80 s⁻¹.

Yeast pyruvate decarboxylase is a well-studied enzyme which decarboxylates pyruvate via an enzyme-bound TPP with CO₂ and acetalddehyde as products. In the yeast pyruvate decarboxylase reaction, the initial 3 carbon adduct, lactyl-TPP, has been viewed as a tertiary malonyl nitrile. This adduct should decarboxylate at a fast rate. The rate-limiting step for this enzyme was found not to be the decarboxylation step but the conversion of the active aldehyde HETPP to...
acetaldehyde and the release of this product (39, 40). Thus the pyruvate decarboxylase mechanism is somewhat analogous to the POX case reported in this paper.

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REFERENCES
1. Hager, L. P. (1967) J. Biol. Chem. 229, 251–263
2. Williams, F. R., and Hager, L. P. (1966) Arch. Biochem. Biophys. 116, 168–176
3. Deeb, S. S., and Hager, L. P. (1964) J. Biol. Chem. 239, 1024–1031
4. Cunningham, C. C., and Hager, L. P. (1975) J. Biol. Chem. 250, 7139–7146
5. Anraku, Y., and Gennis, R. B. (1987) Trends Biochem. Sci. 12, 262–266
6. O’Brien, T. A., Schrock, H. L., Russell, P., Blake, R., and Gennis, R. B. (1976) Biochim. Biophys. Acta 452, 13–29
7. Raj, T., Russell, P., Flygare, W. H., and Gennis, R. B. (1977) Biochim. Biophys. Acta 481, 42–49
8. Russell, P., Hager, L. P., and Gennis, R. B. (1977) J. Biol. Chem. 252, 7877–7882
9. Russell, P., Schrock, H. L., and Gennis, R. B. (1977) J. Biol. Chem. 252, 7883–7887
10. Stevens, D. J., and Gennis, R. B. (1980) J. Biol. Chem. 255, 379–383
11. Grabau, C., and Cronan, J. E., Jr. (1984) J. Bacteriol. 160, 1088–1092
12. Grabau, C., and Cronan, J. E., Jr. (1986) Biochemistry 25, 3748–3751
13. Grabau, C., and Cronan, J. E., Jr. (1986) Nucleic Acids Res. 14, 5549–5566
14. O’Brien, T. A., Blake, R., and Gennis, R. B. (1977) Biochemistry 16, 3105–3109
15. O’Brien, T. A., and Gennis, R. B. (1980) J. Biol. Chem. 255, 3302–3307
16. Recny, M. A., and Hager, L. P. (1983) J. Biol. Chem. 258, 5180–5185
17. Hamilton, S. E., Recny, M. A., and Hager, L. P. (1986) Biochemistry 25, 8178–8183
18. Cunningham, C. C., and Hager, L. P. (1971) J. Biol. Chem. 246, 1575–1582
19. Schrock, H. L., and Gennis, R. B. (1977) J. Biol. Chem. 252, 5990–5995
20. Blake, R., Hager, L. P., and Gennis, R. B. (1978) J. Biol. Chem. 253, 1963–1971
21. O’Brien, T. A., and Gennis, R. B. (1979) Biochemistry 18, 804–809
22. Schrock, H. L., and Gennis, R. B. (1980) Biochim. Biophys. Acta 614, 215–220
23. Recny, M. A., Grabau, C., Cronan, J. E., Jr., and Hager, L. P. (1985) J. Biol. Chem. 260, 14287–14291
24. Mather, M. W., and Gennis, R. B. (1985) J. Biol. Chem. 260, 10395–10397
25. Blake, R. C. (1977) Pyruvate Oxidase: Mechanism of Lipid Activation. Ph.D. thesis, University of Illinois, Champaign-Urbana, IL
26. Blake, R., and Hager, L. P. (1982) in flavins and flavoproteins (Massey, V., and Williams, C. H., eds) pp. 864–868, Elsevier North-Holland, Inc., Amsterdam
27. Mather, M. W., and Gennis, R. B. (1985) J. Biol. Chem. 260, 1575–1582
28. Recny, M. A. (1983) Studies on the Flavin Binding Properties of Native and Protease-activated Pyruvate Oxidase from Escherichia coli. Ph.D. thesis, University of Illinois, Champaign-Urbana, IL
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
30. Recny, M. A., and Hager, L. P. (1982) J. Biol. Chem. 257, 12878–12886
31. Grusy, K. J., Halkides, C. J., and Frey, P. A. (1987) Biochemistry 26, 7575–7585
32. Ho, C., and Sturtevant, J. M. (1963) J. Biol. Chem. 238, 3499–3501
33. Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610–7616
34. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695
35. Abita, J.-P., Parniak, M., and Kaufman, S. (1984) J. Biol. Chem. 259, 14560–14566
36. Edsall, J. T., and Wyman, J. (eds) (1958) Biophysical Chemistry, vol. 1, p. 584, A P Press, New York
37. Jean, Y., and Volatron, F. (1982) Chem. Phys. 65, 107–111
38. Kluger, R., Chin, J., and Smyth, T. (1981) J. Am. Chem. Soc. 103, 884–888
39. Crosby, J., and Lieber, G. E. (1970) J. Am. Chem. Soc. 92, 5707–5716
40. Kuo, D. J., and Jordan, F. (1983) J. Biol. Chem. 258, 13415–13417