Activation of Thiamin Diphosphate and FAD in the Phosphate-dependent Pyruvate Oxidase from *Lactobacillus plantarum**

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The phosphate- and oxygen-dependent pyruvate oxidase from *Lactobacillus plantarum* is a homotetrameric enzyme that binds 1 FAD and 1 thiamine diphosphate per subunit. A kinetic analysis of the partial reactions in the overall oxidative conversion of pyruvate to acetyl phosphate and CO_2 shows an indirect activation of the thiamine diphosphate by FAD that is mediated by the protein moiety. The rate constant of the initial step, the deprotonation of C2-H of thiamine diphosphate, increases 10-fold in the binary apoenzyme-thiamine diphosphate complex to 10^{-2} s^{-1}. Acceleration of this step beyond the observed overall catalytic rate constant to 20 s^{-1} requires enzyme-bound FAD. FAD appears to bind in a two-step mechanism. The primarily bound form allows formation of hydroxyethylthiamine diphosphate but not the transfer of electrons from this intermediate to O_2. This intermediate form can be mimicked using 5-deaza-FAD, which is inactive toward O_2 but active in an assay using 2,6-dichlorophenolindophenol as electron acceptor. This analogue also promotes the rate constant of C2-H dissociation of thiamine diphosphate in pyruvate oxidase beyond the overall enzyme turnover. Formation of the catalytically competent FAD-thiamine-pyruvate oxidase ternary complex requires a second step, which was detected at low temperature.

Pyruvate oxidase (POX)[1] from *Lactobacillus plantarum* (EC 1.2.3.3) is a homotetramer. Each subunit (mass, 65.5 kDa) binds one FAD and one thiamine diphosphate (ThDP) in the presence of Mn^{2+} or Mg^{2+}. In the presence of oxygen and phosphate, the POX holoenzyme catalyzes the oxidative decarboxylation of pyruvate (1–3) according to the equation CH_3-$COCOO^-$+O_2 + HPO_4^{-2} + $2H^+$ → CO_2 + H_2O_2 + CH_3-$COOPO_3H^-$. In the presence of Mn^{2+}, both coenzymes FAD and ThDP can form binary complexes with the apoenzyme that are enzymatically inactive in the native overall oxidation reaction (4).

The important catalytic steps in the POX reaction are: step 1, deprotonation of C2-H of ThDP, the initial step shared by all ThDP dependent enzymes; step 2, binding of pyruvate to the C2 atom of enzyme-bound ThDP; step 3, decarboxylation of pyruvate to hydroxyethyl-ThDP; step 4, oxidation of hydroxyethyl-ThDP by FAD; and step 5, reoxidation of reduced FAD by oxygen. To study the role of both coenzymes in POX catalysis, we have used four different approaches that selectively investigate some of the individual steps enumerated above: (*a*) In the initial reaction (step 1), the deprotonation of the C2-atom of the ThDP in POX holoenzyme as well as in the binary ThDP complex was studied using ^{1}H NMR by following the C2-H/D exchange (5). (*b*) DCPIP is known to accept electrons from hydroxyethyl-ThDP in other ThDP-dependent enzymes (6, 7). Here it was used as an artificial acceptor to assess the mode of electron transfer to O_2. Two alternative donor loci have to be considered, the ThDP adduct itself or $FADH_2$, to which redox equivalents could be transferred from ThDP. We have also used this assay with POX in which normal FAD was substituted with the analogue 5-deaza FAD (5-dFAD), which is essentially incapable of carrying out rapid redox reactions and in particular does not react with O_2. This system was expected to provide information about steps 4 and 5 above. (*c*) Formation of the final product H_2O_2 was followed directly using the peroxidase assay (8). (*d*) The latter method was correlated by direct measurements of the O_2 tension. In addition the different activity assays were used to investigate the recombination of FAD with the binary apo-ThDP complex.

MATERIALS AND METHODS

Chemicals and Proteins

5-dFAD was synthesized from 5-deazariboflavin (9) using the purified FAD-synthetase complex of *Brevibacterium ammoniagenes* (10). FAD was from Boehringer Mannheim, and ThDP and bovine liver catalase (2000 units/mg) (EC 1.11.1.6) were from Sigma. Nitrogen 4.0 was from Messer-Griesheim. All other chemicals were of analytical grade and from Merck or Boehringer Mannheim. Quartz double distilled water was used throughout.

Pretreatment of POX Holoenzyme

Highly purified pyruvate oxidase (10 units/mg) from *L. plantarum* (EC 1.2.3.3) was a generous gift from Boehringer Mannheim. 10 mg of lyophilized enzyme were saturated with 100 μM FAD and 1 mM ThDP/Mn^{2+} in 1 ml of 0.2 M potassium phosphate buffer, 20% glycerol, pH 6.0, for 3 h at 20 °C. Subsequently the enzyme solution was dialyzed against 2×200 ml of 0.2 M potassium phosphate, 20% glycerol, pH 6.0, for 12 h at 4 °C. The absorption ratio of $\epsilon_{278/250}$ was 2.0 ± 0.1.

Preparation of Apoenzyme

Apoenzyme was prepared according to a modified method first established by Strittmatter (11) and Sedewitz *et al.* (1). Lyophilized POX (5 mg) was dissolved in 0.2 ml of 0.2 M potassium phosphate buffer, 20% glycerol, pH 6.0. Subsequently 0.2 ml of 3 M sodium bromide was added, and the solution was gently shaken at 0 °C for 2 min. 1.6 ml of a 50% saturated ammonium sulfate solution adjusted to pH 3.0 with sulfuric acid were added dropwise under gentle stirring at 0 °C. The precipi-

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[1] The abbreviations used are: POX, pyruvate oxidase; ThDP, thiamine diphosphate; 5-dFAD, 5-carba-5-deaza-FAD; DCPIP, 2,6-dichlorophenolindophenol.

FIG. 1. **¹H NMR spectra for the determination of exchange rates.** The ¹H NMR spectra were recorded from the supernatants obtained upon centrifugation of the solutions from the quenched flow experiments. The exchange reactions were initiated by a 1:1 mixing of a sample solution that contained 2 mM ThDP (*A*), 6.5 mg/ml apo-ThDP complex (*B*), or 9 mg/ml POX holoenzyme (*C*) with D₂O, respectively (for further details see "Materials and Methods"). The exchange times displayed in the spectra were 5 min for ThDP (30% exchange) and for the apo-ThDP complex (95% exchange) and 50 ms for the POX holoenzyme (100% exchange).
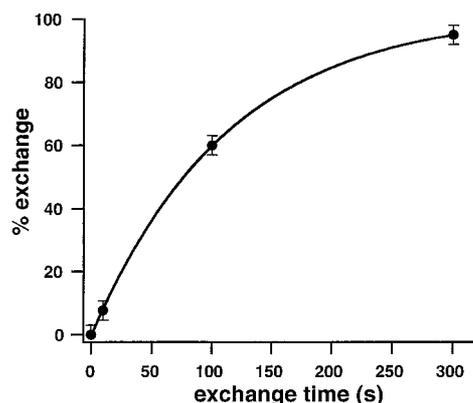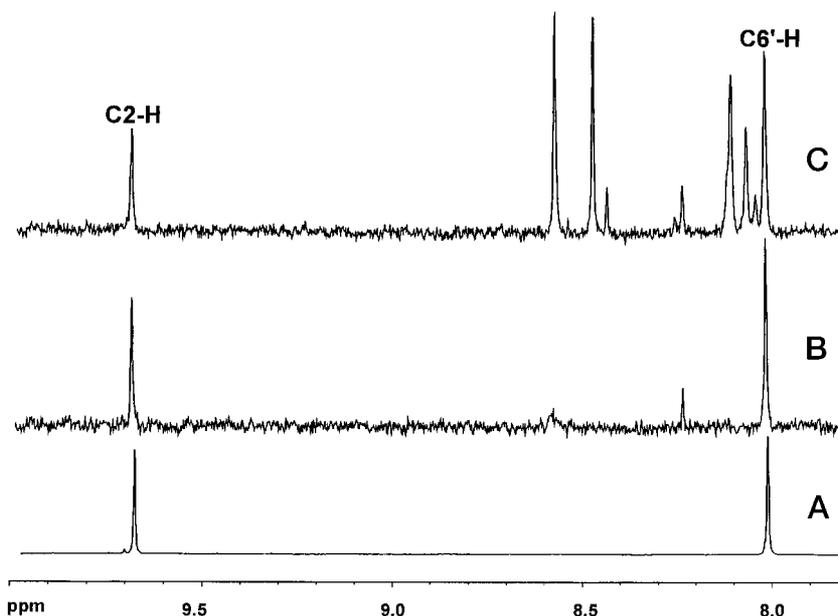

FIG. 2. **Kinetics of H/D exchange of ThDP C2-H in the binary apo-ThDP complex of POX.** Sodium phosphate buffer, pH 6.0, at 4 °C was used. The decay in integral intensity of the C2-H signal was fitted to a single exponential reaction (●). The average of duplicates is shown. The *error bars* show the deviation between two independent measurements. The relative amount of exchange was calculated according to the following equation: % exchange = $(A2_t - A2_\infty) \times 100/(A6' - A2_\infty)$, where $A2_t$ is the integral of the signal of the C2-H proton at the time t, $A2_\infty$ is the integral after complete exchange (50% of the integral area), and $A6'$ is that of the C6' proton that was used as a standard for the integral area for 1 proton because it is not exchanged.

TABLE I
Pseudo first order rate constants for C2-H/D exchange in free and enzyme-bound ThDP at pH 6.0 and 4 °C

Sample	Rate constant	k_cat
	s^{-1}	s^{-1}
Free ThDP in 50 mM phosphate	9.5×10^{-4}	
POX holoenzyme in 50 mM phosphate	314	2
POX holoenzyme in phosphate-free solution	20	
POX holoenzyme recombined with 5-dFAD in 50 mM phosphate	8	
Apo-ThDP complex in 50 mM phosphate	10^{-2}	

tated apoenzyme was sedimented by 15 min of centrifugation at 40000 × g at 4 °C. The yellow supernatant, which contained FAD and ThDP, was discarded, and the pellet was resuspended in 0.2 ml of 0.2 M potassium phosphate, 20% glycerol, pH 6.0. The ammonium sulfate precipitation step was repeated to completely remove the enzyme-bound coenzymes. The resuspended cofactor-free apoenzyme (1 ml) was dialyzed four times for 4 h against 250 ml of the same buffer at 4 °C. Traces of insoluble material were separated by centrifugation at 20000 × g for 10 min at 4 °C. The clear solution was stored at 4 °C. The absorption ratio of $\epsilon_{278/250}$ was 3.3 ± 0.1.

Determination of Protein Concentration

Protein concentrations were determined spectrophotometrically. For the holoenzyme extinction coefficients of $\epsilon_{278} = 1.65$ cm²/mg or of $\epsilon_{460} = 0.235$ cm²/mg were used. Concentration of the apoenzyme was calculated using an extinction coefficient of $\epsilon_{278} = 1.07$ cm²/mg (4).

Assays

Determination of the Proton/Deuterium Exchange—The kinetics of H/D exchange of the C2-H of ThDP were measured by ¹H NMR as described recently (5). The exchange reactions were initiated by mixing equal volumes of a sample solution containing 10 mg/ml apo-ThDP-

POX, holoenzyme or 1 mM ThDP alone in 0.1 M sodium phosphate buffer, pH 6.0, with D₂O in a quenched flow apparatus (model RQF-3, Kin Tek Althouse) at 4 °C. The given pH values are the respective pH meter readings. Incubation times were varied between 2 and 2000 ms. Experiments with longer times were performed by manual mixing. The exchange reactions were stopped by addition of a final concentration of 0.1 M hydrochloric acid and 5% trichloroacetic acid. This procedure also rapidly and completely denatures and precipitates the protein and releases the cofactors ThDP and FAD. After separation of the denatured protein by centrifugation, the ¹H NMR spectra of the supernatant containing ThDP and FAD were recorded in a 5-mm NMR tube on a Bruker ARX 500-MHz NMR spectrometer. As shown in Fig. 1, the signals used for quantification of the H/D exchange at C2 of ThDP do not interfere with the signals of FAD. To obtain the exchange rate, the relative decay in integral intensity of the C2-H signal at 9.68 ppm was fitted to a pseudo first order reaction (Fig. 2). The signal of the C6' proton at 8.01 ppm was used as a nonexchanging internal standard.

DCPIP Assay—Reduction of DCPIP can be followed by a decrease in absorbance at 600 nm. The assay buffer contained 40 mM pyruvate in 50 mM potassium phosphate, 10% glycerol, pH 6.0. Activity measurements were usually performed at 25 °C but at were performed at 10 °C for the recombination experiments. POX activity was calculated using $\epsilon_{600} = 17.7$ cm²/μmol.

Determination of H₂O₂ Production—Conditions were as in the DCPIP assay. Formation of 4-(*p*-benzochinone-monoimino)-phenazone from the reaction of H₂O₂ with 4-aminoantipyrine (2 mM) and 2,4-dichlorophenolsulfonic acid (7 mM) in the presence of horseradish peroxidase (4 units/ml) is followed spectrophotometrically at 546 nm (8). For the calculation of the specific POX activity $\epsilon_{546} = 16.5$ cm²/μmol was used.

Determination of O₂ Consumption—Conditions were as in the DCPIP assay. Oxygen tension was measured with a temperature-controlled O₂ electrode (Rank Brothers, Cambridge, UK). The reaction was started by addition of POX.

FIG. 3. **Time course of recombination of 5-dFAD and FAD with the binary apo-ThDP complex.** The reaction was followed by fluorescence (*A*) or activity (*B*) measurements at 10 °C. 100 μM (final concentration) 5-dFAD or FAD was added to the apo-ThDP complex (200 μg/ml) in 50 mM potassium phosphate buffer, pH 6.0. To determine the fraction of POX recombined with 5-dFAD or FAD either the time-dependent decrease of the fluorescence (FAD, λ_{em} = 527 nm and λ_{exc} = 455 nm; 5-dFAD, λ_{em} = 464 nm and λ_{exc} = 406 nm) was followed or samples were withdrawn at indicated time points to determine the activity in the DCPIP assay (*panel B* shows the time-dependent increase of activity for 5-dFAD recombination; the reactivation for FAD is displayed in Fig. 5). The *lines* represent fits as described under "Results."
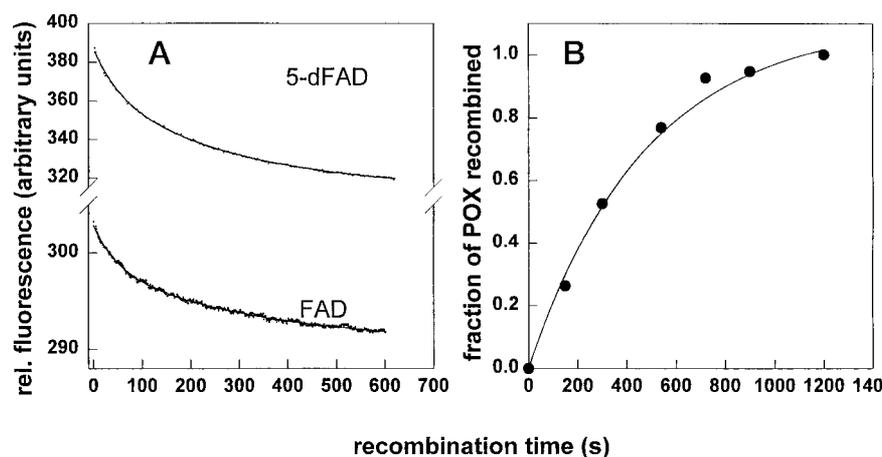
Determination of the Recombination Kinetics

To follow recombination of FAD with the binary apo-ThDP complex, 1 μM apoenzyme was preincubated with 1 mM ThDP/Mn^{2+} in 0.2 M potassium phosphate buffer, 20% glycerol, pH 6.0, for 1 h at 10 °C, and subsequently 100 μM FAD was added. At the indicated times, samples were withdrawn to determine the activity either with the DCPIP or the H$_2$O$_2$ or the O$_2$ detecting assays. For the incubations at both 25 and 10 °C, the assay temperature was 10 °C.

Production of Assay Solutions with Different Oxygen Concentrations

The assay mixtures were purged with nitrogen 4.0 for various times. The residual oxygen concentration was measured with an oxygen electrode from Metra Meβ- und Frequenztechnik (Radebeul, Germany).

Detection of the Reaction Product in the DCPIP Assay

This was achieved by ^{1}H NMR spectroscopy. The reaction was carried out with a small excess of DCPIP (1 mM) with respect to pyruvate (0.75 mM). The POX concentration in the incubation mixture was 1 mg/ml. Reaction products were separated from protein by centrifugation with Amicon Microcon-10 filters for 15 min at 7000 $\times$ g at 4 °C. ^{1}H NMR spectroscopy was performed on a Bruker ARX 500 NMR Spectrometer. To suppress the water signal, water presaturation was used.

Spectroscopy

UV-visible absorption was recorded with a Kontron Uvicon-941 double beam spectrophotometer and fluorescence spectroscopy with a Hitachi F-3010 fluorescence spectrophotometer.

RESULTS

Kinetics of H/D Exchange at the C2 Atom of ThDP in POX—The formation of all reaction intermediates in the POX reaction, detectable by different activity assays, requires the deprotonation of the C2-H of ThDP. This is the initial and key reaction for all ThDP-dependent enzymes. The low rate of this reaction in the free coenzyme ThDP was measured by H/D exchange and would not allow catalysis to proceed at the observed rate of the enzyme reaction (Table I). In the POX holoenzyme this reaction is accelerated by 4 orders of magnitude compared with that of free ThDP (Table I). This exchange rate reflects not only the deprotonation rate at the C2-H of ThDP but also the required exchange of the base with the solvent. Therefore the observed rate could be slower but never faster than the microscopic deprotonation rate for the C2-H proton. The value of the exchange rate, however, exceeds the catalytic rate for this enzyme (k_{cat} = 2 s^{-1} at 4 °C). In the binary apo-ThDP complex this exchange rate is very low (Table I). Interestingly, the second substrate phosphate further increases the rate of the H/D exchange in the holoenzyme $\approx$16-fold compared with that measured in a phosphate-free buffer (Table I).

Preparation and Some Properties of 5-dFAD-POX—The binary apo-ThDP complex binds 5-dFAD, an analogue of FAD

TABLE II
Comparison of K_m and k_{cat} obtained for POX with the DCPIP assay and the H$_2$O$_2$ detecting assay, respectively (pH 6.0 and 25 °C)
K_m values were determined at saturating concentrations of the other substrates.

	DCPIP assay	H$_2$O$_2$ detecting assay
k_{cat} (s^{-1})	17.9	9.6
K_m pyruvate (mM)	1.6	0.4
K_m phosphate (mM)	4.5	2.3
K_m O$_2$ (mM)		0.07
K_m DCPIP (mM)	0.020	

that is unreactive toward O$_2$ but that is isoelectronic with the latter. The process is accompanied by a biphasic decrease of the fluorescence emission of the 5-deaza-isoalloxazine (phase 1, $\approx$70% of the total amplitude with k_1 = 0.018 s^{-1}, and phase 2, $\approx$30% with k_2 = 0.0025 s^{-1}, Fig. 3*A*). It should be noted that the recombination of native FAD proceeds with essentially the same rate constants ($\approx$67% with k_1 = 0.022 s^{-1} and $\approx$33% with k_2 = 0.0025 s^{-1}) using the fluorescence decrease of FAD as a probe (Fig. 3*A*). Binding of 5-dFAD can also be monitored by following the increase of activity in the DCPIP assay that accompanies it (Fig. 3*B*). The rate of this process (k = 0.0022 s^{-1}) is essentially the same as that obtained for the slow phase of the fluorescence changes. This suggests that 5-dFAD binds by the same mode as native FAD, and this is also corroborated by the activity measurements detailed below. When bound to the binary apo-ThDP complex, 5-dFAD accelerates the H/D exchange at the C2-H of ThDP beyond the overall catalytic rate (Table I).

POX Catalysis in the Presence of DCPIP—DCPIP can act as an artificial electron acceptor in ThDP-dependent enzymes (6, 7, 13), and, *e.g.* in pyruvate decarboxylase from yeast (EC 4.1.1.1.), it reacts specifically with the α-carbanion of hydroxyethyl-ThDP (6, 7). In the POX reaction, DCPIP acts as an artificial electron acceptor too. Importantly, the product is acetyl phosphate, *i.e.* the same as obtained in the native reaction. This was shown by ^{1}H NMR spectroscopy, which gave a signal for the product at the chemical shift observed for acetylphosphate (2.1 ppm). We have thus employed DCPIP to assess the question of whether this intermediate is oxidized in the POX catalysis as well. In the presence of saturating pyruvate (40 mM) and phosphate (42 mM) and an O$_2$ concentration of 0.25 mM, the reaction with DCPIP has a K_m value of $\approx$20 μM. Because a nonspecific inactivation by the reaction of DCPIP with sulfhydryl groups was observed with other enzymes (7, 14, 15) and in order to verify whether this would apply also in the present case, 0.25 μM POX was incubated in the absence and presence of 40 μM DCPIP for 1 h, and subsequently the activity

FIG. 4. **O$_2$ and catalase sensitivity of the assays.** Dependence of the relative activities of POX in the DCPIP assay (*open symbols*) and the H$_2$O$_2$ detecting assay (●) on the O$_2$ concentration (*A*) and catalase concentration (*B*) in the assay mixture is shown. The assay solution was 40 mM pyruvate in 50 mM potassium phosphate buffer, 10% glycerol, pH 6.0, and contained either DCPIP (○ 40 μM, □ 16 μM) or 2 mM 4-aminoantipyrin, 7 mM 2,4-dichlorophenolsulfonic acid, and 4 units/ml horseradish peroxidase (●).
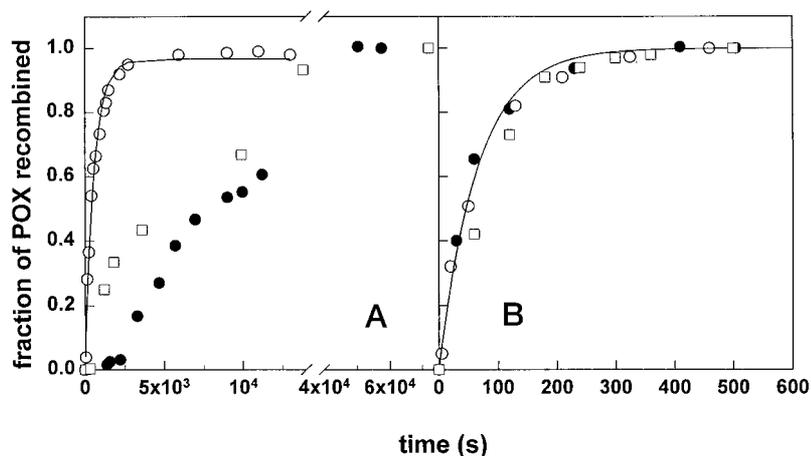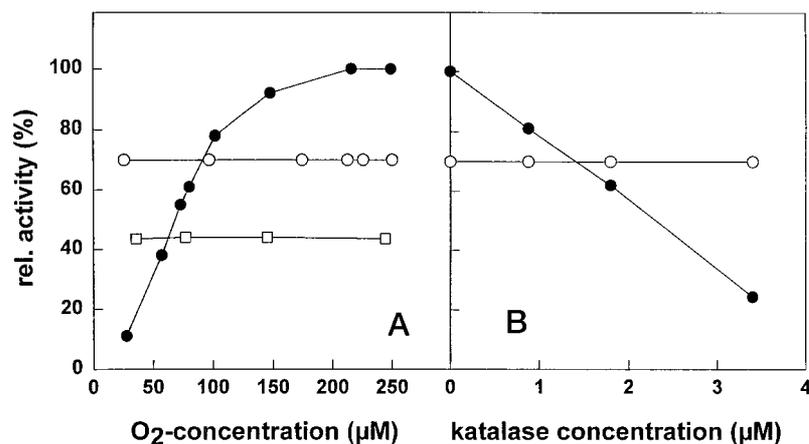
FIG. 5. **Recombination of FAD with the binary apo-POX-ThDP complex.** The experiments were carried out at either 10 (*A*) or 25 °C (*B*). The fraction of recombined POX was determined by measuring the activity with the DCPIP (○, a value of 1.0 corresponds to a specific activity of 15 units/mg), the H$_2$O$_2$ (●, a value of 1.0 corresponds to a specific activity of 8 units/mg), or the O$_2$ (□) detecting assay. Further conditions are described in the legend to Fig. 4 (DCPIP assay with 40 μM DCPIP). The *lines* represent fits according to a pseudo first order reaction with rate constants of $k = 0.017$ s^{-1} (*A*) and $k = 0.0017$ s^{-1} (*B*).

was measured. No effect of the preincubation was observed, and thus a nonspecific inactivation by DCPIP can be excluded for POX. In a phosphate-free solution (same pH value and ionic strength, complete DCPIP assay mixture) DCPIP was not reduced by POX. The binary apo-ThDP complex similarly is inactive in the absence and presence of phosphate. Solely in the presence of enzyme-bound ThDP, Mg^{2+}, FAD, and phosphate, DCPIP is reduced efficiently in the POX reaction. Notably, the k_{cat} and K_m values obtained in the DCPIP assay are about 2-fold increased compared with those measured in the H$_2$O$_2$ assay (Table II).

In POX catalysis either FADH$_2$ or hydroxyethy-ThDP could serve as electron donors for DCPIP. The final product H$_2$O$_2$ could also oxidize DCPIP, either directly or via mediation by POX. In the absence of POX, the rate of DCPIP (red) oxidation by H$_2$O$_2$ is 2.7×10^{-4} M^{-1} s^{-1} and thus too slow to affect the process. Variation of O$_2$ concentration in the range 25–250 μm and the presence of catalase (0–3.5 μM; Fig. 4) have no effect on the rate constant of the DCPIP assay. We thus conclude that H$_2$O$_2$-dependent DCPIP oxidation does not influence the rate constants measured in this assay. However, the H$_2$O$_2$ peroxidase assay shows the expected O$_2$ and catalase dependence (Fig. 4).

Kinetics of FAD Association Followed by Activity Measurements and Effect of Temperature—The binding of FAD to the binary POX-ThDP complex was investigated at different temperatures using the assays described under "Materials and Methods." At 25 °C the three methods revealed essentially the same result (Fig. 5*B*). The data points could be fitted to a monophasic, pseudo first order process with $k = 0.016$ s^{-1} (100 μM FAD).

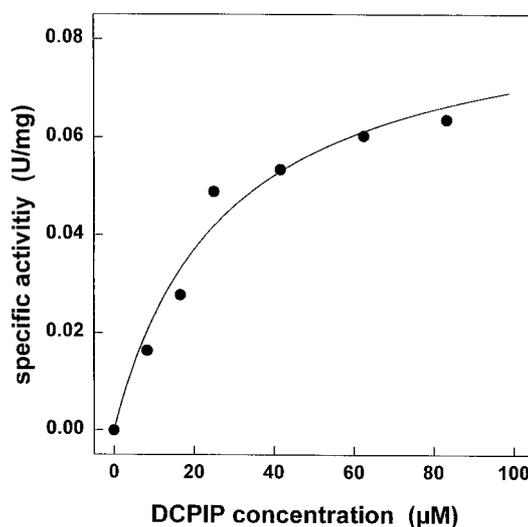Unexpectedly, at 10 °C the kinetics of recombination with

FIG. 6. **Dependence of the relative activity of POX holoenzyme containing 5-dFAD on the DCPIP concentration in the assay.** The activity was measured at 25 °C in 50 mM potassium phosphate buffer, pH 6.0, containing 40 mM pyruvate.

FAD differ significantly depending on the type of assays used (Fig. 5*A*). The rate estimated using the DCPIP method can be described by a pseudo first order process with $k = 0.0017$ s^{-1} and is much faster than those obtained by the H$_2$O$_2$ or O$_2$ detecting assays. The rate derived from the decrease of [O$_2$] also can be approximated by a first order process; it is, however, slower by approximately 1 order of magnitude ($k = 0.0001$ s^{-1}). The release of H$_2$O$_2$ as detected with the peroxidase assay
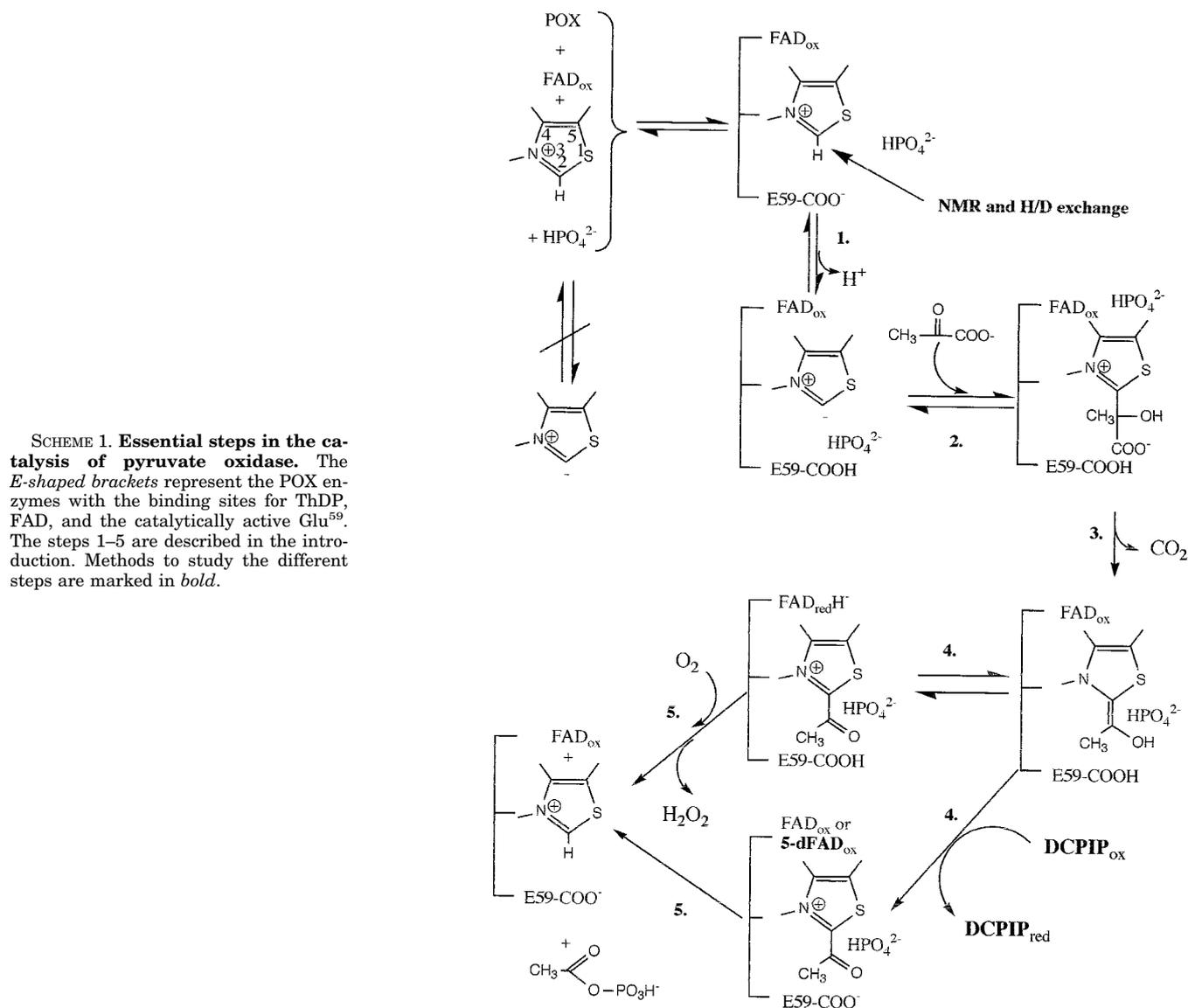
SCHEME 1. **Essential steps in the catalysis of pyruvate oxidase.** The *E-shaped brackets* represent the POX enzymes with the binding sites for ThDP, FAD, and the catalytically active Glu[59]. The steps 1–5 are described in the introduction. Methods to study the different steps are marked in *bold*.

exhibits a pronounced lag phase extending for up to 1000 s and subsequently proceeds at a rate similar to that observed with the oxygen electrode.

Catalytic Properties of 5-dFAD-POX—The ternary complex of 5-dFAD, ThDP, and POX is completely inactive in the H_2O_2 detecting assay but active in the test using DCPIP. The dependence of the rate in the latter assay from DCPIP concentration follows a saturation behavior with a K_m of ≈ 24 μM. At saturating substrate concentrations, the 5-dFAD-POX has 0.4% activity of native enzyme (Fig. 6). The possibility that this 0.4% activity results from traces of unremoved FAD can be ruled out because apoenzyme did not show any residual activity. Furthermore, the procedure for the synthesis of 5-dFAD (9, 10) excludes production of any normal FAD. 5-dFAD containing holo-POX was completely inactive in the H_2O_2 assay. Incubation of the 5-dFAD containing POX with FAD has no effect on the observed rate in the DCPIP assay. Similarly, incubation of native POX with 5-dFAD does not affect the POX activity. This indicates that no dissociation/exchange of both FAD and 5-dFAD from the ternary complex occurs.

<center>DISCUSSION</center>

The essential steps constituting the cycle catalyzed by POX from *L. plantarum* are shown in Scheme 1. Deprotonation of

the ThDP-C2-H is mandatory for the nucleophilic attack of the coenzyme ThDP $C2^-$ on the pyruvate carbonyl, which leads to formation of the enzyme-bound intermediate hydroxyethyl-ThDP. The rate of this deprotonation for free ThDP and in the binary apo-ThDP complex is too slow by about 3 orders of magnitude to account for the POX reaction to proceed at its observed overall catalytic rate of 2 s^{-1} at 4 °C (Table I). In native holoenzyme, *i.e.* in the ternary complex POX-FAD-ThDP, the rate of C2-H deprotonation has a higher value than k_{cat}, but in the presence of phosphate a further 16-fold increase of the C2-H dissociation rate can be observed (Table I). H^+ abstraction does not appear to be mediated by a direct interaction of the FAD with C2-H of the enzyme-bound ThDP but rather by interactions with functional groups of the protein, which are operative only in the POX-FAD-ThDP ternary complex. In the crystal structure the distance of the closest FAD atom to C2-H is 11 Å (16, 17). However, based on the structural homology of the ThDP binding site to other ThDP enzymes, we assume that Glu[59] is the residue that mediates this activation in POX. In POX the crucial interaction could be that of the ThDP-N1' with the conserved Glu[59] (16, 17). This would be analogous to what is observed with pyruvate decarboxylase and transketolase where the same type of interaction occurs (5).

This interpretation is consistent with the observation that in the ternary complex with 5-dFAD the rate of the H/D exchange at C2-H is accelerated by 3 orders of magnitude compared with the binary apo-ThDP complex (Table I). The substantial increase in rate observed in the presence of phosphate cannot be interpreted in molecular terms at the present time and might be due to a variety of effects.

The next important steps in catalysis are the formation of the pyruvate-ThDP adduct, which is followed by its decarboxylation to form the hydroxyethyl-ThDP as shown in Scheme 1. DCPIP can react specifically and rapidly with the latter intermediate. In POX, DCPIP reduction requires both coenzymes and the cosubstrate phosphate. Therefore, the redox partner for DCPIP in the POX reaction could be either $FADH_2$ or hydroxyethyl-ThDP. Because no competition between DCPIP and O_2 was observed (Fig. 4) and because the k_{cat} value for the DCPIP assay is 2-fold increased compared with that determined with the H_2O_2 detecting assay (Table II), we conclude that different steps in catalysis are observed with the DCPIP and the H_2O_2 detecting assays and that DCPIP does not react competitively with $FADH_2$. It has been discussed (17) that the distance between the cofactors ThDP and FAD exclude a direct electron transfer from the $C2\alpha$ of hydroxyethyl-ThDP to N5 of the isoalloxazine (16). More likely, oxidation occurs via transfer of two single electrons to the isoalloxazine (16). We conclude that DCPIP reacts with the enzyme-bound intermediate hydroxyethyl-ThDP. The occurrence of further intermediates during the electron transfer to FAD clearly cannot be excluded; an intermediate could be inferred from the requirement of FAD for the DCPIP reduction.

Further evidence for the proposed mode of reaction of DCPIP arises from the experiments with 5-dFAD. This analogue cannot transfer electrons to O_2. As expected, the 5-dFAD containing holoenzyme is inactive in the H_2O_2 detecting assay but reduces DCPIP (Fig. 6). The observation that in the presence of 5-dFAD, only 0.4% activity is observed in the DCPIP assay, although the H/D exchange for the ThDP C2-hydrogen is fast, points to a strong deceleration of a later step in the formation of hydroxyethyl ThDP in the presence of this FAD analogue. As pointed out under "Results," it can be ruled out that traces of native FAD are responsible for the residual activity. In the absence of phosphate no DCPIP activity is observed. Although phosphate stimulates the C2-H exchange of the enzyme-bound ThDP (Table I), this effect cannot be responsible for the essential function of phosphate. It is likely that phosphate is a cosubstrate in the formation of the product acetyl phosphate in the DCPIP reaction.

The differences among the kinetics of FAD recombination with the binary apo-ThDP complex at 10 °C using the different assays (Fig. 5) suggest that the final catalytically competent complex is formed via at least one intermediate form. This is assumed to be a holoenzyme in which formation of hydroxyethyl-ThDP but not transfer of electrons to O_2 is possible. At 25 °C the lag phase in the recombination kinetics is not observable. This is compatible with the occurrence of a second step with a high activation energy during FAD binding. The possibility that the peroxidase reaction in the H_2O_2 detecting assay is rate-limiting at low temperatures can be ruled out, because all measurements of the FAD recombination process at 10 and 25 °C were performed at 10 °C.

The crystal structure for POX (16) shows that the isoalloxazine ring of the FAD is surrounded by six aromatic amino acids. Along the N10-N5-axis, the planar structure of the isoalloxazine ring is bent by 15 ° along the side chain of valine 265, which increases the redox potential of $FADH_2$ (17). We suggest a model in which FAD binds in a two-step mechanism. The first leads to an intermediate that is competent exclusively in the formation of hydroxyethyl-ThDP. For this step FAD can be substituted by 5-dFAD. The nature of the second, temperature-sensitive step is still elusive at present. It could represent structural changes of the FAD binding site followed by the distortion of the planar structure of the isoalloxazine ring. $FADH_2$, exclusively bound in this final conformation, is capable of performing the natural reaction, the reduction of O_2 to H_2O_2.

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