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Single Step Analysis of Catalysis of Pyruvate Oxidase from Lactobacillus plantarum. Kinetics, Mechanism and Regulation.

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Introduction

Pyruvate oxidase [*Lp*POX; E.C. 1.2.3.3] from *Lactobacillus plantarum* is a homo-tetrameric flavoenzyme with a subunit molecular weight of 65.5 kDa composed of 603 residues. Each subunit contains one tightly and noncovalently bound flavin adenin dinucleotide (FAD), thiamin diphosphate (ThDP) and a bivalent metal ion (Mg^{2+}) for anchoring the diphosphate moiety of the ThDP. In the presence of phosphate and oxygen, POX catalyzes the oxidative decarboxylation of pyruvate yielding hydrogen peroxide, carbon dioxide and the energy storage metabolite acetylphosphate (1-3).

Catalysis of *Lp*POX can be subdivided in several steps. i) Initially the 4'-imino function of the enzyme-bound ThDP deprotonates C2-ThDP forming an ylide species, which ii) attacks the carbonyl group of the substrate pyruvate in a nucleophilic manner, iii) lactyl-ThDP decarboxylates to hydroxyethyl-ThDP, iv) which is subsequently oxidized by FAD under formation of acetyl-ThDP and $FADH_2$, v) the reduced flavin is transferring the electrons to the final electron acceptor O_2 releasing H_2O_2. vi) Finally the acetyl-ThDP is cleaved phosphorolytically to acetylphosphate and ThDP (see scheme 1).

In this paper we present results of presteady-state and steady-state kinetic studies to elucidate the partial step reactions of *Lp*POX catalysis. Within the scope of our experiments, we combined quenched-flow, stopped-flow / sequential stopped-flow with single wavelength and photodiode array data acquisition, kinetic solvent isotope effect (KSIE) experiments and ^{1}H-NMR to characterize kinetically the partial steps i) deprotonation of C2-H (ThDP), ii) decarboxylation, iii) FAD reduction, iv) $FADH_2$ reoxidation and v) phosphorolysis / hydrolysis.

Results and Discussion

H/D exchange kinetics on the C2-atom of the coenzyme ThDP in LpPOX

The deprotonation of the C2 of ThDP is the initial reaction for all ThDP dependent enzymes. For a large number of these it has been shown that the protein component accelerates the deprotonation reaction of C2 of enzyme-bound ThDP by several orders of magnitude with respect to free ThDP (4). In the process, a highly conserved glutamate interacts with the N1' of the pyrimidine moiety of ThDP and thereby

increases the basicity of the 4'-aminogroup facilitating proton abstraction at C2 (4-6). The formation of all catalytic intermediates in the *Lp*POX reaction is dependent on this initial reaction. In view of this, the deprotonation rate of the enzyme-bound ThDP in *Lp*POX has been estimated by measuring the rates of H/D exchange (methods described in 4). As shown in table 1, this rate in the POX holoenzyme is accelerated by five orders of magnitude comparing to that of free ThDP and is larger than the maximal catalytic velocity of this enzyme (k_{cat} = 2 s^{-1} at 4 °C). Interestingly, the substrate phosphate additionally increases this dissociation rate by more than one order of magnitude, whereas the dissociation rate of the C2-H in the binary apo-ThDP complex is very low. The coenzyme FAD, which accelerates the deprotonation of the C2 of ThDP after its binding to the protein, can be substituted by 5-deaza-FAD. In the 5-deaza-FAD-ThDP-POX complex a very fast deprotonation of C2-H (ThDP) also occurs.

Table 1
Pseudo first-order Rate Constants for the Deprotonation of C2 in free and enzyme-bound ThDP at pH 6.0 and 4 °C

Sample	Rate constant (s^{-1})
Free ThDP in 50 mM potassium phosphate	$9.5*10^{-4}$
POX-holoenzyme in 50 mM potassium phosphate	314
POX-holoenzyme in phosphate free solution	20
POX-holoenzyme recombined with 5-deaza-FAD in 50 mM potassium phosphate	8
Apo-ThDP complex in 50 mM potassium phosphate	10^{-2}

Kinetics of the FAD reduction during LpPOX catalysis in the presence of phosphate
This reaction was studied in air-saturated potassium phosphate buffer (0.2 M, pH 6.0) and initiated by mixing the protein solution with the substrate pyruvate. Spectral changes were monitored at 457 nm (FAD absorption) using the stopped-flow instrument. As shown in Fig. 1, the course of enzyme-bound FAD reduction consists of several phases. First, in the pre steady-state phase, there is an initial, very rapid decrease in absorbance (Fig.1, insert). This is followed by a small, but significant increase. The time course of the decrease in absorbance mainly reflects the electron transfer from HEThDP to FAD and all preceding steps in *Lp*POX catalysis, such as binding of pyruvate and the decarboxylation of the lactyl-ThDP intermediate. The following small increase in absorbance is attributed to the reoxidation of reduced FAD by oxygen during the first catalytic cycle. It is followed by an additional slow step before the free enzyme is generated, which can reenter the catalytic cycle. This

slow step should be the phosphorolysis of the acetyl-ThDP intermediate to yield acetylphosphate. After the pre steady-state phase as described, the enzyme enters a steady-state in which a balance of reduced and oxidized FAD species is present. The position of this balance depends on the concentration ratios of the substrates , as well as on the corresponding rate constants as detailed below. In the presence of excess pyruvate the consumption of O_2 then shifts the equilibrium towards complete *Lp*POX-FAD reduction. A mechanism that is in accordance with the experimental data is shown in scheme 1.

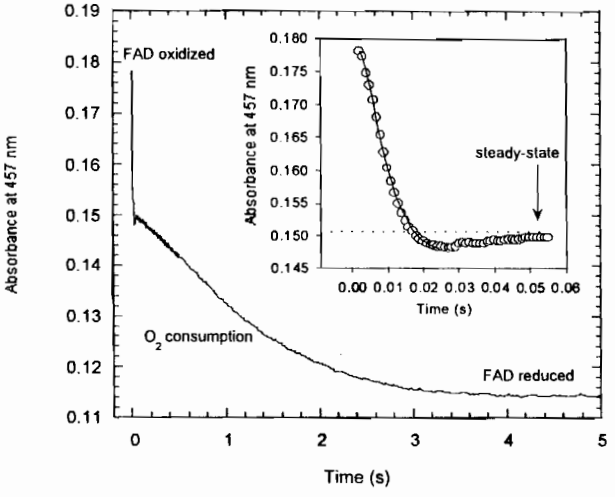


Figure 1: Progress curve of the pyruvate oxidase reaction with pyruvate monitored at 457 nm starting from the oxidized enzyme. The reaction of 20 µM *Lp*POX (active site concentration) in 0.2 M potassium phosphate buffer, pH 6.0 (air saturated) with 20 mM pyruvate at 25 °C was monitored with a stopped-flow machine with an optical path length of 10 mm. Insert : Initial phase (0-60 ms). The decrease in absorbance was fitted to an equation for a consecutive reaction mechanism (A⇒B⇒C) with rates of the decarboxylation and the electron transfer $k_{dec} = 200\ s^{-1}$ and $k_{trans} = 200 s^{-1}$.

Electron transfer from $FADH_2$ to acetyl-ThDP is negligible (under the chosen experimental conditions) because in the absence of oxygen the substrate pyruvate completely reduces the enzyme-bound FAD to $FADH_2$ (data not shown). Therefore, at pyruvate saturation conditions, the equilibrium position between FAD and $FADH_2$ in the steady-state depends on the rate of decarboxylation (k_{dec}) of the lactyl-ThDP

intermediate , the rate of electron transfer from HEThDP to FAD (k_{trans}) , as well as on the rate of the reoxidation by oxygen (k_{reox}) and the rate of the phosphorolysis of the acetyl-ThDP intermediate (k_{phos}) (Eq. 1).

$$\frac{[FAD]}{[FADH_2]} = \frac{k_{reox}}{k_{dec}} + \frac{k_{reox}}{k_{trans}} + \frac{k_{reox}}{k_{phos}} \qquad (1)$$

At pyruvate saturation, the initial decrease in absorbance is mainly determined by the electron transfer from HEThDP to FAD and the preceding steps of decarboxylation of the lactyl-ThDP intermediate. The latter is reflected by the initial lag-phase in the FAD reduction. The increase in absorbance following the first rapid decrease reflects the reoxidation of $FADH_2$ and the phosphorolysis of the acetyl-ThDP intermediate. It depends on the ratio of the rates of phosphorolysis to those of the preceding steps of catalysis. The slower the rate of phosphorolysis, the higher an increase in absorbance can be expected. In that case, where the reaction is started from the completely reduced species by adding oxygen (data not shown), the equilibrium position between the oxidized and the reduced FAD species is obtained with a significantly slower rate (k = 70 s^{-1}) compared to that observed upon starting the reaction from the oxidized FAD species with pyruvate. This indicates, that decarboxylation as well as the electron transfer from HEThDP to FAD occur with a significantly higher rate than the reoxidation and the phosphorolysis.

From the starting point of the steady-state shown in figure 1, a ratio of oxidized *vs* reduced species $FAD/FADH_2$ =1.3 was estimated. Considering that this value correlates with a rate constant of the decarboxylation, electron transfer and phosphorolysis, respectively and the value of the total of rates of the reoxidation and the phosphorolysis (k_{obs}=70 s^{-1}), values of k_{reox} =34 s^{-1} and of k_{phos}=36 s^{-1} can be calculated. This clearly indicates that the rate limiting steps in *Lp*POX catalysis are the reoxidation reaction by oxygen and the phosphorolysis of the acetyl-ThDP intermediate, respectively.

No flavosemiquinone species was detectable during *Lp*POX catalysis under steady-state conditions. In stopped-flow experiments with an instrument equipped with a diode array photometer (acquisition time t = 0.7 ms), no radical flavin intermediate is populated during the electron transfer from HEThDP to FAD.

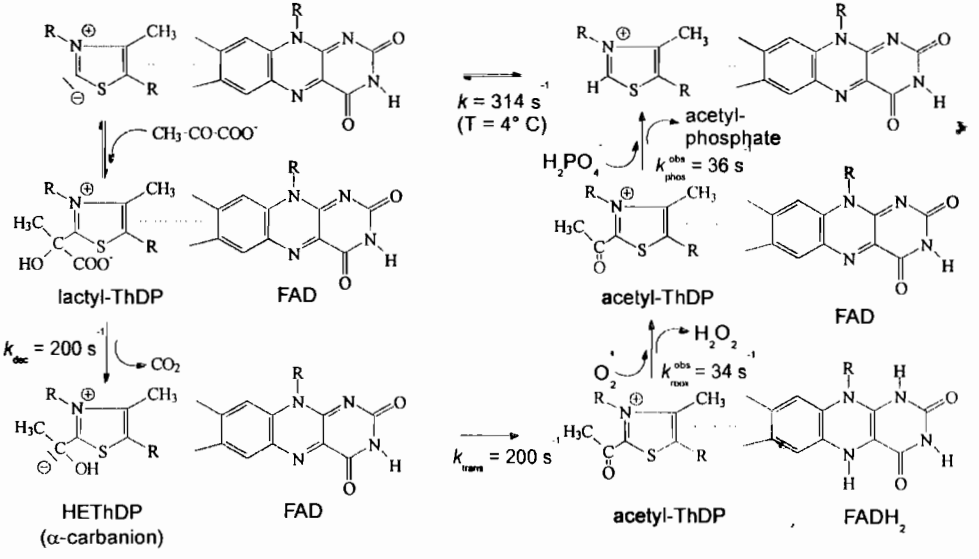
To address the question, whether the electron transfer in *Lp*POX proceeds in a two step single electron transfer or in a hydride transfer, kinetic solvent isotope effect experiments were performed. Single wavelength (λ = 457nm) stopped-flow experiments were carried out for all substrates under saturating conditions using different mole fractions of deuterium oxide. No kinetic solvent isotope effect could be determined in the electron transfer reaction under the experimental conditions used.

Conclusions

A summary of the mechanistic interpretations is depicted in scheme 1 which represents the whole catalytic cycle of *Lp*POX in kinetic terms.

The reduction of enzyme-bound FAD through the HEThDP intermediate proceeds via a two step single electron transfer mechanism with k_{obs} = 200 s^{-1} at saturating conditions for both pyruvate and phosphate. Hydride transfer is improbable due to the absence of a kinetic solvent isotope effect. In line with this *Lp*POX bound 5-d-FAD, a good hydride acceptor is not reduced by the HEThDP intermediate (5).

Reoxidation of $FADH_2$ by O_2 yielding H_2O_2 takes place with a pseudo-first-order rate of k_{obs} = 34 s^{-1} in air saturated buffer, whereas the phosphorolysis of the acetyl-ThDP intermediate occurs with a rate of k_{obs} = 36 s^{-1} at phosphate saturation concentration conditions. In competition to phosphorolysis, enzyme-bound acetyl-ThDP is hydrolyzed with a rate of k = 0.03 s^{-1} .



Scheme 1: Catalytic cycle of *Lp*POX

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