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Phosphate mediates electron transfer in pyruvate oxidase from *Lactobacillus plantarum*

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Introduction

Pyruvate oxidase from *Lactobacillus plantarum* (*Lp*POX, EC 1.2.3.3) is homotetrameric flavoenzyme with a subunit molecular mass of 65.5 kDa composed 603 residues. Each subunit contains one tightly and noncovalently bound FA thiamin diphosphate (ThDP), the biologically active form of vitamin B_1, and Mg^{2+} anchoring the diphosphate moiety of ThDP. In the presence of phosphate and oxyg *Lp*POX catalyses the oxidative decarboxylation of pyruvate yielding carbon dioxi acetylphosphate and hydrogen peroxide.
The catalytic cycle of *Lp*POX comprises i) the deprotonation at the C2 of ThDP, the covalent binding of pyruvate to ThDP, iii) the decarboxylation of the ther formed 2-lactyl-ThDP (LThDP) intermediate to yield the carbanion/enamine of hydroxyethyl-ThDP (HEThDP), iv) an intramolecular two step electron transfer fr HEThDP to FAD and finally v) the reoxidation of $FADH_2$ by oxygen and vi) phosphorolysis/hydrolysis of the 2-acetyl-ThDP (AcThDP) intermediate (1).
A kinetic analysis of single steps of catalysis using FAD absorbance revealed phosphorolysis of the AcThDP intermediate as well as the reoxidation of the redu FAD to be partially rate-limiting. In the presence of phosphate no transient rad FAD species can be observed in the course of the reductive half-reaction (2).
Here, we present stopped flow and titrimetric results of the influence of the subst phosphate on the reductive and oxidative half-reaction as well as the stabilisatio radical FAD species in the enzyme.

Results and discussion

Reductive half-reaction in the presence and absence of phosphate

The time course of the anaerobic reduction of enzyme-bound FAD in *Lp*POX by substrate pyruvate is clearly dependent on the presence of phosphate (Figure 1). I presence, the progress curve shows a pronounced *lag* phase followed by a monoph rapid decrease in absorbance at 457 nm. With phosphate absent, however, the

course of the reductive half reaction is composed of a *lag* phase and *two* distinguished consecutive phases.

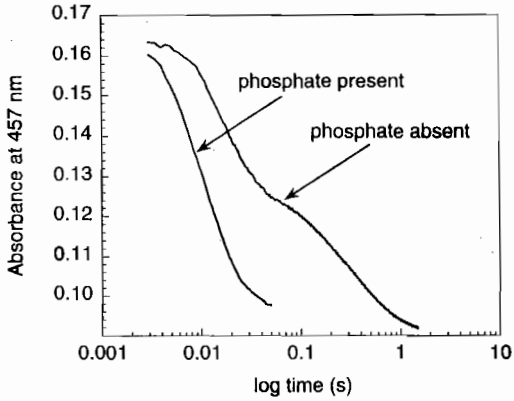

Figure 1: Time course of the *Lp*POX reaction with pyruvate at saturating concentration under anaerobic conditions in the presence and absence of phosphate at 25 °C.

A kinetic analysis of single steps in catalysis (2) revealed the reversible covalent binding of pyruvate to ThDP (k_{on}, k_{off}) as well as the decarboxylation of LThDP (k_{dec}) to be nearly independent of phosphate, whereas the intramolecular redox reaction of HEThDP and FAD is remarkably slower and composed of two phases (k_{red1}, k_{red2}) with no phosphate (Table 1).

Table 1: Rate Constants of Elementary Steps of *Lp*POX Catalysis in Dependence on the Presence of Phosphate.

	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	k_{dec} (s^{-1})	k_{red1}(s^{-1})	k_{red2} (s^{-1})
*Lp*POX in 0.1 M PIPES, pH 6.0 at 25 °C	$(2.1 \pm 0.2) \cdot 10^5$	14 ± 6	82 ± 5	138 ± 12	3.5 ± 0.1
*Lp*POX in 0.2 M KPP,	$(6.5 \pm 0.2) \cdot 10^4$	20 ± 3	112 ± 20	k_{red}	

In order to prove, whether the two step reduction of FAD by HEThDP is associated with the transient occurence of radical FAD species, the time-resolved absorbance spectra of enzyme-bound FAD in the course of the reductive half-reaction were recorded. As depicted in figure 2A, the initial rapid phase of the reaction is accompanied by an increase at $\lambda > 500$ nm and a blue-shift of λ_{max} from 405 to 378 nm. The spectra may be interpreted as being composed of the oxidized FAD and the half-reduced FAD (semiquinone). In the second phase of the reaction (figure 2B), the reduced FAD is formed.

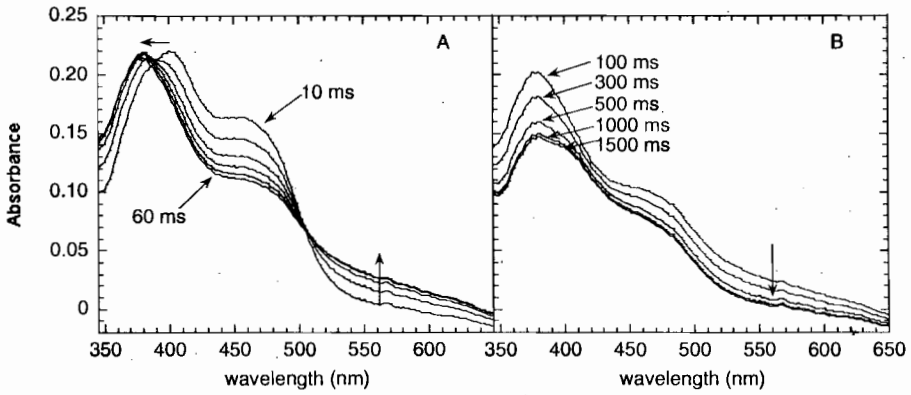

Figure 2: Time-resolved absorbance spectra of enzyme-bound FAD in *Lp*POX in the course of the reaction with pyruvate under anaerobic conditions in 0.1 M PIPES, pH 6.0 at 25 °C. A: 0-60 ms. B: 100-1500 ms.

Oxidative half-reaction in the absence and presence of phosphate

The reoxidation of the reduced FAD in *Lp*POX by oxygen (data not shown) is nearly independent of the presence of phosphate ($k_{obs} = 70$ s^{-1} with phosphate, $k_{obs} = 50$ s^{-1} without phosphate) thus supporting our suggested specific role of phosphate in the intramolecular electron transfer.

Anaerobic titration of enzyme-bound FAD with dithionite

A titration of the FAD in *Lp*POX with sodium dithionite showed (Figure 3), that the FAD semiquinone is formed both in the presence and the absence of phosphate with a comparable stabilisation of about 55 %.

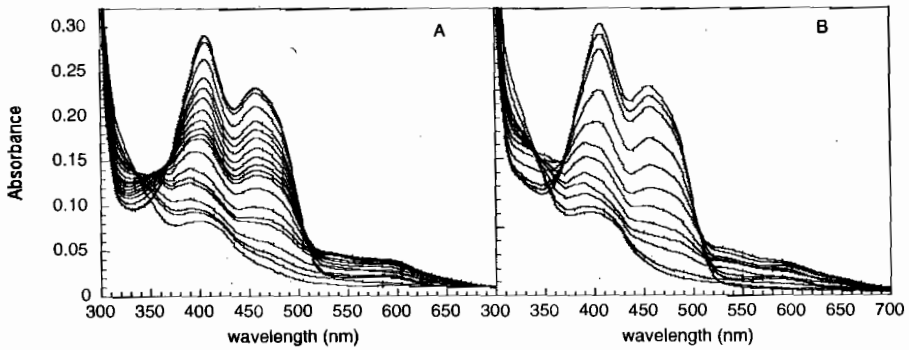

Figure 3: Anaerobic reduction of enzyme-bound FAD in *Lp*POX by sodium dithionite in either 0.2 M KPP, pH 6.0 (A) or 0.1 M PIPES, pH 6.0 (B).

Conclusions

The electron transfer from the HEThDP intermediate to FAD in *Lp*POX is dependent on the presence of phosphate. In its presence, reduction of enzyme-bound FAD proceeds via a two step single electron transfer with $k_{obs} = 422\ s^{-1}$, but no radical FAD species can be detected by using time-resolved spectroscopy. In the absence of phosphate, however, the reduction of enzyme-bound FAD is clearly biphasic and slowed. The time-resolved spectra provide evidence for a stepwise electron transfer from HEThDP to FAD with transient formation of radical FAD and an oxyethyl-ThDP' species. Since the thermodynamic stabilisation of radical flavin species by *Lp*POX is independent of the presence of phosphate, the latter appears to mediate directly or facilitate electron transfer from HEThDP to FAD.

References

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