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Substrate activation behaviour of pyruvate decarboxylase from *Pisum sativum* cv. Miko

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Abstract The substrate activation behaviour of pyruvate decarboxylase from germinating seeds of *Pisum sativum* is characterised kinetically via stopped-flow measurements and discussed with respect to other species. The involvement of SH-groups in this process is demonstrated by reference experiments with chemically modified enzyme.

Key words: Substrate activation; Pyruvate decarboxylase; SH-group; Modification; Thiamine diphosphate; *Pisum sativum* cv. Miko

1. Introduction

Pyruvate decarboxylase (PDC, EC 4.1.1.1) is a key enzyme in the anaerobic metabolism of yeast, various bacteria and plant seeds. It catalyses the non-oxidative decarboxylation of pyruvate into acetaldehyde and CO_2. PDC occurs in plant seeds only during germination, when the plant embryo is completely surrounded by an oxygen-impermeable testa.

The phenomenon of substrate activation was described for all PDC species investigated so far, except for that from the bacterium *Zymomonas mobilis*. Substrate activation is thoroughly kinetically characterised for PDC from brewer's yeast [1]. By HMB-modification experiments it was also found that SH-groups are somehow involved in this process [2]. On the basis of the 3-D structure model of yeast PDC, Baburina et al. [3] suggested Cys^{221} as a binding site for the substrate acting as activator. We first discovered that pyruvate decarboxylase from pea seeds is activated by its substrate pyruvate via steady-state measurements [4]. In this paper we describe studies on substrate activation of this enzyme species using stopped-flow technique. The resulting rate and equilibrium constants are discussed and compared with results obtained with PDC from brewer's yeast. Reference experiments were done with 4-hydroxymercuri benzoate-modified PDC to determine the role of SH-groups in the activation process for the pea enzyme.

2. Materials and methods

2.1. Enzyme preparation

Enzyme preparation was done according to our method published recently [4]. Elimination of residual DTE and buffer exchange was performed simultaneously by gel filtration on Sephadex G-25 (5 ml Hitrap columns; Pharmacia). *SH-groups* at the surface of the enzyme protein were modified with an excess of HMB (corresponding concentrations are specified in the figure legends) for 30 min at room temperature in 20 mM Pipes, pH 6.0, at a PDC concentration of 10 μg/ml.

2.2. Protein concentration

Protein concentration was determined spectrophotometrically at 280 nm using the absorption coefficient of 71 000 $M^{-1}\cdot cm^{-1}$ calculated from the corresponding nucleotide sequence [5] following the method of Gill and von Hippel [6]. HMB concentration was determined spectrophotometrically at 232 nm using the absorption coefficient of 16 900 $M^{-1}\cdot cm^{-1}$ [7].

2.3. Substrate activation

Substrate activation was followed via pyruvate consumption with a stopped-flow spectrophotometer (Applied Photophysics) at 340 nm and 30°C with the coupled optical assay of Holzer et al. [8] in 0.1 M sodium citrate, pH 6.0, for the native enzyme and in 20 mM Pipes, pH 6.0, at 25°C for the HMB-modified enzyme. One syringe contained ADH (2 mg/ml), NADH (0.15 mg/ml) and various pyruvate concentrations in the buffer mentioned above; the other syringe contained the enzyme in buffered solution. The progress curves were analysed by fitting the data to a coupled first- and zero-order reaction according to the equation:

$$Abs. = A + B\cdot t + C_{exp}(-k_{act}\cdot t)$$

3. Results and discussion

A simple model for the substrate activation of PDC is illustrated in the scheme below. The substrate is rapidly bound to the enzyme's regulatory site to give an intermediary ES complex. Another, slower reaction step follows the conversion of PDC into its fully activated state (the further reaction sequence is omitted [1,9]).

$$E + S \underset{k_{-a1}}{\overset{k_{a1}}{\rightleftharpoons}} ES \underset{k_{-a2}}{\overset{k_{a2}}{\rightleftharpoons}} ES^*$$

A typical progress curve of the catalysed reaction illustrating substrate activation of PDC from *Pisum sativum* is shown in Fig. 1A. During the first 5–6 s the reaction velocity is steadily increasing. However, pea PDC starts with an initial rate of about 25% of maximum reaction rate whereas PDC from brewer's yeast is almost inactive (initial rate < 5% [10]) at the beginning of the catalytic reaction. By analysing the progress curves measured at various substrate concentrations (according to [1]) we were able to calculate the dissociation constants K_{a1} and K_{a2} as well as the rate constants k_{a2} and k_{-a2} (see the scheme) from the plot of activation rate constants versus substrate concentration for both enzyme species

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Abbreviations: ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase; HMB, 4-hydroxymercuri benzoate; DTE, 1,4 dithioerythritol

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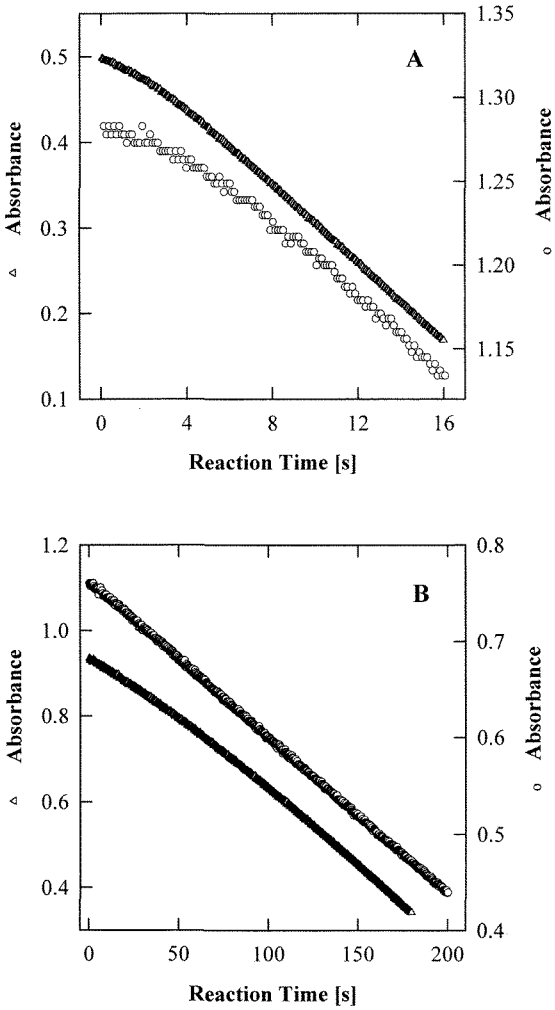

Fig. 1. Progress curves of the PDC-catalysed reaction for the enzymes from pea seeds (△) and brewer's yeast (○). The lag phase in product formation corresponds to substrate activation. A: Native. B: HMB-modified. Measurements at 340 nm and 30°C (A) and 25°C (B), respectively, with the coupled optical test of Holzer et al. [8] in 0.1 M sodium citrate, pH 6.0 (A), and 20 mM Pipes, pH 6.0 (B). Pyruvate concentration 2 mM, PDC concentration about 10 μg/ml, HMB modification for 30 min at room temperature, with 2 HMB per subunit for yeast PDC and 6 HMB for pea PDC.

(Fig. 2). The values summarised in Table 1 illustrate that substrate activation of PDC is faster in the pea than yeast enzyme. The value for K_{tot} – the dissociation constant for the whole activation process – is about one order of magnitude lower for the pea enzyme than that for the yeast PDC, indicating a higher affinity of the regulatory site of pea PDC for the substrate pyruvate. Thus, this enzyme is catalysing the decarboxylation of pyruvate more efficiently at lower substrate concentrations. For other plant PDCs slower activation rates had been found. For the species from wheat and maize this process lasts several minutes [11,12].

Table 1
Comparison of dissociation and rate constants for substrate activation of PDC from *Pisum sativum* and from brewer's yeast

	Pyruvate decarboxylase		
	From pea seeds	From brewer's yeast	
		Own data	Data from [1]
k_{a2} (1/s)	1.15	0.48	0.46
k_{-a2} (1/s)	0.03	0.05	0.04
K_{a1} (mM)	3.31	5.52	8.00
K_{a2} (mM)	26.00	100.00	87.00
K_{tot} (mM)	0.09	0.64	0.76

For definition of constants see the scheme.

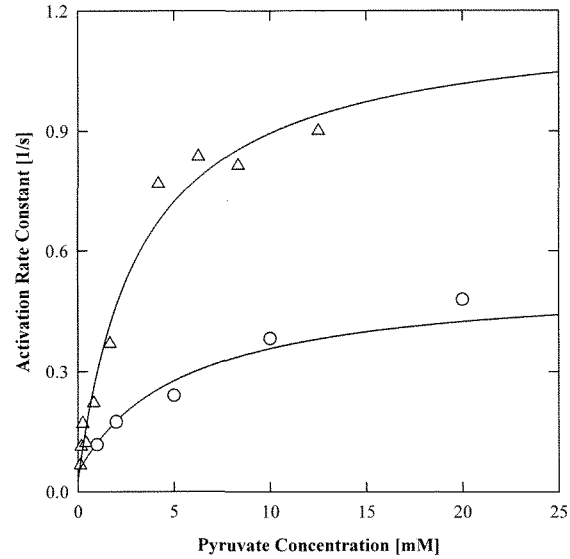

Fig. 2. Dependence of activation rate constant on substrate concentration for PDC from *Pisum sativum* (△) and from brewer's yeast (○), respectively. Experimental conditions as in Fig. 1.

Baburina et al. [3] favoured Cys^{221} as a pyruvate binding site for the activation of PDC from brewer's yeast based on structural analysis and investigations of Cys mutants. By modification of cysteine residues at the surface of this PDC with 4-hydroxymercuribenzoate (HMB) and 3-bromopyruvamide we had already demonstrated in 1988 that these side chains are involved in the substrate activation process [2]. After complete modification of all accessible SH-groups (6

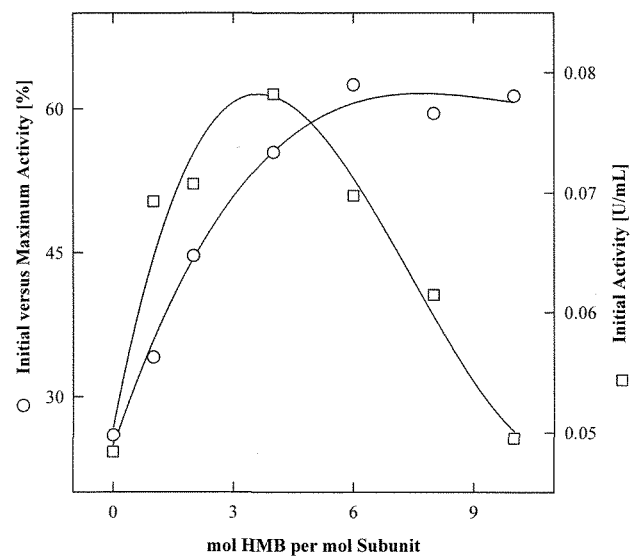

Fig. 3. Absolute (□) and relative (○) initial reaction velocities for PDC from *Pisum sativum* in dependence on HMB concentration. Experimental conditions as in Fig. 1B.

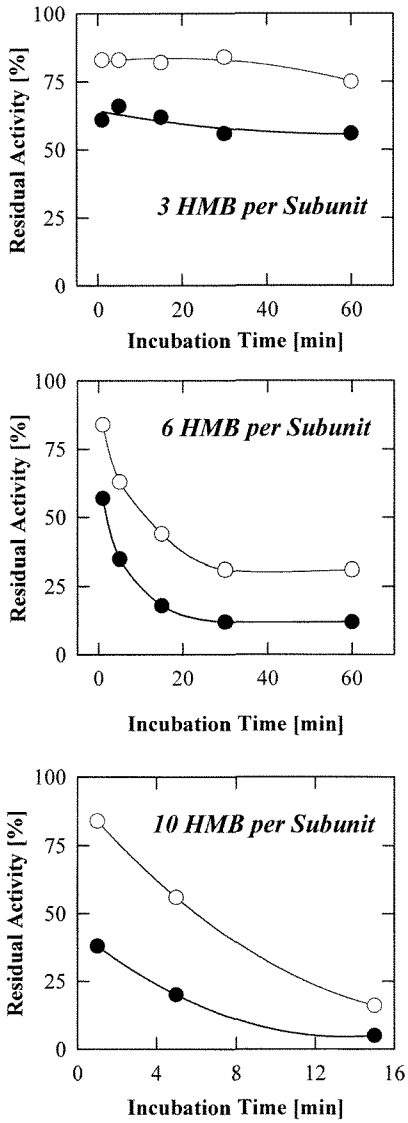

Fig. 4. Relative residual (●) and DTE-restored (○) catalytic activity (in percent of the catalytic activity of the unmodified enzyme) of HMB-modified PDC from *Pisum sativum* in dependence on incubation time with HMB. Enzyme concentration 142 μg/ml, 1000-fold excess of DTE over HMB, measurement conditions as in Fig. 1.

per tetramer) the enzyme was permanently activated, but had only 10% of the original catalytic activity (Fig. 1B). It was possible to restore the original catalytic activity and substrate activation behaviour after cleaving the HMB-sulphur bond by treatment with DTE.

We were able to modify 6 out of 16 SH-groups per subunit of pea PDC [4] — 4 times more than in the case of the yeast enzyme. However, contrary to this PDC, even the completely modified pea enzyme was still able to be activated by its substrate pyruvate, although the ratio of initial to maximum reaction rate was increased up to 60% (Fig. 1B). As shown in Fig. 3 this ratio could not be increased by rising the HMB excess during protein modification, but the catalytic activity was further decreased – also contrary to the yeast enzyme – to below 10%. In addition, the extent of activity recovery after thiol reduction by DTE dropped down (Fig. 4). Thus, the native state of pea PDC was by no means restored after DTE treatment.

Summarising the results we may point out that, although substrate activation is faster in the pea enzyme than in the yeast PDC, SH-groups of pea PDC may be involved in this process, but far from the extraordinary role they play in the substrate activation of PDC from brewer's yeast.

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