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Phosphorylation-activated 6-phosphofructo-2-kinase from mantle tissue of marine mussels

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PKF-2 from mussel mantle was phosphorylated by cAMP-dependent protein kinase. The phosphorylation does not change the enzyme activity at neutral pH values, but at acid pH the activity of the phosphorylated form is higher than the native PFK-2. With respect to the native enzyme, the activation consisted of a reduction in the K_m for Fru-6-P and a decrease in the inhibitory effect of PEP. These results are in keeping with the stabilized concentration of Fru-2,6-P_2 found in the mussel mantle during the physiological hypoxia caused by the closure of the valves.

6-Phosphofructo-2-kinase; Phosphorylation; Mollusc; Mantle tissue; *Mytilus galloprovincialis* Lmk

1. INTRODUCTION

Fructose-2,6-bisphosphate (Fru-2,6-P_2) has been found to be a potent modulator of the glycolytic pathway from different sources [1–3], including several tissues of intertidal molluscs [4–7]. In the adductor muscle of mussels, the transition from aerobiosis (immersed) to hypoxia (emersed), during which mussels close the valves, induces the fall in the Fru-2,6-P_2 concentrations [4,5] and the depression of glycolytic flow (reverse Pasteur effect). During hypoxia, the Fru-2,6-P_2 level in the mantle remains stable [6,7]; however, it is known that the fall in pH produced by the accumulation of propionate and succinate [8], inhibits 6-phosphofructo-2-kinase (PKF-2) and increases the inhibitory effect of citrate and PEP [9].

Phosphorylation and dephosphorylation seems to be a powerful mechanism for controlling the activities of numerous regulatory enzymes in the cell [10]. In molluscs several enzymes of the glycolytic pathway were modulated by phosphorylation/dephosphorylation processes [11,12]. The response to the covalent modification of an enzyme may be different; in this sense, PFK-2 from mammalian livers is inhibited by phosphorylation [1–3] whereas the enzyme from yeast is activated [13].

Abbreviations: Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-6-P, fructose-6-phosphate; Fru-2,6-P_2, fructose-2,6-bisphosphate; FBPase-1, fructose-1,6-bisphosphatase (EC 3.1.3.11); PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.11); PEP, phosphoenolpyruvate.

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In this paper we describe the changes in the kinetic properties caused by the phosphorylation of PFK-2 from the mantle of the common sea mussel *Mytilus galloprovincialis* Lmk. The results are also discussed in relation to the findings about the aerobiosis/hypoxia transition that occurs when mussels close the valves.

2. MATERIAL AND METHODS

Mussels (*M. galloprovincialis*) were obtained from the Ria de Arosa (N.W. Spain). All biochemical and purified enzymes were obtained from Sigma and Boehringer. Radioactive products were from Amersham International. [2-^{32}P]Fru-2,6-P_2 was synthesized as in [14]. PFK-2 from mussel mantle was purified as described previously [15]. Purified PFK-2 from rat liver was kindly donated by R. Bartrons. The PFK-2 activity was measured as in [16]. The phosphorylation was made as follows. (i) Samples with enzymatic activity were incubated at 30°C for 20 min with 1 mM ATP or 0.2 mM [τ-^{32}P]ATP and 1 mM DTT, 1 mM PMSF, 20 mM potassium phosphate buffer (pH 7.5) in the presence of 100 units per assay of catalytic subunit of cAMP-dependent protein kinase. As a control, the same process was carried out for the PFK-2 enzyme from rat liver. (ii) The phosphorylation of the PFK-2/FBPase-2 with [2-^{32}P]Fru-2,6-P_2 was made as in [15]. Protein was measured by the Coomassie blue dye-binding method [17]. SDS-PAGE electrophoresis was performed as in [18].

3. RESULTS

3.1. *Phosphorylation of PFK-2 from mussel mantle*

PFK-2 from the mantle tissue of mussels was phosphorylated in the presence of [τ-^{32}P]ATP by the purified catalytic subunit of cAMP-dependent protein kinase. As the enzyme contained FBPase-2 activity in the same protein [9,15], it was also phosphorylated using [2-^{32}P]Fru-2,6-P_2. The autoradiographs of SDS-PAGE electrophoresis (Fig. 1) of protein phosphorylated by [2-^{32}P]Fru-2,6-P_2 and [τ-^{32}P]ATP showed that the in-

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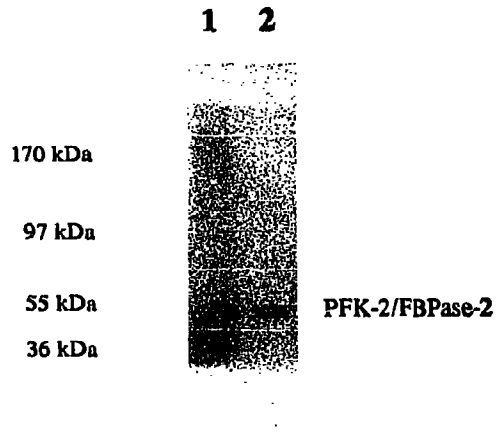

Fig. 1. Phosphorylation of mussel mantle PFK-2/FBPase-2. Lane 1 phosphorylation by cAMP-dependent protein kinase and [γ-^{32}P]ATP. Lane 2 by [2-^{32}P]Fru-2,6-P_2. Phosphorylations were made as described in section 2.

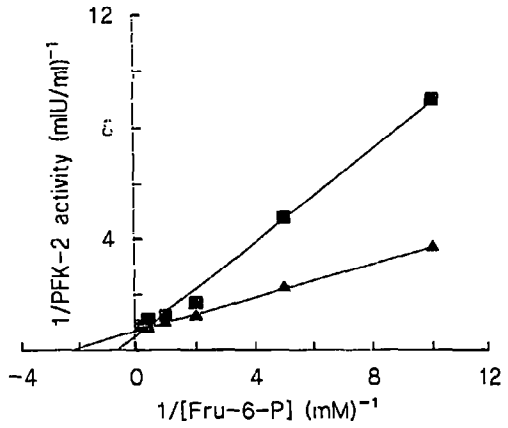

Fig. 3. Variation of PFK-2 activity with respect to Fru-6-P concentration. The assays were carried out at pH 6.5. ATP and P_i were 5 mM. (■) Native enzyme; (▲) PFK-2 phosphorylated by cAMP-dependent protein kinase as in Fig. 2.

corporation of radioactive phosphate occurred in the band corresponding to PFK-2.

3.2. *Effect of pH on the phosphorylated PFK-2*

As shown in Fig. 2 the native PFK-2 possesses an optimum pH between 7.5 and 8.0 units. At alkaline pH, phosphorylated and native forms of PFK-2 showed enzymatic activities of the same order; however, at acid pH, the activity of the phosphorylated PFK-2 is higher than that of the native form. The same kinetic parameters for Fru-6-P were obtained by the phosphorylated and native forms of PFK-2 at basic pH; nevertheless, when the pH falls, the affinity of phosphorylated PFK-2 for Fru-6-P is greater than that of the native enzyme (at pH 6.5, native PFK-2 K_m=1.7 mM; phosphorylated PFK-2 K_m = 0.4 mM (Fig. 3)). The pH of the assay does not appear to modify the kinetic parameters (K_m and V_{max}) for the ATP of both forms of PFK-2.

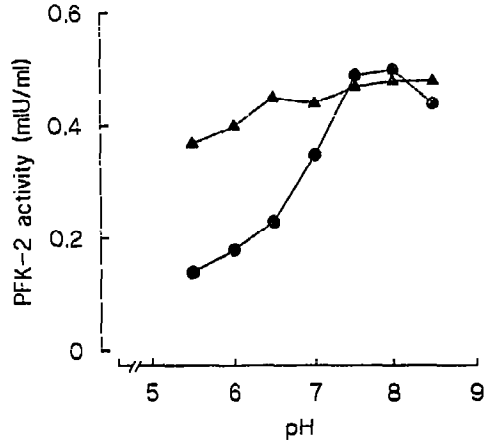

Fig. 2. Effect of pH on the PFK-2 activity. Fructose-6-P and ATP were 0.5 mM in reaction mixture. (●) Native enzyme; (▲) PFK-2 phosphorylated by cAMP-dependent protein kinase as described in section 2 using 1 mM ATP.

3.3. *Effect of citrate and PEP on PFK-2*

Only citrate and PEP inhibit both forms of PFK-2. At pH 8.0 the inhibition by citrate and PEP is similar for the two forms of the enzyme (Table I); however at pH 6.5, the native form shows a high sensitivity to the PEP when compared with the phosphorylated form of the enzyme (Fig. 4).

4. DISCUSSION

As occurs in other sources [1–3], the PFK-2 from mussel mantle is phosphorylated by cAMP-dependent protein kinase. When assayed at optimum pH, no changes were detected in the kinetic constants when comparing the phosphorylated and the native forms of the enzyme, a result similar to that obtained for the L

Table I

Effect of citrate and PEP on the PFK-2 activity

Modulator	Native PFK-2 mIU/ml	Phosph. PFK-2 mIU/ml
A. pH 8.0		
None	0.65	0.63
Citrate 1 mM	0.30	0.28
PEP 0.1 mM	0.36	0.33
B. pH 6.5		
None	0.35	0.47
Citrate 1 mM	0.03	0.04
PEP 0.1 mM	0.04	0.11

The assays were carried out at pH 8.0 and 6.5. The reaction mixture contained 0.5 mM Fru-6-P, 0.5 mM ATP and 5 mM P_i. PFK-2 was phosphorylated as described in Fig. 2.

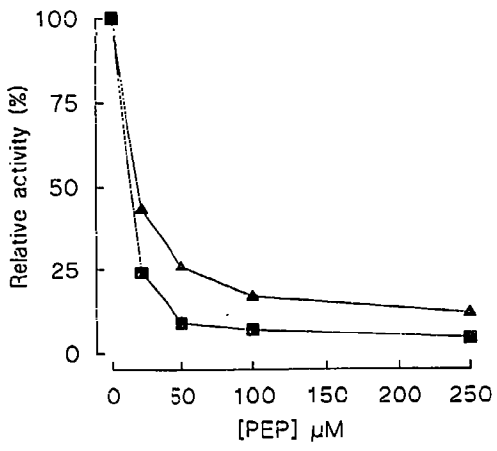

Fig. 4. Variation of the PFK-2 activity with respect to PEP concentration. The reaction mixture contained 0.5 mM Fru-6-P, 0.5 mM ATP and 5 mM P_i. The assays were carried out at pH 6.5. (■) Native enzyme; (▲) PFK-2 phosphorylated by cAMP-dependent protein kinase as in Fig. 2. The activities corresponding to 100% values were 0.35 for native PFK-2 and 0.47 for the phosphorylated form.

(liver) isozyme of PFK-2 from mammalians [19]. At acid pH, the activity of phosphorylated PFK-2 is higher than the native enzyme, similar behaviour to that of the H isozymes [20–22], and contrary to that showed by the L forms [19]. The decrease in the K_m value for Fru-6-P showed by the phosphorylated enzyme measured at pH 6.5 without modifications of other kinetic parameters with respect to the native enzyme, and the descent in sensitivity with respect to the PEP, suggests that the phosphorylation activates the enzyme from mussel mantle; similar results were obtained with PFK-2 from myocardium [20,21] and *Saccharomyces cerevisae* [23].

These results are in keeping with the modulation of the Fru-6-P/Fru-1,6-P_2 cycle, and the stable levels of Fru-2,6-P_2 [6] in mussel mantle during the transition from aerobiosis to hypoxia. The fall in pH that occurs in hypoxia [8] inhibits PFK-1 increasing the allosteric inhibition by ATP [24,25], and increases the K_m for Fru-6-P of native PFK-2. The data suggest that the synthesis of Fru-2,6-P_2 may be depressed and the positive effect on 6-phosphofructo-1-kinase (PFK-1) diminished [26]. The inhibition of PFK-2 may be reversed by the phosphorylation of the enzyme at acid pH, increasing the Fru-2,6-P_2 levels and supporting the activation of PFK-1 by the hypoxia-increased levels of AMP [24,6]. On the other hand, high levels of Fru-2,6-P_2 inhibit fructose-1,6-bisphosphatase (FBPase-1) [27].

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