The reaction mechanism of phosphomannomutase in plants

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Abstract The enzyme phosphomannomutase catalyzes the interconversion of mannose-1-phosphate (Man-1-P) and mannose-6-phosphate (Man-6-P). In mammalian cells the enzyme has to be activated by transfer of a phosphate group from a sugar-1.6-P_2 (Guha, S.K. and Rose, Z.B. (1985) *Arch. Biochem. Biophys.* 243, 168). In contrast, in the red alga *Galdieria sulphuraria* the co-substrate (Man-1.6-P_2 or Glc-1.6-P_2) is converted to the corresponding sugar monophosphate while the substrate is converted to the sugar bisphosphate in each reaction cycle. Evidence is presented that the same reaction mechanism occurs in spinach and yeast.

Key words: *Galdieria sulphuraria*; Rhodophyta; Phosphomannomutase; Mannose-1,6-bisphosphate; Reaction mechanism

1. Introduction

The enzyme phosphomannomutase (PMM) catalyzes the interconversion of mannose-1-phosphate (Man-1-P) and mannose-6-phosphate (Man-6-P). It requires bisphosphate sugars as cofactors and belongs to the family of 'glucose–bisphosphate enzymes' [1]. Although the importance of the sugar bisphosphate is generally accepted, little is known about its precise role and the reaction mechanism of phosphomutases in general. The unicellular acido- and thermophilic red alga *Galdieria sulphuraria* Merola possesses a phosphoglucomutase which is specific for glucose phosphates and a bifunctional PMM which can use mannose phosphates and glucose phosphates as substrates [2]. Both enzymes have an absolute requirement for $MgCl_2$ and a sugar bisphosphate for activity. In the course of these experiments we obtained indications that the reaction mechanism of the algal PMM differs from the enzyme from animals.

2. Material and methods

Galdieria sulphuraria (Galdieri) Merola (strain 074) from the culture collection of the University of Naples [3] was used. Culture conditions have been described previously [4].

2.1. Enzyme purification

PMM from *Galdieria*, spinach, yeast, and pig brain was purified using the method described previously [2,5].

2.2. Enzyme assays

One unit of activity is defined as 1 μmol/min. Phosphomannomutase was measured in a coupled reaction at room temperature according to Murata [6]. The reaction was started by the addition of 0.4 mM Man-1-P. K_M and V_{max} values were determined from a linear direct plot [7]. Because Man-1.6-P_2 is not commercially available, we estimated the K_m for Man-1.6-P_2 from the linear phase of the reaction with Glc-1.6-P_2. In order to identify the reaction products of PMM in the presence of an excess of Glc-1.6-P_2, purified PMM from *G. sulphuraria*, pig brain, and yeast was incubated with 1 mM Glc-1.6-P_2 and 0.1 mM Man-1-P for 3 h at room temperature. The reaction products were separated by TLC at pH 10 as described [8]. The corresponding regions for Man-1-P, Man-6-P, and Glc-6-P were scraped off, the sugar phosphates eluted, and identified enzymatically. The concentration of Glc-6-P was determined by the addition of Glc-6-P dehydrogenase and NADP. For Man-6-P determination PGI and PMI were included and for Man-1-P purified PMM from *G. sulphuraria* was added.

3. Results and discussion

When purified PMM from *Galdieria sulphuraria* was assayed in the presence of Glc-1-P and Glc-1.6-P_2, the reaction rate was linear from the beginning (Fig. 1). However, when the assay was started with Man-1-P the PMM reaction exhibited a lag phase (Fig. 1). The duration of the lag phase depended on the amount of enzyme present and was usually between 3 and 5 min. This lag phase was not caused by activating reaction products, such as NADPH and gluconate-6-P (data not shown). Therefore, we concluded that in the course of the PMM reaction Glc-1.6-P_2 is replaced by Man-1.6-P_2. This conversion is rate limiting for the whole reaction and thus causes the lag phase at the beginning of the reaction. Only when all Glc-1.6-P_2 molecules are finally converted to Man-1.6-P_2 the reaction can proceed with the maximum rate.

The following experiments give an indirect proof that Glc-1.6-P_2 is indeed converted to Man-1.6-P_2 by the PMM.

(1) The PMM reaction with Man-1-P as substrate was monitored until the reaction rate was linear. Subsequently, the enzyme was denatured by boiling for 5 min. When native PMM was again added to this mixture, the reaction progressed without a lag phase.

(2) When purified PMM was incubated in the presence of Man-1-P and an excess of Glc-1.6-P_2, the product of the reaction was Glc-6-P ($>95\%$) and not Man-6-P ($<5\%$) while all Man-1-P was consumed. This can only be explained by the conversion of Glc-1.6-P_2 to Glc-6-P and the production of Man-1.6-P_2.

The K_M for Glc-1.6-P_2 with Glc-1-P as substrate was 3 μM, similar to the value obtained for Man-1.6-P_2 with Man-1-P as substrate (4 μM).

When Fru-1.6-P_2 was used as cosubstrate for the PMM assay, a lag phase was observed with Man-1-P and Glc-1-P. In this case none of the sugars used as substrates was identical with the sugar used as co-substrate. Because all reactions exhibited lag phases, the conversion of the co-substrate into the sugar which corresponds to the substrate can be regarded as a general phenomenon. If this assumption is true, the addition of two different substrates and only one co-substrate to the bifunctional PMM would lead to a decrease in activity. Under these conditions, the chances that the substrate and the corresponding co-substrate are present at the PMM molecule at

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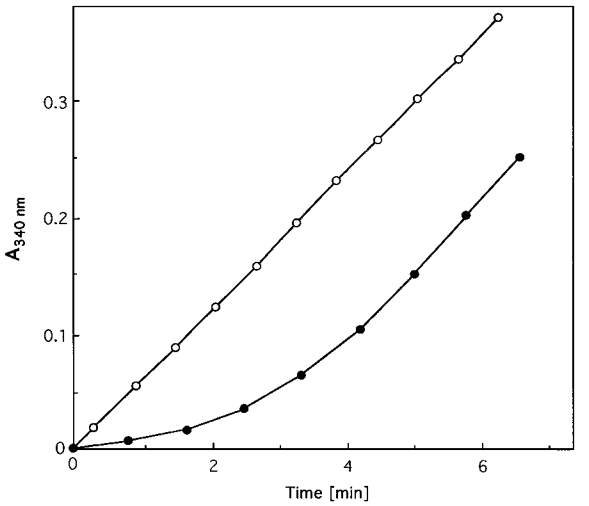

Fig. 1. Reaction of PMM from *G. sulphuraria* with Glc-1.6-P_2 and Glc-1-P (○) or Man-1-P (●).

the same time are only 50%. This was confirmed in an experiment where PMM was started with Glc-1-P and Man-1-P at the same time and Glc-1.6-P_2 as co-substrate. The activity with both substrates was only 0.36 U/ml compared to 0.49 U/ml when Glc-1-P or 0.45 U/ml when Man-1-P was added alone.

For bacteria [9] and mammalian tissue [5,10,11] it is generally accepted that phosphomutases have to be phosphorylated in order to be active. Only very few phosphomutases have been isolated in this phosphorylated form, e.g. the PGM from rabbit muscle where no Glc-1.6-P_2 is needed in the assay [12,13]. It is assumed that the addition of sugar bisphosphates is only necessary to regenerate the active enzyme when the enzyme was dephosphorylated during isolation [9].

For the PMM from *Galdieria sulphuraria* a sugar bisphosphate was essential for activity indicating that the enzyme could have been dephosphorylated during isolation. In order to determine whether the phosphorylation state of the PMM from *Galdieria sulphuraria* caused the lag phase of the reaction with Man-1-P we pre-incubated the enzyme for 10 min with Glc-1.6-P_2. After the coupling enzymes and Man-1-P were added, the reaction exhibited the usual lag phase. This shows that Glc-1.6-P_2 alone is not sufficient to activate the enzyme.

In order to compare the results obtained with the phosphomannomutase from *G. sulphuraria* with the enzyme from other organisms we tested the PMM activity in partially purified preparations of spinach, pig brain, and yeast. Similar to the enzyme from *G. sulphuraria*, the PMM from spinach and yeast showed a lag phase with Man-1-P and Glc-1.6-P_2. When the yeast enzyme in this assay mixture was denatured after several minutes and the mixture supplemented with new coupling enzymes and the PMM from *G. sulphuraria*, no lag phase was observed. This indicates that the yeast enzyme converted Glc-1.6-P_2 to Man-1.6-P_2. Thus, the reaction mechanism of the PMM from plants and yeast are apparently the same. A similar experiment was carried out with the enzyme from pig brain which does not exhibit a lag phase with Man-1-P and Glc-1.6-P_2. When the mammalian enzyme was denatured after several minutes of reaction and PMM from *G. sulphuraria* was added, a lag phase was observed. In addition, we identified the reaction products of the enzyme from yeast and pig brain when Man-1-P and Glc-1.6-P_2 were present. As shown for the algal enzyme, the PMM from yeast produced mainly Glc-6-P and not Man-6-P when high concentrations of Glc-1.6-P_2 were offered. In contrast, the PMM from pig brain yielded exclusively Man-6-P (data not shown). Therefore, unlike the enzyme from yeast or plants the PMM from animals did not convert Glc-1.6-P_2 to Man-1.6-P_2. This strongly suggests that here the co-substrate is only required for the activation of the enzyme as has been described [5,10,11].

4. Conclusions

Several reaction mechanisms have been proposed for phosphomutases, especially phosphoglucomutases. All of these involve two binding sites for sugar phosphates [13,14] and include the phosphorylated enzyme at some point of the reaction. It is not known, however, whether the dephosphorylated enzyme is a part of the reaction mechanism or occurs only during enzyme purification. It is also still a matter of debate whether the active phosphomutase is an enzyme–monophosphate or an enzyme–bisphosphate complex.

The reaction mechanism proposed for the PGM from rabbit muscle [10,13] does not involve free Glc-1.6-P_2 (Fig. 2A). The sugar bisphosphate remains bound to the enzyme and is only released once in about 20 turnovers. It is added to the assay in order to activate any dephosphorylated enzyme. The first step of the reaction is the formation of an enzyme–phosphate complex. By reacting with the substrate the enzyme–bisphosphate is generated. Subsequently, the product of the reaction and the phosphorylated enzyme are released. This model does not apply to the PMM from yeast and *G. sulphu-*

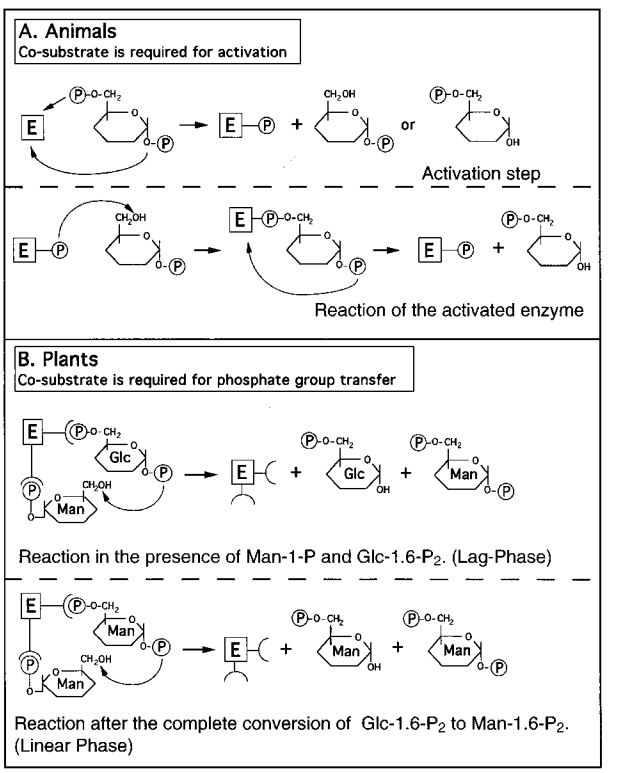

Fig. 2. Reaction mechanisms of phosphomannomutase as proposed for rabbit muscle by Lowry and Passonneau [10] and by Rose [1] (A) and for the PMM from plants and yeast (B).

raria because here in each reaction cycle the co-substrate yields the product while the substrate is converted to the sugar bisphosphate. A model meeting these conditions is depicted in Fig. 2B. We propose that the enzyme is not covalently phosphorylated because the enzyme–phosphate complex is broken down in every reaction cycle. When Glc-1.6-P_2 is used as co-substrate and Man-1-P as substrate, the Glc-1.6-P_2 pool is eventually completely converted into Man-1.6-P_2. This explains the lag phase of the reaction because the specific sugar bisphosphate first has to be generated. Only when all Glc-1.6-P_2 molecules have been converted into Man-1.6-P_2 the reaction can proceed with maximal activity. The duration of the lag phase, thus, depends on the amount of Glc-1.6-P_2 in the assay and the activity of the PMM.

In conclusion, while the function of the co-substrate in animal tissue is the activation of the PMM, for plants and yeast the co-substrate is directly involved in the reaction mechanism.

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