Investigation of the role of the substrate metal ion in the yeast inorganic pyrophosphatase reaction

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The substrate activities of a series of tripositive metal ion–pyrophosphate complexes with yeast inorganic pyrophosphatase were examined. While the Michaelis constants for these complexes were shown to be between one and two orders of magnitude greater than that of the natural substrate, $[Mg(H_2O)_4PP_i]^{2-}$, the turnover numbers were in general comparable to that of $[Mg(H_2O)_4PP_i]^{2-}$. These data suggest that the nature of the metal ion cofactor effects substrate binding but in most cases not catalysis. Thus, the role of the metal ion in catalysis is probably restricted to that of an electron sink.

Yeast inorganic pyrophosphatase *Metal ion cofactor* *Substrate activation*

1. INTRODUCTION

Yeast inorganic pyrophosphatase catalyzes the hydrolysis of pyrophosphate (PP_i) to orthophosphate (P_i). Previous studies have shown that 3 divalent cations per active site are required for catalysis ([1] and unpublished). Two of these metal ions bind directly to the enzyme while the third cofactor coordinates with the PP_i (unpublished). The in vivo substrate, $[Mg(H_2O)_4PP_i]^{2-}$ is fully ionized [2] and P^1,P_2-bidentate in structure [2,3]. Previously we examined the role of the substrate metal ion in substrate binding [3]. We examine here the potential role of the substrate metal ion in activation of the substrate for phosphoryl transfer to water by using a series of tripositive metal ion–PP_i complexes as probes of enzyme substrate specificity.

2. MATERIALS AND METHODS

Pyrophosphatase was purified according to the modified [4] method of [5]. The enzyme used in these experiments migrated as a single band on SDS–polyacrylamide gel electrophoresis gels (7.5% acrylamide) and had an activity of 690 μmol P_i/min per mg protein at pH 7.5. $Na_4[^{32}P]PP_i$ was purchased from New England Nuclear and all buffers were obtained from Sigma. All metal ions (Alfa Ventron) were used as their perchlorate salts except Mg^{2+} and Al^{3+} which were used as their chloride salts. Stock solutions of $MgPP_i$ or $M(III)PP_i$ complexes were prepared by adding one equivalent of metal ion to PP_i. Aliquots from freshly prepared stock solutions were added to reaction mixtures 50 mM in K^+–Pipes (pH 7.0) (1,4-piperazinediethanesulfonate) and 1 mM in $MgCl_2$. Reactions were initiated by addition of pyrophosphatase to the 1 ml reaction mixtures at 25°C and terminated by the addition of 60 μl of 6 N HCl. Reaction mixtures were assayed for $^{32}P_i$ as in [2]. Control reactions, lacking enzyme, were run concurrently in order to insure that P_i formation was exclusively enzyme catalyzed.

Initial velocity data were gathered using metal–PP_i complexes at concentration ranges 0.5–5-fold their K_m value. K_m and V_m values were evaluated from Lineweaver-Burk plots in which the inverse of the initial velocity was plotted against the inverse of the initial total PP_i concentration. The K_i value of $ScPP_i$ was calculated from initial velocity data obtained from an experiment in which $ScPP_i$ was tested as a competitive inhibitor vs $MgPP_i$.

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3. RESULTS AND DISCUSSION

The present studies were designed to probe the role of the metal ion cofactor associated with PP_i in the pyrophosphatase-catalyzed hydrolysis reaction by examining the specificity of the enzyme towards a series of tripositive metal ion–PP_i complexes. The in vivo cofactor for pyrophosphatase is Mg^{2+}. Not only does an Mg^{2+} coordinate to PP_i to form the active substrate, P^1,P^2-bidentate $[Mg(H_2O)_4PP_i]^{2-}$ [2,3] but two additional Mg^{2+} coordinate to cofactor sites present on the enzyme. Previous studies have shown that only Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} can function in all 3 cofactor roles [2,6]. Tripositive metal ions are not only unable to function in all 3 roles [6] but also have been shown to be incapable of serving in the roles of the two enzyme bound cofactors [7]. The M^{3+} ions are in fact competitive inhibitors vs Mg^{2+} for the two enzyme cofactor sites and have stability constants which range from 1×10^4–1×10^6 M^{-1} [7]. Thus, in order to test the substrate activity of the M(III)PP_i complexes Mg^{2+} had to be included in the reaction mixture to fulfill the requirements at the two cofactor sites on the enzyme. Because the stability constant of $MgPP_i$ ($\sim 1 \times 10^6$ M^{-1}) is so much smaller than that of the M(III)PP_i complexes ($\sim 1 \times 10^{17}$ M^{-1}) [8] compared to stability constants describing Mg^{2+} binding to the enzyme ($\sim 3 \times 10^3$–3×10^4 M^{-1}) vs M^{3+} binding to the enzyme ($\sim 1 \times 10^4$–1×10^6 M^{-1}), M^{3+} and PP_i could be added in 1:1 ratio at concentrations ranging from 0.070–3.0 mM to reaction mixtures containing 1 mM $MgCl_2$ with practically exclusive formation of M(III)PP_i and enzyme–Mg complexes. Table 1 shows V_m and K_m values measured for PP_i varied in constant ratio with M^{3+} in the presence of 1 mM Mg^{2+} as well as the V_m and K_m values for PP_i varied in constant ratio with Mg^{2+} in the presence of 1 mM excess Mg^{2+}. In any given experiment we can calculate on the basis of the reported $MgPP_i$ and M(III)PP_i stability constants that the concentration of $MgPP_i$ present in the reaction mixture containing the M^{3+} is $\sim 1 \times 10^{10}$-fold less than that in the same reaction mixture lacking M^{3+}. Thus, if the observed P_i formation derives primarily from the turnover of the $MgPP_i$ present in the M(III)PP_i reaction mixture then the apparent K_m measured should be at a minimum (since we are ignoring input from the competitive inhibition by the M(III)PP_i complex) 1×10^{10} times that of the true K_m for $MgPP_i$ (0.01 mM) or $\sim 1 \times 10^8$ mM. As indicated in table 1 the apparent K_m values measured fall in the range of 0.1–1.0 mM and therefore we can conclude with reasonable certainty that the P_i formation observed in the presence of the M^{3+} ions derives predominantly from the substrate activity of the M(III)PP_i complexes and that these apparent K_m values actually represent true K_m values for these complexes.

Table 1

The relative V_m and K_m values measured for pyrophosphate in the presence of 1 mM free Mg^{2+} and a stoichiometric amount of Mg^{2+} or tripositive metal ion

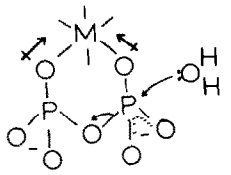
	Relative V^a_{max}	K^a_m (mM)	Ionic radius[c]
$Mg(H_2O)_4PP_i$	1.00	0.01	0.720
$TbPP_i$	1.00	0.48	0.923
$LuPP_i$	1.00	0.21	0.861
$DyPP_i$	0.93	0.27	0.910
$ErPP_i$	0.88	0.55	0.890
$YbPP_i$	0.87	0.15	0.868
$EuPP_i$	0.83	0.55	0.905
$HoPP_i$	0.79	0.26	0.900
$LaPP_i$	0.77	0.40	1.032
YPP_i	0.74	0.28	0.900
$NdPP_i$	0.59	0.38	0.985
$CePP_i$	0.55	0.55	1.010
$GdPP_i$	0.52	1.0	0.938
$InPP_i$	0.29	0.17	0.810
$AlPP_i$	0.18	0.16	0.500
$ScPP_i$	0.0000	0.008[b]	0.745

[a] SE $\sim \pm$ 10%

[b] K_i value for $ScPP_i$ as a competitive inhibitor vs $MgPP_i$ at pH 7.0

[c] Effective ionic radius of the hydrated metal ion [10]

The K_m values for the M(III)PP_i complexes fall in a range 1–2 orders of magnitude larger than the K_m value of $MgPP_i$. This result is not surprising since the M(III)PP_i complex possess only a single negative charge while the natural substrate, $MgPP_i$ possesses a net of two negative charges. What we did find surprising is that with the exception of the $ScPP_i$ turnover numbers for the M(III) complexes (table 1) are either identical to or quite close to that of $MgPP_i$. This result is in contrast to the data obtained for yeast hexokinase which showed that



Scheme 1.

M^{3+} complexes of ATP are tight inhibitors but are not substrates for the enzyme [9]. Stability constant data (K_s for M(III)PP_i $\approx 1 \times 10^{17}$ while K_s for binding PP_i to M(III)PP_i $\approx 1 \times 10^{1}$–1×10^{2} M^{-1} and K_s for binding M(III) to M(III)PP_i $\approx 1 \times 10^{2}$–1×10^{3}) [8] suggest that under conditions where M^{3+} and PP_i are present in a 1:1 ratio the predominant complex in solution is M(III)PP_i. Unlike the $MgPP_i$ complex, however, the M(III)PP_i complex does not necessarily have a coordination number of 6 but may, for example, exist in solution and perhaps be absorbed onto the enzyme in the form M(III)$(H_2O)_nPP_i$ where $n = 4$–7. In addition, because the radius of the M^{3+} ion is significantly larger than that of Mg^{2+} we might expect that the geometries of the chelate rings formed between the M^{3+} ions and PP_i will differ significantly from that of $MgPP_i$. Since neither difference in the M(III)PP_i vs $MgPP_i$ complex is recognized by the enzyme it may be reasonable to conclude that the role of the substrate metal ion is, as indicated in scheme 1, simply that of an electron sink. Since the metal ion is coordinated to both phosphoryl moieties it can serve to activate the phosphorus for nucleophilic attack by water as well as stabilize the phosphate anion displaced during the reaction.

Why $ScPP_i$ is not a substrate for pyrophosphatase is presently unclear to us. Of the tripositive metal ions Sc^{3+} is closest to Mg^{2+} in size and like $MgPP_i$, $ScPP_i$ binds very tightly to the enzyme. Substrate activity may be in some way related to the ratio of charge to radius of the metal ion.

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