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THE REGULATION OF *myo*-INOSITOL-1-PHOSPHATE-SYNTHASE ACTIVITY FROM *NEUROSPORA CRASSA* BY PYROPHOSPHATE AND SOME CATIONS

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Summary

The effect of several cations on the inhibition by PP_i of the enzyme *myo*-inositol-1-phosphate synthase (1L-*myo*-inositol-1-phosphate lyase (isomerizing) EC 5.5.1.4) from *Neurospora crassa* was studied. The study was undertaken in an attempt to explain how *myo*-inositol biosynthesis can occur in the presence of an intracellular PP_i concentration which exceeds the K_i for the enzyme by 350-fold.

The inhibition of enzyme activity by PP_i, at pH 7.7, was reversed, in decreasing order of effectiveness, by Mn^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+} and Zn^{2+}. The concentration of Mg^{2+} shown to be effective in reversing inhibition is well within the range demonstrable in *N. crassa* mycelia. It was shown that inhibition of the enzyme by PP_i occurs only in the presence of NH_4^+, an activator of the enzyme, and that inhibition is pH dependent.

The data presented suggest that the in vivo regulation of *myo*-inositol biosynthesis occurs as a consequence of the modulation of *myo*-inositol-1-phosphate synthase activity by PP_i, pH, and several cations.

myo-Inositol-1-phosphate synthase (1L-*myo*-inositol-1-phosphate lyase (isomerizing) EC 5.5.1.4), catalyzes the synthesis of L-*myo*-inositol-1-*P* from D-glucose-6-*P* with an absolute requirement for NAD^+. Previously it was reported that with a partially-purified enzyme preparation from *N. crassa*, PP_i acts as a competitive inhibitor and that the enzyme has an apparent K_m of 1.9 mM and a K_i of 4.6 μM [1]. In addition, the suggestion was made that PP_i might contribute to the regulation of *myo*-inositol (inositol) biosynthesis in *N. crassa*. We subsequently presented experimental evidence in favor of a regulatory role for PP_i in the in vivo biosynthesis of inositol by showing that a four-fold decrease in the PP_i pool is accompanied by a four to five-fold increase in the rate of conversion of [U-^{14}C] glucose to [^{14}C] inositol [2]. Determination of the PP_i pool in wild-type mycelia, gave a value of 6.29 μmol/g dry

weight or approximately 1.7 mM, assuming 4 ml of intracellular water per g dry weight of cells [2]. However, since the intracellular PP_i concentration is 350-fold greater than the K_i value for the enzyme, it is difficult to understand how inositol can readily be synthesized in vivo.

This report deals with a more detailed study of the factors which influence the inhibition of *myo*-inositol-1-phosphate synthase by PP_i, in order to better understand the regulation of inositol biosynthesis in *N. crassa*.

Materials and Methods

Organism and growth conditions

Wild type *N. crassa* strain RL 21a was maintained on slants of glycerol-sucrose-complete agar [3]. To obtain enzyme preparations, fresh conidia were inoculated into a 12 l carboy containing 10 l of Vogel minimal medium [4] plus 2% sucrose and grown, harvested and extracted as previously described [5]. For the determination of Mg^{2+}, K^+ and pH, cells were grown on the same medium for 48 h with constant agitation at 29° C. Mycelia were then harvested by filtration, washed twice with distilled water and the excess water was eliminated with the aid of filtration and hand pressure.

Purification and assay of the enzyme

myo-Inositol-1-phosphate synthase was purified approximately 400-fold by a slight modification of the method previously described [6]. The procedure was the same as before the protamine sulfate treatment step, then a first precipitate obtained with protamine sulfate (0.5 mg protamine sulfate per 10 mg protein) was discarded. The supernatant was treated with 1.5 mg protamine sulfate per 10 mg protein and the enzyme was recovered in the precipitate. This was dissolved in 1.0 M NaCl, treated with $(NH_4)_2SO_4$ and the bulk of the activity recovered in the 40—55% $(NH_4)_2SO_4$ precipitate. Enzyme activity was assayed according to the method of Piña et al. [1], with exceptions to the experimental conditions used, as indicated in the text. The enzymatic product, inositol-1-*P*, was hydrolyzed with commercial alkaline phosphatase EC 3.1.3.1. (Sigma Chemical Co., St. Louis, Mo.) to form free *myo*-inositol. In experiments in which the activity of *myo*-inositol-1-phosphate synthase was determined at an acidic pH, the incubation mixture was first adjusted to pH 8.0 before treatment with alkaline phosphatase. In addition, it was routinely demonstrated that the quantity of alkaline phosphatase added to enzyme reaction mixtures was not a limiting factor in the quantitative conversion of inositol-1-*P* to free inositol.

Analytical methods

Inositol was determined by the method described by McKibbin [7] using *Saccharomyces carlsbergensis* strain 4228. Mg^{2+} was determined by the fluorometric method of Schachter [8], pH by the freezing method described by Conway and Downey [9] and K^+ by conventional flame photometry.

Results

Reversal of PP_i inhibition by cations

myo-Inositol-1-phosphate synthase inhibition by PP_i has been shown to be reversible by Mg^{2+} and Mn^{2+} [1]. Table I shows the results of a more systematic study on the reversal of PP_i inhibition of the enzyme by various cations. Of the cations tested Mn^{2+} was clearly the most effective. In comparison, the effectiveness of Mg^{2+} was moderate in magnitude. However, it must be noted that Mg^{2+} is present at somewhat higher intra-cellular concentration than other divalent cations tested. Cu^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+} inhibit the activity to some extent in addition to partially overcoming PP_i inhibition.

The results presented in Fig. 1 clearly indicate that the H^+ concentration strongly influences the degree of PP_i inhibition of the enzyme, with less than 10% enzyme inhibition evident at pH 9.0 in Tris/HCl buffer, and nearly no inhibition at pH 6.0 in Tris/Acetate buffer.

Ammonium ion requirement for PP_i inhibition

The catalytic activity of *myo*-inositol-1-phosphate synthase from yeast [10] *N. crassa* [1] is known to be stimulated by NH_4^+. Therefore, NH_4^+ have

TABLE I

REVERSAL OF PP_i INHIBITION OF *myo* - INOSITOL-1-PHOSPHATE SYNTHASE BY SEVERAL METALS

a, enzymatic activity without metals and PP_i was taken as 100%. This activity in the presence of 0.1 mM PP_i was 19%; b, the metal and/or the inhibitor were added in such a way to keep the volume of the incubation mixture [1] to 1 ml. The salts used were; $MgCl_2$, $MnSO_4$, $CoCl_2$, $CuSO_4$, $ZnSO_4$, $CaSO_4$, $Ba(OH)_2$, $FeSO_4$ and $FeCl_3$.

Metal added[b]	Final metal concentration (mM)	% Enzymatic activity[a]	
		Without PP_i	Plus 0.1 mM PP_i
Mg^+	1.0	97	79
	0.1	—	41
Mn^{2+}	0.005	104	27
	0.05	100	44
	0.1	—	52
	1.0	95	96
Co^{2+}	0.1	82	19
	1.0	70	60
Cu^{2+}	0.1	94	26
	1.0	54	42
Zn^{2+}	0.01	74	20
	0.1	39	24
Ca^{2+}	0.1	99	19
	1.0	86	32
Ba^{2+}	0.005	100	25
	0.05	104	19
Fe^{2+}	0.1	86	48
	1.0	62	64
Fe^{3+}	0.1	86	52
	1.0	72	88

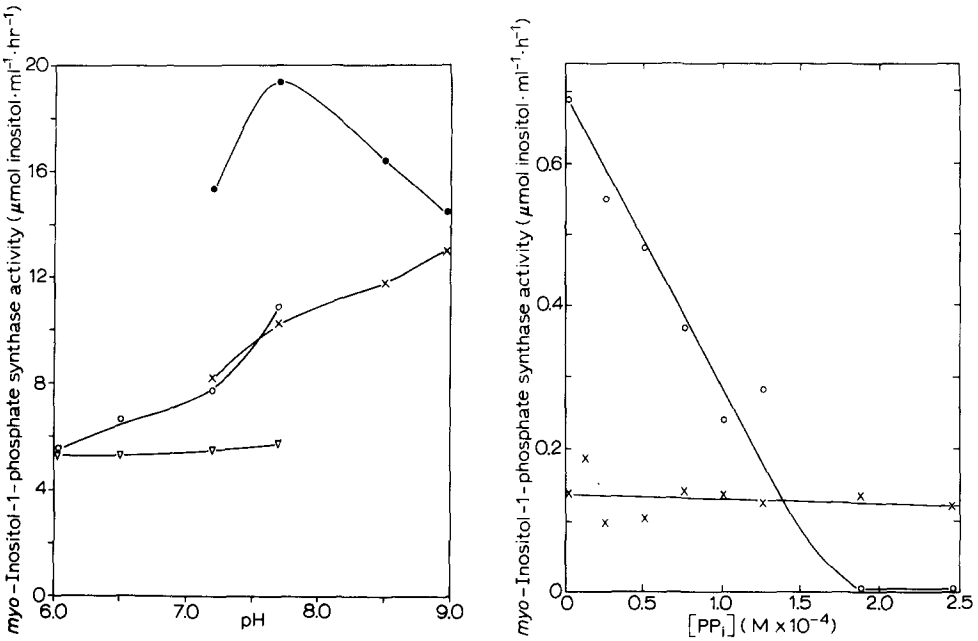

Fig. 1. Influence of the pH on the inhibition of *myo*-inositol-1-phosphate synthase by PP_i. The incubation mixture [1] for the enzyme reaction was kept constant except for the 0.1 M Tris/HCl buffer pH 7.7 which was modified in its pH or substitued by 0.1 M Tris/acetate buffer. The reactions were carried out with: ○———○ Tris/acetate; △———△, Tris/acetate plus 0.1 mM PP_i; ●———●, Tris/HCl and x———x, Tris/HCl plus 0.1 mM PP_i.

Fig. 2. Effect of NH_4^+ ions on the inhibition of *myo*-inositol-1-phosphate synthase by PP_i. The partially purified enzyme, approximately 1 ml, was dialyzed an additional 12 h against 2 l of 0.005 M Tris/HCl buffer pH 7.7 containing 0.25 mM EDTA with 4 changes of dialysis buffer. The final concentration of NH_4^+ in the reaction mixture was 14 mM [1]; ○———○, with NH_4^+; x- - - - -x, without NH_4^+.

routinely been incorporated into enzyme reaction mixtures in most studies. The data presented in Fig. 2 show that PP_i acts as an inhibitor of the enzyme only when it is present simultaneously with NH_4^+. In the absence of these ions, no inhibition of the enzyme by PP_i is demonstrable, but the activity is only 20% of the total activity obtained in its presence (Fig. 2). NH_4^+ in excess (140 mM) does not overcome PP_i inhibition [1].

Intracellular levels of various cations

After demonstrating that cations such as Mg^{2+} and H^+ can markedly affect the extent of PP_i inhibition of the enzyme, it was deemed significant to determine the levels of several ions in *N. crassa* mycelia. The results presented in Table II show the range of concentration of K^+, H^+, Mg^{2+}, PP_i and D-glucose-6-*P*.

In a previous study [1], K_i for PP_i of $4.6 \cdot 10^{-6}$ M was determined with enzyme reaction mixtures lacking added Mg^{2+}. Fig. 3 shows the results of experiments conducted to determine the effect of Mg^{2+}, at the prevailing intracellular concentration, on the PP_i K_i for the enzyme. The presence of Mg^{2+} resulted in a change of almost three orders of magnitude in the K_i to $2 \cdot 10^{-3}$ M. In these experiments, no precipitation occurred in enzyme reaction mixtures, even at the higher concentrations of Mg^{2+} and PP_i utilized.

TABLE II

AMOUNT OF SEVERAL IONS IN THE *N. CRASSA* MYCELIA

a, concentration of the different ions was calculated assuming the absence of an extracellular compartment in the used sample and the presence of 4.0 ml of intracellular water per g dry weight cells; b, mean ± standard error; c, calculated from the pH value obtained of 6.13 ± 0.03; d, values reported in ref. 2.

Ion	Amount detected (equivalents for K^+) (μmol per g of dry wt)	Number of determinations	Concentration[a] (mM)
K^+	519 ± 23[b]	10	130
H^+	—	12	$7.42 \cdot 10^{-4}$ [c]
Mg^{2+}	25.2 ± 0.7	7	6.3
PP_i [d]	6.75 ± 0.65	14	1.7
D-Glucose-6-*P* [d]	5.28 ± 0.29	5	1.3

Discussion

The results of our previous studies on the metabolism of inositol in *N. crassa* [2] strongly support the notion that the regulation of its biosynthesis by PP_i occurs in vivo. This is also in keeping with the earlier finding that the K_i for PP_i of the enzyme is $4.6 \cdot 10^{-6}$ M with Tris/HCl buffer and $1.1 \cdot 10^{-6}$ M with histidine buffer [1]. However, with inhibition constants of this order of magnitude, intracellular concentrations of 1.7 mM for PP_i, a known competitive inhibitor [1] and 1.3 mM for D-glucose-6-*P* (K_m, 1.9 mM), it became difficult to understand how effective inositol synthesis could occur in vivo.

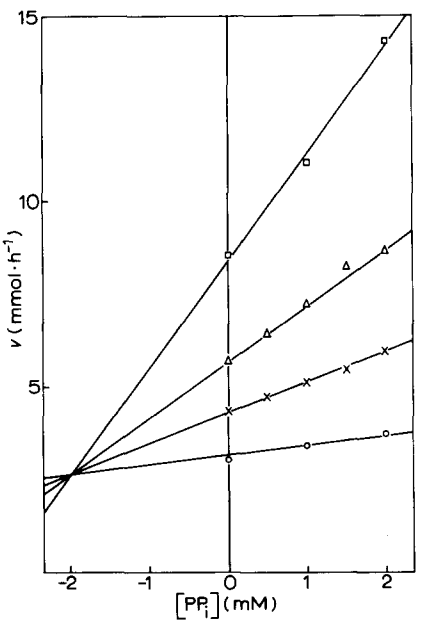

Fig. 3. K_i of PP_i for *myo*-inositol-1-phosphate synthase in the presence of Mg^{2+}. The K_i was obtained by the method described by Dixon [11]. Mg^{2+} was used at a final concentration of 6.25 mM. The concentration of D-glucose-6-*P* was varied as follows: o———o, 10 mM; x- - - - -x, 5 mM; Δ———Δ, 3.3 mM and □———□, 2.5 mM.

The reversal of PP_i inhibition of glucocycloaldolase by several cations, at pH 7.7, is evident from the results of the current study (Table I), in addition to those previously reported [1]. Mg^{2+} and Mn^{2+} in particular, effectively reversed PP_i inhibition. Under the standard conditions for enzyme assay used and at concentrations comparable to those found intracellularly, Mg^{2+} essentially reversed the inhibition evident in its absence. In addition, when enzyme reaction mixtures contained substrate, PP_i and Mg^{2+} at concentrations closely approximating intracellular levels, reversal of inhibition by the cations resulted in a marked change in the apparent K_i of approximately three orders of magnitude to $2 \cdot 10^{-3}$ M (Fig. 3). Since these experiments were conducted at higher Mg^{2+} (6.25 mM) than PP_i (2 mM) concentrations and since Mg^{2+} has a high affinity for PP_i (K_s of P_2O_7 $Mg^{2-} = 2 \cdot 10^{-6}$ [13] we can consider that the inhibitory species under these conditions is not free PP_i but $Mg_2P_2O_7$ (or some related complex) which shows a lower affinity for the enzyme than the free anion. From the examination of Fig. 3 we can see that the slopes of the lines are not linearly related to $1/S$, instead they give a parabolic relationship suggesting that the inhibitory species combines with more than one form of the enzyme.

Another factor which seems to play an important role in the in vivo regulation of this enzyme is H^+ concentration. At pH 6.13 (the value obtained from mycelium homogenates) the enzyme has a low activity and is practically insensitive to PP_i (Fig. 1), nevertheless, we can not exclude the possibility of in vivo local changes in pH which would favor both enzyme activity and PP_i modulation.

It would seem reasonable to conclude that under the prevailing intracellular cation concentrations established (Table II), regulated inositol biosynthesis occurs as a consequence of the modulation of *myo*-inositol-1-phosphate synthase activity by pH and various ions.

PP_i, a well known chelating agent, was shown to be a competitive inhibitor of *myo*-inositol-1-phosphate synthase [1]. Other chelating agents such as citrate and succinate do not inhibit this enzyme activity [1]. Another one, such as EDTA, inhibit only at high concentrations [12]. In addition, the inhibitory effect of PP_i is readily reversed by several divalent cations. However, in the current study, no direct evidence was obtained to conclude that the cations effective in reversing inhibition formed complexes with PP_i. With the available data it is not yet possible to ascertain the mechanism through which PP_i inhibits the enzyme.

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