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INORGANIC PYROPHOSPHATE–PHOSPHOHYDROLYTIC ACTIVITY IN HUMAN SERUM

CATALYTIC PROPERTIES OF THE IONIC SPECIES OF PP_i AND $MgPP_i$ COMPLEXES

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SUMMARY

The kinetics of the enzyme-catalyzed hydrolysis of inorganic pyrophosphate (PP_i) by human serum has been investigated as a function of the ionic species of PP_i and Mg at pH 9.0 (37 °C): Mg^{2+}, $MgPP_i^{2-}$, and PP_i^{4-}.

Hyperbolic activity–substrate curves are obtained when $MgPP_i^{2-}$ and PP_i^{4-} are used as substrates; the activity–substrate curve for total PP_i is sigmoid.

On the basis of experiments in which $MgPP_i^{2-}$ and PP_i^{4-} are assumed to be alternative substrates for the same site, a catalytic mechanism is proposed: PP_i^{4-} is the real substrate; Mg^{2+} does not directly modify the binding or the hydrolysis of PP_i^{4-}, but leads to a decrease in the activity through formation of $MgPP_i$ complexes. Neither Mg^{2+} nor $MgPP_i$ complexes bind to the enzyme.

INTRODUCTION

In previous experiments on the PP_i–phosphohydrolytic activity in dialysed serum from healthy, human individuals the activity on the substrate, PP_i, as well as on the modifier, Mg, gave a sigmoidal dependence curve (Hørder, M., unpublished).

Sigmoidal activity–substrate and activity–modifier curves are not necessarily indicative of multiple interacting binding sites and conformational changes in the enzyme molecule during binding of substrate and modifiers[1]. Kinetic and binding studies on the specific inorganic pyrophosphate phosphohydrolases (EC 3.6.1.1) from different sources[2–4] have demonstrated that binding of Mg by PP_i may lead to non-hyperbolic activity–substrate curves. Enzymes that require metals for catalysis, and that have substrates with chelating properties, seem to display this allosteric-like behaviour[5].

The present work was undertaken to study the PP_i–phosphohydrolytic activity

of serum in terms of free and Mg complex species of PP_i in an attempt to deduce the nature of and the catalytic properties of possible enzyme–Mg–PP_i complexes.

MATERIALS AND METHODS

Serum

Pooled serum from healthy human individuals was dialysed against four changes of 155 mM NaCl, pH 7.5 at 4 °C for 48 h.

Chemicals

A-R grade $MgSO_4 \cdot 7\ H_2O$, $Na_4P_2O_7 \cdot 10\ H_2O$ from Merck Chemical Co. and $C_4H_{11}NO_2$ from Koch and Light Laboratories were used. Fresh solutions of buffers, PP_i and Mg were made up daily in double distilled water. The pH of buffers and incubation mixtures were adjusted at 37 °C.

Determinations of enzyme activities

Two ml of incubation mixture contained 0.6 ml of serum, 50 mM $C_4H_{11}NO_2$. Concentrations of Mg, PP_i and the ionic species of these were as noted in Results. The pH during incubation at 37 °C was 9.0. The PP_i–phosphohydrolytic activity was followed by the release of inorganic phosphate[6] determined after 0, 3, 6 and 10 min of incubation to insure that zero-order kinetics were obeyed. All experiments were performed at least twice. Enzyme activities are expressed as μmoles of PP_i hydrolysed ($\equiv$ μmoles of $P_i/2$ released) per unit of time in the following unit: $((\mu moles \cdot min^{-1} \cdot g^{-1}) \times 10^3)$.

Calculations

At pH 9.0 (37 °C) the following ionic species of PP_i and Mg are in equilibrium:

$$PP_i^{4-} + Mg^{2+} \rightleftharpoons MgPP_i^{2-} \qquad (1)$$
$$K_1 = 10^{5.41}\ M^{-1}$$

$$MgPP_i^{2-} + Mg^{2+} \rightleftharpoons Mg_2PP_i^{0} \qquad (2)$$
$$K_2 = 10^{2.34}\ M^{-1}$$

The ionic, protonated species HPP_i^{3-} and $MgHPP_i^{1-}$ are negligible and can be ignored. The values of the association constants, K_1 and K_2, were taken from the literature[7] and used together with the conservation equations for Mg and PP_i to calculate the concentrations of Mg^{2+}, PP_i^{4-}, $MgPP_i^{2-}$, $Mg_2PP_i^{0}$ (refs 4 and 7).

The numerical values of K_m, inhibitor constants and V were determined arimethically by statistical treatment of data[8].

RESULTS

Enzyme activity at constant concentrations of total PP_i and total Mg

When total Mg concentrations were fixed at different values from 0.05 to 3.00 mM and the concentrations of total PP_i varied, plots of the activity against total PP_i (Fig. 1) deviated from the hyperbolic form except at very low [total Mg]. Sigmoid inhibition curves were also found when [total Mg] varied while [total PP_i]

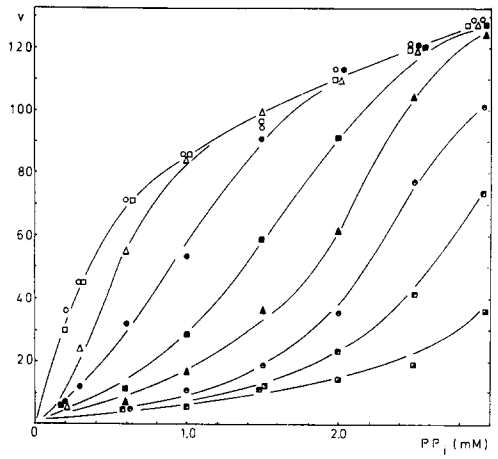

Fig. 1. The effects of the concentration of total PP_i, PP_i, on the PP_i–phosphohydrolytic activity of serum, v, ($(\mu moles \cdot min^{-1} \cdot g^{-1}) \times 10^3$) at nine different constant concentrations of total Mg: ○, 0.05 mM; □, 0.10 mM; △, 0.30 mM; ●, 0.60 mM; ■, 1.00 mM; ▲, 1.50 mM; ◓, 2.00 mM; ◪, 2.50 mM; ◪, 3.00 mM.

was constant (Fig. 2). The inhibition curves from Fig. 2 were replotted according to the Hill equation[9] in the form of $\log_{10}(V/v_i - 1)$ against $\log_{10}$ [total Mg], where V is the activity at the highest [total PP_i]:[total Mg] ratio and v_i is the activity at increasing concentrations of [total Mg]. Straight line plots were obtained at six constant concentrations of total PP_i, with slopes decreasing from 2.6 at 3.00 mM total PP_i to 0.6 at 0.6 mM total PP_i.

The enzyme activity at constant [total Mg]:[total PP_i] ratios

When the enzyme activity was determined at five different constant [total Mg]:

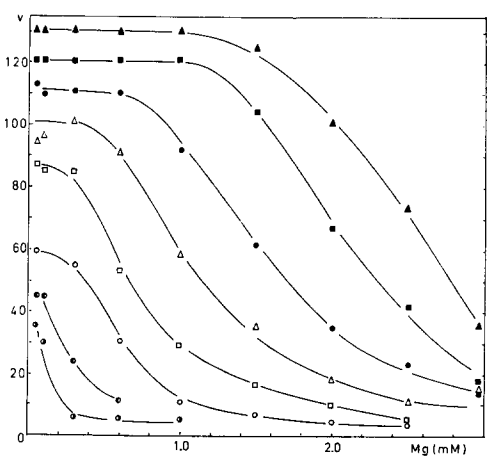

Fig. 2. The effects of the concentration of total Mg, Mg, on the PP_i–phosphohydrolytic activity of serum, v, ($(\mu moles \cdot min^{-1} \cdot g^{-1}) \times 10^3$), determined at eight different constant concentrations of total PP_i: ◑, 0.20 mM; ◐, 0.30 mM; ○, 0.60 mM; □, 1.00 mM; △, 1.50 mM; ●, 2.00 mM; ■, 2,50 mM; ▲, 3.00 mM.

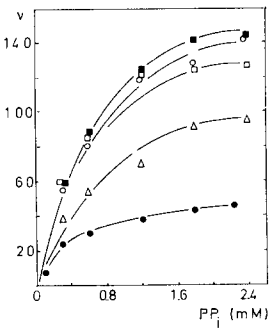

Fig. 3. The effects of the concentration of total PP_i, PP_i, on the PP_i–phosphohydrolytic activity of serum, v, ($(\mu moles \cdot min^{-1} \cdot g^{-1}) \times 10^3$), determined at five different constant [Mg]:[PP_i] ratios: ■, 1:10; □, 1:4; ○, 1:2; △, 2:3; ●, 1:1.

[total PP_i] ratios from 0.1 to 1.0, the activity–substrate ([total PP_i])curves, (Fig. 3) were hyperbolic. Double-reciprocal plots were linear, but did not intersect at a common point. The calculated values for $K_m^{[\text{total } PP_i]}$ were 1.8 mM at a [total Mg]:[total PP_i] ratio of 1.0 and decreased to 0.6 mM at a ratio of 0.1. The corresponding values for V were 76 and 155 ($(\mu moles \cdot min^{-1} \cdot g^{-1}) \times 10^3$), respectively.

The enzyme activity as a function of the concentrations of the ionic species of PP_i and Mg

(a) When determined at five constant concentrations of Mg^{2+}, and considering $MgPP_i^{2-}$ as the substrate, the activity decreased with increasing [Mg^{2+}], (Fig. 4).

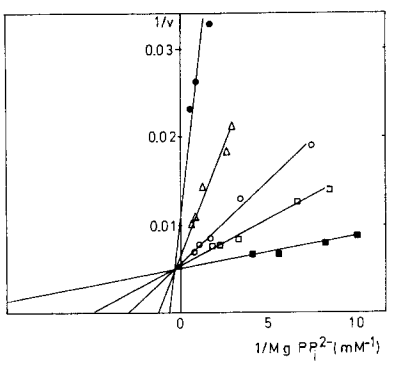

Fig. 4. The effects of the concentration of $MgPP_i^{2-}$ on the PP_i–phosphohydrolytic activity of serum, v, ($(\mu moles \cdot min^{-1} \cdot g^{-1}) \times 10^3$) plotted in the form $1/v$ against $1/[MgPP_i^{2-}]$ at five different constant concentrations of free (Mg^{2+}): ■, 0.4 μM; □, 1.1 μM; ○, 3.5 μM; △, 6.5 μM; ●, 56.0 μM.

The secondary plots[10,11]: slopes of lines against [Mg^{2+}] and intercepts of lines with the v-axis against [Mg^{2+}], were both linear and intersected the [Mg^{2+}] axis. These intercepts gave the apparent dissociation constants of [Mg^{2+}] with regard to the enzyme, $K_i^{(\text{slope})} = 0.55$ μM, and to the enzyme–$MgPP_i^{2-}$ complex, $K_i^{(\text{intercept})} = 17.5$ μM.

From the values of the apparent dissociation constants for Mg^{2+}, and of the co-ordinates of the common crossover point, ($1/v$, $1/[MgPP_i^{2-}]$), of the intersecting

lines in Fig. 4 were next calculated the apparent kinetic constants $K_m^{[MgPP_i^{2-}]} = 0.078$ mM and $V = 194$ (μmoles·min^{-1}·g^{-1}) × 10^3) (refs 10 and 11).

Plots of $\log_{10}(V/v_i - 1)$ against $\log_{10}[Mg^{2+}]$, where V is the calculated maximal activity for the hydrolysis of $MgPP_i^{2-}$, and v_i is the actual activity as a function of $[Mg^{2+}]$ at six constant concentrations of $MgPP_i^{2-}$ from 0.05 to 1.50 mM, were linear and had slopes of 0.8 to 1.0.

(b) The double-reciprocal plots, shown in Fig. 5, describe the activity as a

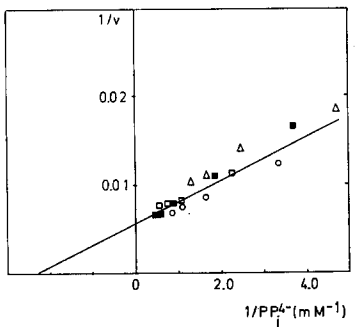

Fig. 5. The effects of the concentration of PP_i^{4-} on the PP_i–phosphohydrolytic activity of serum, v, (μmoles·min^{-1}·g^{-1}) × 10^3) plotted in the form $1/v$ against $1/[PP_i^{4-}]$ at four different constant concentrations of free Mg^{2+}: ■, 0.4 μM; □, 1.1 μM; ○, 3.5 μM; △, 6.5 μM.

function of $[PP_i^{4-}]$ at four constant concentrations of Mg^{2+}. The values for $K_m^{[PP_i^{4-}]}$ and V were calculated for each Mg^{2+} concentration and for all the points at a time; the results did not differ significantly, and were $K_m^{[PP_i^{4-}]} = 0.422$ mM (S.D. = 0.081 mM) and $V = 176$ (μmoles·min^{-1}·g^{-1}) × 10^3) (S.D. = 11).

(c) In a series of experiments $[PP_i^{4-}]$ was kept constant while $[MgPP_i^{2-}]$ was varied (Fig. 6) and in another set of experiments $[MgPP_i^{2-}]$ was constant while $[PP_i^{2-}]$ was varied, (Fig. 7). This was done by chosing appropriate concentrations of total Mg and total PP_i; it was ensured that the Mg^{2+} concentration was less than 7 μM and varied within 10% at each experimental condition.

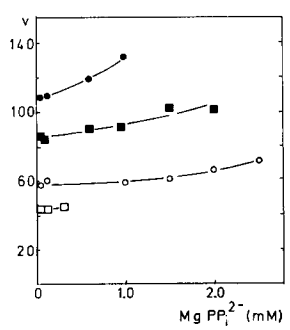

Fig. 6. The effects of the concentration of $MgPP_i^{2-}$ on the PP_i–phosphohydrolytic activity of serum, v, (μmoles·min^{-1}·g^{-1}) × 10^3) at four different constant concentrations of PP_i^{4-}: □, 0.25 mM; ○, 0.50 mM; ■, 1.00 mM; ●, 2.00 mM.

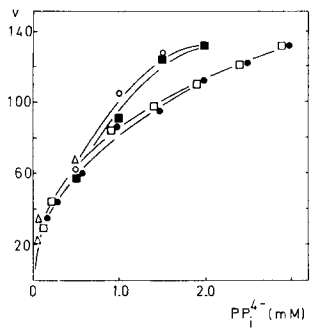

Fig. 7. The effects of the concentration of PP_i^{4-} on the PP_i–phosphohydrolytic activity of serum, v, ($(\mu moles \cdot min^{-1} \cdot g^{-1}) \times 10^3$) at five different constant concentrations of $MgPP_i^{2-}$: ●, 0.05 mM; □, 0.09 mM; ■, 1.00 mM; ○, 1.50 mM; △, 1.9 mM.

DISCUSSION

This investigation has shown, that the sigmoidal dependence of PP_i–phosphohydrolytic activity of serum at pH 9.0 (37 °C) on [total PP_i] and [total Mg] disappears if the ionic species of PP_i and $MgPP_i$ complexes are considered as substrates.

The value of 0.8 to 1.0 for the Hill-coefficient from the effect of Mg^{2+} on the activity makes it unlikely, that Mg^{2+} binding induces conformational changes in the enzyme.

The results are thus consistent with results from studies on other enzymes possessing PP_i–phosphohydrolytic activity[3,12,13], which also display sigmoid activity–substrate curves because the modifier, Mg, and substrate, PP_i, combine with each other.

If the values of the kinetic constants are taken as expressions for the ability to function as substrate it appears that the enzyme apparently has a 5-fold higher affinity for $MgPP_i^{2-}$ than for PP_i^{4-}; and since they have nearly identical values for V, they are hydrolysed at an equal rate.

The only product from the hydrolysis of $MgPP_i^{2-}$ and PP_i^{4-} will be P_i. Considering these two ionic species as alternative substrates, present in the same reaction mixture, and using the values for the kinetic constants determined for each of them, the following expression for the combined rate of hydrolysis is obtained[14]:

$$v = \frac{(V MgPP_i^{2-}/K_m MgPP_i^{2-}) \cdot [MgPP_i^{2-}] + \left(V PP_i^{4-}/K_m{}^{PP_i^{4-}}\right) \cdot [PP_i^{4-}]}{1 + [MgPP_i^{2-}]/K_m MgPP_i^{2-} + [PP_i^{4-}]/K_m{}^{PP_i^{4-}}}$$

$$= \frac{24\,871 \cdot [MgPP_i^{2-}] + 417 \cdot [PP_i^{4-}]}{1 + [MgPP_i^{2-}] \cdot 12.8 + [PP_i^{4-}] \cdot 2.3}$$

From the above it is to be expected that the combined rate of hydrolysis will be determined by the concentration of $MgPP_i^{2-}$. This assumption seems to condradict the experimental evidence, presented in the Figs 6 and 7, from which it appears that the combined rate is primarily a function of the concentration of PP_i^{4-}, when both are present at the same time.

An explanation of this discrepancy may be found in the role played by Mg.

The kinetic treatment of the inhibition studies in terms of Mg^{2+} might lead to the assumption, that Mg^{2+} is an effective, competitive inhibitor ($K_i^{slope} = K_i^{E\ Mg}$) of the binding of $MgPP_i^{2-}$, but not PP_i^{4-}, to the enzyme. However, a different and more simple mechanism may be proposed: if PP_i^{4-} is considered as the only real substrate, the role played by Mg^{2+} is to decrease the concentration of the substrate (and thus the activity) by the formation of Mg–PP_i complexes. Neither these nor Mg^{2+} will bind to the enzyme according to the mechanism proposed; consequently the apparent kinetic constants for the interaction of Mg^{2+} and $MgPP_i^{2-}$ solely reflect the formation of Mg–PP_i complexes.

The last explanation is consistent with the experimental observations and has also been suggested on the basis of ligand-binding studies[15] on other enzymes possessing PP_i–phosphohydrolytic activity. Some limitations can still be put upon the observations. The concentrations of the ionic species of PP_i and Mg are not determined, but calculated on the assumption[16] that the values of the association constants[7] for Mg–PP_i complex species, originally determined at 25 °C and at a ionic strength of 0.1, may be applied to the conditions of these experiments at 37 °C and a lower ionic strength. Other competing equilibria must also be considered; divalent cations, tightly bound to proteins, may not have been removed completely by dialysis, and may be in competition with Mg for PP_i. On the other hand Mg may bind to polyelectrolytes as proteins[17] and buffer components[18] although the association constants for such complexes are low in comparison to those of the Mg–PP_i complexes[7].

REFERENCES

1 Westley, J. (1969) *Enzymic Catalysis*, pp. 169–178, Harper and Row Publishers, New York and London
2 Josse, J. and Wong, S. C. K. (1971) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn, Vol. IV, pp. 499–541, Academic Press, New York and London
3 Rapoport, T. A., Höhne, W. E., Reich, J. G., Heitmann, P. and Rapoport, S. M. (1972) *Eur. J. Biochem.* 26, 237–246
4 Klemme, J. and Gest, H. (1971) *Proc. Natl. Acad. Sci U.S.* 68, 721–725
5 London, W. P. and Steck, T. L. (1969) *Biochemistry* 8, 1767–1779
6 Hørder, M. (1972) *Analyt. Biochem.* 49, 37–48
7 Lambert, S. M. and Watters, J. I. (1957) *J. Am. Chem. Soc.* 79, 5606–5608
8 Wilkinson, G. N. (1961) *Biochem. J.* 80, 324–332
9 Koshland, Jr, D. E. (1970) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn. Vol. I, pp. 341–396, Academic Press, New York and London
10 Cleland, W. W. (1963) *Biochim. Biophys. Acta* 67, 173–187
11 Cleland, W. W. (1963) *Biochim. Biophys. Acta* 67, 188–194
12 Butterworth, P. J. (1968) *Biochem. J.* 110, 671–675
13 Nayudu, P. R. V. and Miles, P. L. (1969) *Biochem. J.* 115, 29–35
14 Pocklington, T. and Jeffery, J. (1969) *Biochem. J.* 112, 331–334
15 Fernley, H. N. and Bisaz, S. (1968) *Biochem. J.* 107, 279–283
16 Josse, J. (1966) *J. Biol. Chem.* 241, 1948–1957
17 Pedersen, K. O. (1972) *Scand. J. Clin. Lab. Invest.* 30, 321–329
18 Hanlon, D. P., Watt, D. S. and Westhead, E. W. (1966) *Analyt. Biochem.* 16, 225–233