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INORGANIC PYROPHOSPHATE-PHOSPHOHYDROLYTIC ACTIVITY ASSOCIATED WITH HUMAN PLACENTAL ALKALINE ORTHOPHOSPHATASE

NATURE OF THE SUBSTRATE AND ROLE OF MAGNESIUM

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SUMMARY

The nature of the real substrate and the role of magnesium in PP_i-phosphohydrolysis by human placental alkaline orthophosphatase (EC 3.1.3.1) has been investigated.

The dependence of activity on the concentrations of total PP_i and total Mg^{2+} was sigmoid, whereas hyperbolic curves were obtained if PP_i^{4-} and $MgPP_i^{2-}$ each were considered as substrate.

Models for the PP_i-phosphohydrolytic mechanism, assuming PP_i^{4-} and $MgPP_i^{2-}$ as substrates, separately, as well as competitive substrates all fitted well with the experimental data.

The simple model, having PP_i^{4-} as the substrate, was the only one in which Mg^{2+} was non-inhibitory, and thus consistent with the role of Mg^{2+} in phosphomonoester hydrolysis by alkaline orthophosphatases. In proposed models with $MgPP_i^{2-}$ as the substrate, Mg^{2+} was a competitive, non co-operative inhibitor.

INTRODUCTION

It now is well established that mammalian alkaline orthophosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) possess PP_i-phosphohydrolytic activity [1]; however, the question of why Mg^{2+} stimulates the orthophosphatase activity and inhibits the associated PP_i-phosphohydrolytic activity [2] remains unanswered; neither has there been presented an unambiguous account of the catalytic roles of the equilibrium species in mixtures of Mg^{2+} and PP_i [1].

The discrepancies in interpretations partly stem from the insufficient information about the structural and catalytic effects of divalent cations on mammalian alkaline orthophosphatases; in that respect the placental enzyme is studied at greatest detail [3–5]. In previous reports [6–9] on the PP_i-phosphohydrolytic activity of human placental alkaline orthophosphatases the catalytic active reactants were assumed to be identical with the concentrations of total Mg^{2+} and total PP_i in the incubation mixtures.

The present study was therefore aimed at study the PP_i-phosphohydrolytic activity of alkaline orthophosphatase, purified from human placenta, in terms of the equilibrium species, Mg^{2+}, $MgPP_i^{2-}$, and PP_i^{4-}. Several models for the reaction mechanism have been suggested and evaluated, and a PP_i-phosphohydrolytic mechanism, that ascribed to Mg^{2+} a role compatible with its role in phosphomonoester hydrolysis, has been proposed.

MATERIALS AND METHODS

Preparation of enzyme from placenta

Fresh placentas were collected just after delivery; after perfusion of the vessels a crude homogenate was prepared from tissue slices in a Potter–Elvehjem homogenizer. The extract was purified, as previously described [10], by partial differential centrifugation, treatment with detergent, and gel filtration. During purification a constant ratio between ortho- and PP_i-phosphohydrolytic activities was found, and the specific activities of the enzymes were increased approx. 55 times.

Determination of enzyme activities

All measurements were performed at pH 9.0 and at 37 °C; detailled descriptions of the composition of reaction mixtures, conditions of incubation and of determination of the activities (μmoles of PP_i hydrolysed $\cdot min^{-1} \cdot l^{-1}$) has previously been given [11]. Calculations of the concentrations of the equilibrium species in mixtures of Mg^{2+} and PP_i from the values of the association constants for Mg^{2+}–PP_i complexes [11], and calculations of kinetic constants by statistical treatment of data [12], were performed with programs written in Fortran and implemented on the 1130 IBM computer.

RESULTS

Activities versus [total PP_i] and [total Mg^{2+}]

From the plots in Fig. 1 of activities against total Mg^{2+} concentration at different, fixed concentrations of total PP_i it appeared, that the activities decreased once

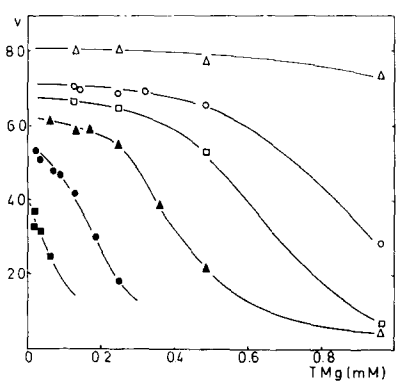

Fig. 1. The dependence of the PP_i-phosphohydrolytic activity of human placental alkaline orthophosphatase, v, (μmoles $\cdot min^{-1} \cdot l^{-1}$) on the concentration of total Mg^{2+} (TMg) determined at six constant concentrations of total PP_i (mM): ■, 0.12; ●, 0.25; ▲, 0.48; □, 0.77; ○, 0.96; △, 1.92.

the total Mg^{2+}/total PP_i concentration ratios exceeded approx. 1:2. Replots of the data were made according to the Hill equation [13] in the form of $\log_{10}(V/v_i - 1)$ vs $\log_{10}$ (total Mg^{2+} concentration), where V were the activities for total Mg^{2+} concentration going towards zero and v_i the actual activities when inhibition occurred; straight line plots were obtained with slopes decreasing from 4.3 at 0.96 mM total PP_i to 1.6 at 0.25 mM total PP_i.

Plots of activity vs total PP_i concentration at fixed concentrations of total Mg^{2+} from 0.05 mM to 1.00 mM and varying concentrations of total PP_i up to 2.00 mM were sigmoid with points of inflection at approximately equimolar total Mg^{2+}/total PP_i ratios, and showing no peaks of activities.

Activities versus the equilibrium species in mixtures of Mg^{2+} and PP_i

PP_i^{4-} *considered as substrate.* Activity vs PP_i^{4-} plots at seven fixed concentrations of free Mg^{2+}, merged into one hyperbolic curve (Fig. 2) and a similar hyperbolic curve was also obtained for five, fixed concentrations of $MgPP_i^{2-}$ from 0.03 to 0.47 mM.

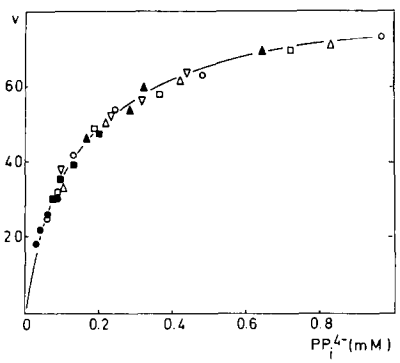

Fig. 2. The PP_i-phosphohydrolytic activity, v, (μmoles·min^{-1}·l^{-1}) as a function of the concentration of free PP_i^{4-} at seven constant concentrations of free Mg^{2+} (μM): ▽, 0.3; △, 0.5; □, 1.2; ▲, 1.8; ○, 3.4; ■, 9.5; ●, 44.

For each of the fixed concentrations of free Mg^{2+} and of $MgPP_i^{2-}$ the connected values of activity and concentration of PP_i^{4-} were utilized to estimate the apparent kinetic constants; the obtained values were of the same order of magnitude for each of the 12 set of data; the mean values were not significantly different and the following common means were determined: $K_m^{PPi4-} = 143\ \mu M$ and $V^{PPi4-} = 85$ (μmoles·min^{-1}·l^{-1}).

$MgPP_i^{2-}$ considered as substrate. Activity vs $MgPP_i^{2-}$ concentration plots at fixed concentrations of free Mg^{2+} (Fig. 3) indicated that free Mg^{2+} was an inhibitor. Replots [14] of the slopes and intercepts of the double reciprocal plots in Fig. 3 against free Mg^{2+} are presented in Fig. 4. From Figs 3 and 4 emerged the following apparent kinetic constants for $MgPP_i^{2-}$ as the substrate and Mg^{2+} as a competitive inhibitor:

$K_m^{MgPPi2-} = 4.1\ \mu M$, $V^{MgPPi2-}$ (uninhibited) = 85 (μmoles·min^{-1}·l^{-1}) and $K_i^{Mg2+} = 0.12\ \mu M$.

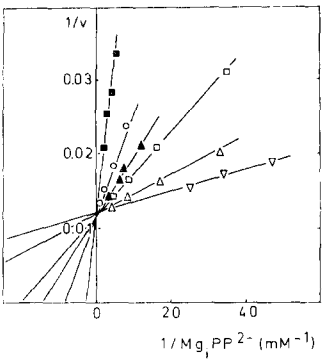

Fig. 3. Double-reciprocal plot of the effects of the concentration of the complex $MgPP_i^{2-}$ on the PP_i-phosphohydrolytic activity, v, (μmoles·min^{-1}·l^{-1}) at six constant concentrations of free Mg^{2+} (μM): ▽, 0.3; △, 0.5; □, 1.2; ▲, 1.8; ○, 3.4; ■, 9.6.

Secondary plots according to the Hill equation [13] in the form of $\log_{10}(V/v_i - 1)$ vs $\log_{10}$ (Mg^{2+} concentration), where V is the apparent $V^{MgPP_i^{2-}}$ (uninhibited) and v_i the actual activities as a function of Mg^{2+} concentration at five constant concentrations of $MgPP_i^{2-}$ from 0.03 to 0.47 mM, were linear with a mean slope of 0.82 (S.D., 0.11).

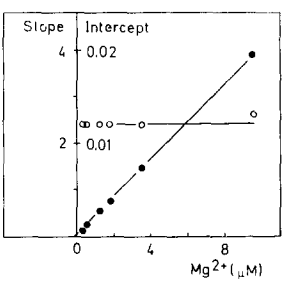

Fig. 4. Replots, based on the data of the double reciprocal plots in Fig. 3, showing slopes (●) and intercepts with $1/v$-axis (○) versus free Mg^{2+}.

The fit of the experimental data to models for PP_i-phosphohydrolysis

Two different reaction mechanisms, Models A and B, were suggested above; the rate laws for these, in which either PP_i^{4-} or $MgPP_i^{2-}$ was the substrate, are given in Table I. From the rate laws and from the values of the kinetic constants, determined together with the establishment of the models, were calculated the activities as functions of the concentration of total Mg^{2+} at one fixed concentration of total PP_i. The results, which appear in Fig. 5, represent a qualitative comparison of the activities, calculated on basis of the assumed reaction mechanisms, with the concentrations of the equilibrium species; the figure demonstrates the direct conformity of the activity profile for Model A with the profile for the concentration of PP_i^{4-}, and also the necessity of free Mg^{2+} as an inhibitor in Model B, having $MgPP_i^{2-}$ as the substrate.

TABLE I

RATE LAWS AND ROLES OF REACTANTS IN THE PROPOSED MODELS FOR PP_i-PHOSPHOHYDROLYSIS

The rate laws, v_t, express the expected total activities and are derived from the equations below, in which PP_i^{4-} and $MgPP_i^{2-}$ are considered separately as the substrates:

$$v_{PP4-} = \frac{V^{PP4-}}{1 + K_m^{PP4-}/[PP^{4-}]}$$

$$v_{MgPP2-} = \frac{V^{MgPP2-}}{1 + K_m^{MgPP2-}/[MgPP^{2-}]\,(1 + [Mg^{2+}]/K_i^{Mg2+})}$$

$$v'_{PP4-} = \frac{V^{PP4-}}{1 + K_m^{PP4-}/[PP^{4-}]\,(1 + [MgPP^{2-}]/K_m^{MgPP2-})}$$

$$v'_{MgPP2-} = \frac{V^{MgPP2-}}{1 + K_m^{MgPP2-}/[MgPP^{2-}]\,(1 + [PP^{4-}]/K_m^{PP4-})}$$

$$v''_{PP4-} = \frac{V^{PP4-}}{1 + K_m^{PP4-}/[PP^{4-}]\,(1 + [MgPP^{2-}]/K_m^{MgPP2-} + [Mg^{2+}]/K_i^{Mg2+})}$$

$$v''_{MgPP2-} = \frac{V^{MgPP2-}}{1 + K_m^{MgPP2-}/[MgPP^{2-}]\,(1 + [PP^{4-}]/K_m^{PP4-} + [Mg^{2+}]/K_i^{Mg2+})}$$

Model	Rate law*	Roles of equilibrium species of Mg^{2+} and PP_i
A	$v_t = v_{PP4-}$	PP_i^{4-} is active substrate: no catalytic role of Mg^{2+} and $MgPP_i^{2-}$
B	$v_t = v_{MgPP2-}$	$MgPP_i^{2-}$ is active substrate: Mg^{2+} a competitive inhibitor. PP_i^{4-} has no catalytic role.
I	$v_t = v'_{PP4-} + v'_{MgPP2-}$	PP_i^{4-} and $MgPP_i^{2-}$ are both active substrates, competing for the same site. Mg^{2+} has no catalytic role.
II	$v_t = v'_{PP4-} + v''_{MgPP2-}$	PP_i^{4-} and $MgPP_i^{2-}$ are both substrates and binds to different conformational forms of the enzyme that are in rapid equilibrium. Mg^{2+} is an inhibitor, but competes only with $MgPP_i^{2-}$.
III	$v_t = v''_{PP4-} + v''_{MgPP2-}$	PP_i^{4-} $MgPP_i^{2-}$, and Mg^{2+} compete for the same site on the enzyme. PP_i^{4-} and $MgPP_i^{2-}$ are alternative substrates, and Mg^{2+} is a competitive inhibitor.
IV	$v_t = v''_{MgPP2-}$	PP_i^-, $MgPP_i^{2-}$, and Mg^{2+} compete for the same site on the enzyme. $MgPP_i^{2-}$ is the only active substrate.

The consistence of both models with the experimental data also appear from the figure. The possibility that PP_i^{4-} and $MgPP_i^{2-}$ were hydrolysed simultaneously could not, on basis of the experimental data, be precluded; the rate laws for such reaction mechanisms are given in Table I. To evaluate the different models in a quantitative manner experiments were performed having total Mg^{2+}/total PP_i concentration ratios from 0.2 to 2.0; from the rate laws in Table I, from the values of the kinetic constants, and from the concentrations of the equilibrium species the activities at reaction conditions similar to those used in experiments were calculated. The results obtained by experiments were compared with the calculated activities in Table II; a meaningfull convergence was found with all models except the one, in which free Mg^{2+} had no inhibitory role, if $MgPP_i^{2-}$ was one of the substrates.

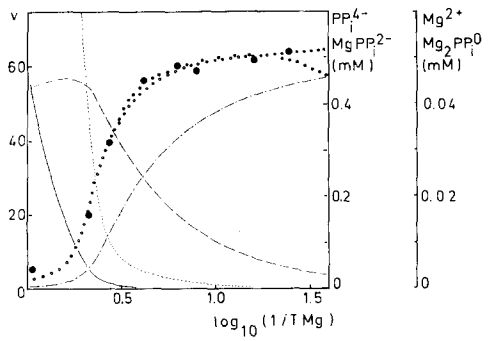

Fig. 5. Comparison of the variation in the concentration of the equilibrium species in mixtures of Mg^{2+} and PP_i with the measured PP_i-phosphohydrolytic activity, v, (●) and with activities calculated from the rate laws of Model A (small open circles) and Model B (small closed circles) all as function of total Mg^{2+} concentration, (TMg) (mM). Concentration of total PP_i was 0.48 mM. The concentrations indicated with the species on the right axis refer to the following lines: Mg^{2+}, (· · · · · ·): $Mg_2PP_i^0$; (———); $MgPP_i^{2-}$, (— — —); PP_i^{4-}, (— · —).

TABLE II

A COMPARISON OF THE ACTIVITIES DETERMINED IN EXPERIMENTS (v_e) WITH THE ACTIVITIES CALCULATED FROM THE RATE LAWS OF THE PROPOSED MODELS (v_t)

Number of values used for calculations were 36. The experimental values (v_e): mean 51 (μmoles · min^{-1} · l^{-1}), S.D. = 18, range 14–81. S.E. (1) is the standard error of the coefficient of regression; S.E. (2) is the standard error of estimate in the v_t direction; r^2 is the coefficient of determination.

Model	r^2	Regression equation	S.E. (1)	S.E. (2)
A	0.981	$v_t = 0.99\ v_e + 0.59$	0.023	2.52
B	0.977	$v_t = 0.96\ v_e + 2.66$	0.027	2.87
I	0.559	$v_t = -0.35\ v_e + 97.41$	0.053	5.64
II	0.959	$v_t = 0.95\ v_e + 6.97$	0.033	3.56
III	0.975	$v_t = 0.96\ v_e + 4.32$	0.026	2.85
IV	0.919	$v_t = 0.81\ v_e + 6.51$	0.041	4.45

DISCUSSION

This investigation has shown that the dependence of the PP_i-phosphohydrolytic activity, associated with human placental alkaline orthophosphatase, on total PP_i and on total Mg^{2+} was sigmoid; it was shown too, that the assumption of the equilibrium species, Mg^{2+}, $MgPP_i^{2-}$ and PP_i^{4-}, as the catalytically active reactants led to a hyperbolic reaction mechanism. This behaviour has been found in connection with enzyme reactions in which a modifier and a substrate combine with the enzyme and with each other [15–17].

The proposed reaction mechanisms were consistent with the experimental results if PP_i^{4-} and $MgPP_i^{2-}$ each was taken as the substrate, and also if the two species were considered as competing substrates. The analysis has shown that free Mg^{2+} had no catalytic role if PP_i^{4-} was the only substrate; otherwise free Mg^{2+} modified the binding of the alternative substrate, $MgPP_i^{2-}$, to the enzyme, acting as a competitive, non co-operative inhibitor. The testing of the models showed, that the first-mentioned,

simple mechanism, having PP_i^{4-} as the only substrate, was as likely as more complicated models. Previously PP_i^{4-} has been suggested as the substrate for PP_i-phosphohydrolytic activities of bovine brain [18] and calf intestinal [19] alkaline orthophosphatases. The further kinetic studies of PP_i^{4-} as the substrate reported here, showed that free Mg^{2+} had no direct catalytic role; this finding seems to be in accordance with results showing, that the effects of Mg^{2+} in phosphomonoester hydrolysis by placental [3] and brain [20] alkaline orthophosphatases were chiefly structural.

Support for $MgPP_i^{2-}$ as the substrate and Mg^{2+} as an inhibitor has come from kinetic interpretation of PP_i-phosphohydrolytic reactions of pig kidney [17] and mouse duodenal [21] alkaline orthophosphatases, although these studies did not test the possibility of PP_i^{4-} as the substrate. The recognition of $MgPP_i^{2-}$ as the substrate for the specific $MgPP_i$-phosphohydrolases (EC 3.6.1.1) could seem to support the mechanism of Model B too, but for the rate increasing effects of free Mg^{2+} in these reactions [16]. Certain reservations in connection with the above interpretations should be made. Although a model with PP_i^{4-} as the substrate seems attractive due to its simplicity and the good fit of experimental data to the model this does not exclude the possibility of more complicated mechanisms; therefore preference for any single of the proposed models should await further evaluation of their validities. The values of the kinetic constants employed to predict the activities in Models no I–III, having PP_i^{4-} and $MgPP_i^{2-}$ as competing substrates, were assumed to be the same as the values determined in Models A and B, having the species as substrates separately; determinations of the values of the kinetic constants by additional experimental procedure [16] is therefore needed.

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