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COMPETITION OF INORGANIC PYROPHOSPHATE AND A PHOSPHOMONOESTER AS SUBSTRATES FOR HUMAN PLACENTAL ALKALINE ORTHOPHOSPHATASE

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SUMMARY

The probability that free PP_i^{4-} and the complex $MgPP_i^{2-}$ are substrates in PP_i phosphohydrolysis by human placental alkaline orthophosphatase (EC 3.1.3.1) has been investigated.

Experiments were performed in which the concentration of Mg, PP_i, and 4-nitrophenylphosphate varied. The hydrolysis of the phosphorylated compounds was determined, both separately and combined. The results were interpreted in terms of the equilibrium species of mixtures containing Mg and PP_i.

It was found that the PP_i species and the phosphomonoester competed as substrates for the same binding site, and that free PP_i^{4-} would be the only PP_i species consistent with all experimental data.

Independent evidence for free PP_i^{4-} as the real substrate was the identity between the value of K_m for the hydrolysis of free PP_i^{4-} and the value of K_i for free PP_i^{4-} as an ordinary, competitive inhibitor of 4-nitrophenylphosphate hydrolysis.

Apparently free Mg^{2+} did not influence the binding of the substrates, 4-nitrophenylphosphate and free PP_i^{4-}, to the enzyme.

INTRODUCTION

Preparations of alkaline orthophosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from human placenta possess PP_i phosphohydrolytic activity [1, 2]. The involvement of a single, bifunctional enzyme may be questioned, however, because Mg enhances phosphomonoester hydrolysis [4–7] but apparently inhibits PP_i phosphohydrolysis [2, 8].

The consideration of the equilibrium species in mixtures of Mg and PP_i, free PP_i^{4-} and $MgPP_i^{2-}$, as the actual substrate showed [8], that free Mg^{2+} would have no influence on the hydrolysis of PP_i^{4-} and would strongly inhibit $MgPP_i^{2-}$ hydrolysis. It was not possible to exclude any of the two species as the actual substrate [8].

The purpose of the present paper was to provide evidence, that free PP_i^{4-} is the actual substrate for PP_i phosphohydrolysis by human placental alkaline orthophosphatase. This was done by mixed substrate experiments in which a phosphomonoester 4-nitrophenylphosphate, was found to compete with only one kind of PP_i species.

MATERIALS AND METHODS

Preparation of enzyme [1] and determination of PP_i phosphohydrolytic activity [8–10] was performed as previously described.

The expressions for enzyme activities were in μmoles of substrate hydrolysed per min (μmoles·min^{-1}·l^{-1}) on basis of the release of 4-nitrophenol (d[4NP]/dt) and inorganic orthophosphate (d[P_i]/dt). There was no indication of transphosphorylation (i.e. (d[4NP]/dt) = (d[P_i]/dt)) with 4-nitrophenylphosphate as the only substrate. The hydrolysis of 4-nitrophenylphosphate, $v_{(4NPP)}$, was expressed by (d[4NP]/dt) and also by (d[P_i]/dt) if 4-nitrophenylphosphate was the only substrate. The hydrolysis of PP_i, $v_{(PP_i)}$, was expressed as (d[P_i]/dt)/2 if PP_i was the only substrate, and in mixtures of substrate as ((d[P_i]/dt)−(d[4NP]/dt))/2. The combined, total hydrolysis of the two substrates at the same time, v_t, was derived from ((d[4NP]/dt) + (d[P_i]/dt))/2.

RESULTS

The dependence of the individual activities on $[Mg]_t$

The hydrolysis of 4-nitrophenylphosphate was determined at five different, constant concentrations from 0.08 to 0.64 mM. At 0.06 mM of $[Mg]_t$ the activities were slight larger than if Mg was not added. A further increase in $[Mg]_t$ up to 0.48 mM had no effect on the activities.

The previous (ref. 8, Fig. 1) sigmoidal decrease in PP_i phosphohydrolytic activity when the $[Mg]_t/[PP_i]_t$ ratio exceeded 1/2 was reconfirmed.

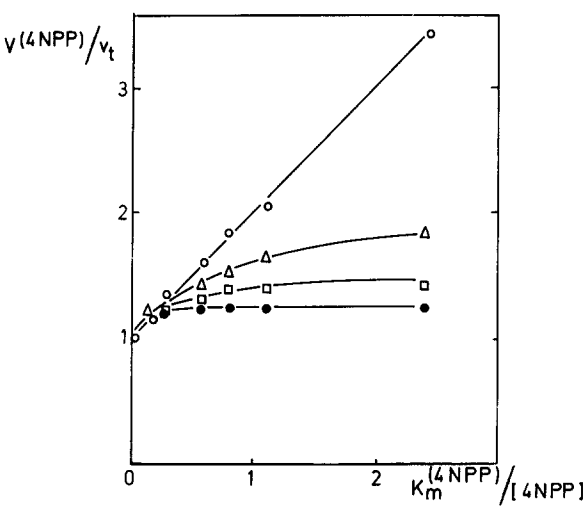

Fig. 1. Effects on the combined activity, v_t, of altering the concentration of 4-nitrophenylphosphate, [4NPP], at various concentrations of total PP_i. The results are given as a double-reciprocal plot, and are normalised, i.e. the activities were couched in fractions of V, and the substrate concentrations in multiples of K_m. The V and K_m values were determined with each series of experiments. Magnesium was not added. The fixed concentrations of total PP_i were (mM): ○, zero; △, 0.12; □, 0.25; ●, 0.35; corresponding to the normalised form ($[PP_i]_t/K_m^{PP_i}$): ○, zero; △, 1.0; □, 2.1; ●, 2.9.

Activities as functions of the concentrations of the substrates, separately and combined

The kinetic parameters, K_m and V, for the hydrolysis of 4-nitrophenylphosphate, were independent of the presence and concentrations of Mg, Table I. K_m values for the hydrolysis of PP_i determined without Mg added were about double the values found at a $[Mg]_t/[PP_i]_t$ ratio of 1/2, whereas V was not influenced by Mg.

TABLE I

COMPILATION OF V AND K_m FOR THE HYDROLYSIS OF PP_i AND 4-NITROPHENYLPHOSPHATE BY HUMAN PLACENTAL ALKALINE ORTHOPHOSPHATASE

Reaction studied		V (μmoles·min^{-1}·l^{-1})		K_m (mM)		n***
Substrate*	Magnesium**	mean	S.D.	mean	S.D.	
PP_i	−	112	11	0.118	0.017	4
PP_i	+	107	16	0.242	0.014	5
4-Nitrophenylphosphate	−	137	23	0.148§	0.029	3
4-Nitrophenylphosphate	+	136	18	0.135§	0.025	9

* Range of concentrations (mM): total PP_i: 0.12–0.96; 4-nitrophenylphosphate: 0.08–1.28.

** Concentrations of total magnesium: (−), not added approximately equal to zero; (+), 0.06–0.48 mM, keeping a $[Mg]_t$: $[PP_i]_t$ ratio of 1:2.

*** n, number of determinations.

§ No difference ($P > 0.02$) between mean values by the rank sum test.

The simultaneous hydrolysis of the two substrates was estimated in Fig. 2 without Mg added, and at a $[Mg]_t/[PP_i]_t$ ratio of 1/2 in Fig. 3. The lines connecting the experimental data, v_t vs 4-nitrophenylphosphate plots, intersected in a single point in both figures. The values of the co-ordinates, that would be expected if the two phos-

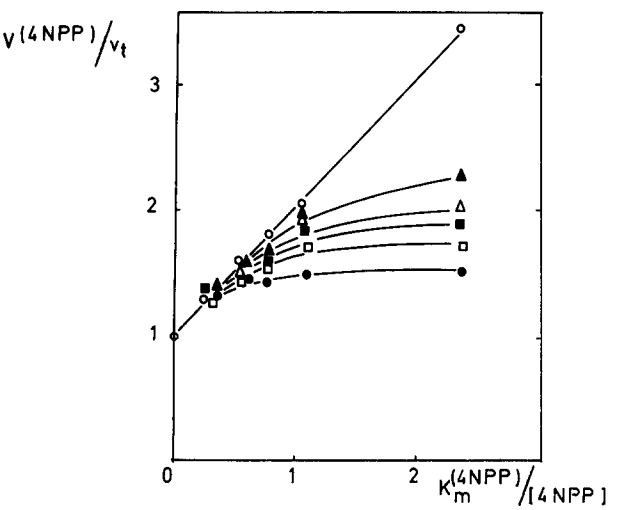

Fig. 2. Effects on the combined activity, v_t, of altering the concentration of 4-nitrophenylphosphate, [4NPP], at various concentrations of total PP_i and at a $[Mg]_t/[PP_i]_t$ ratio of 1/2. The results were, as in Fig. 1, normalised and plotted in a double-reciprocal form. The concentrations of total PP_i were, (mM): ○, zero; ▲, 0.12; △, 0.25; ■, 0.35; □, 0.48; ●, 0.96. Corresponding to the normalised form ($[PP_i]_t/K_m^{PP_i}$): ○, zero; ▲, 0.5; △, 1.1; ■, 1.5; □, 2.0; ●, 4.0.

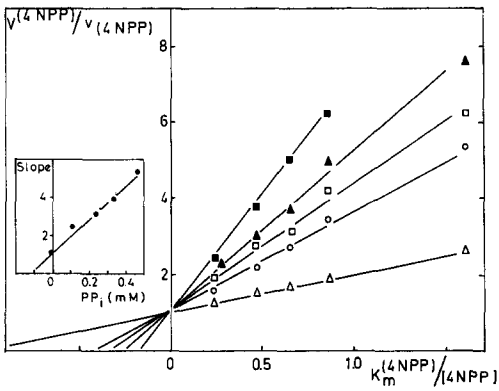

Fig. 3. Double-reciprocal, normalised plot of the activity of 4-nitrophenylphosphate hydrolysis, $v_{(4NPP)}$, against substrate concentration, [4NPP] at five constant concentrations of total PP_i (mM): △, zero; ○, 0.12; □, 0.25; ▲, 0.35; ■, 0.48. Magnesium was not added. Inserted, a secondary plot showing the slopes of the lines in the primary plot against concentrations of total PP_i.

phorylated compounds in the mixture were alternative competing substrates, were (y, x) = (1.3, 0.3). These values, that were in agreement with the experimental data in Figs 2 and 3, were calculated from the kinetic parameters for the hydrolysis of each substrate, given in Table I [11, 12].

TABLE II

RATES OF CATALYSIS OF 4-NITROPHENYLPHOSPHATE AND PP_i, INDIVIDUALLY AND COMBINED, BY HUMAN PLACENTAL ALKALINE ORTHOPHOSPHATASE

Calculated values were determined by substituting kinetic parameters, determined in the same series of experiments and of the order of magnitude given in Table I, into the equation for combined activity with alternative, competing substrates for the same enzyme [11, 12]. The experimental values of the rates of consumption of the substrates, combined and individually, were based on determinations of the release of 4-nitrophenol and inorganic orthophosphate, and expressed as indicated in Materials and Methods. The concentration of 4-nitrophenylphosphate, 0.64 mM, was chosen on the basis of the X-axis co-ordinate of the cross-over points in Figs 2 and 3: 0.26→ [4NPP] ≧ K_m^{4NPP}/0.26 equals 0.6 mM. (See text).

Concentrations of reactants			Rates of catalysis (μmoles·min^{-1}·l^{-1})	
4-Nitrophenyl-phosphate (mM)	Total PP_i (mM)	Mg^{2+} (mM)	Calculated values	Experimental values
0	0.48	0	88	92
0.64	0.48	0	111	99
0.64	0	0	112	103
0	0.48	0.24	74	72
0.64	0.48	0.24	111	104
0.64	0	0.24	112	109
0	0.96	0	98	99
0.64	0.96	0	111	97
0.64	0	0	112	103
0	0.96	0.48	88	92
0.64	0.96	0.48	111	104
0.64	0	0.48	112	107

The X-axis co-ordinate of the crossover point signified that concentration of 4-nitrophenylphosphate at which the combined hydrolysis of the two substrates should no longer depend on the concentration of the PP_i species. This follows from the assumption [11, 12] that the two substrates competed for a single binding site. This was the case, as it appeared from results of the experiments described in Table II.

The effects of the equilibrium species in mixtures of Mg and PP_i on the hydrolysis of 4-nitrophenylphosphate

The hydrolysis of 4-nitrophenylphosphate, in the presence of constant concentrations of PP_i, was followed by the release of 4-nitrophenol. Without added Mg, Fig. 3, and also at a $[Mg]_t/[PP_i]_t$ ratio of 1/2, Fig. 4, an increase in $[PP_i]_t$ led to a decreased rate of 4-nitrophenylphosphate hydrolysis. The features of the double-reci-

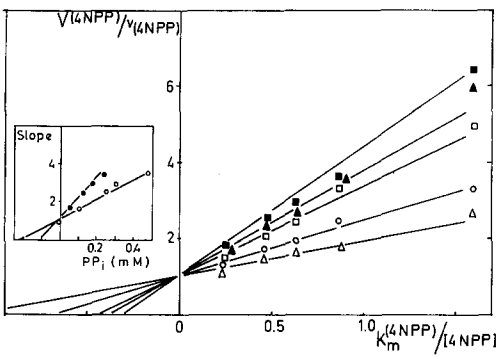

Fig. 4. Double-reciprocal, normalised plot of the activity of 4-nitrophenylphosphate hydrolysis, $v_{(4NPP)}$, against substrate concentration, [4NPP], at the same five constant concentrations of total PP_i as in Fig. 3, but at a $[Mg]_t/[PP_i]_t$ ratio of 1/2. The inserted secondary plot shows the slopes of lines against the concentration of total PP_i (closed circles) and against the calculated concentrations of free PP_i^{4-} (open circles).

procal plots were compatible with the presence of a competitive inhibitor. The numerical values of the inhibition constants were equal to the X-axis intercepts in the inserted, secondary plots [13]. At pH 9 and without added Mg, as in Fig. 3, the only PP_i species present would be free PP_i^{4-}. The K_i of this species was 0.1 mM. A K_m value of the same order of magnitude was previously found for the hydrolysis of PP_i using identical conditions (Table I). The value of K_i, considering the total PP_i as an inhibitor, was about 0.25 mM, Fig. 4. If free PP_i^{4-} was assumed to be the actual inhibitor, the K_i value would be 0.12 mM. From Table I it appeared, that the K_m for the hydrolysis of PP_i at a $[Mg]_t/[PP_i]_t$ ratio of 1/2 was 0.24 mM, which corresponds to a K_m value for free PP_i^{4-} of 0.12 mM.

DISCUSSION

The interpretation of the mixed substrate experiments showed, that 4-nitrophenylphosphate and PP_i were mutual, competitive substrates for the enzyme. Concordant reports of studies, in which varieties of mixed substrate experiments were

performed, have been compiled for kidney and intestinal alkaline orthophosphatases from animal sources [14–16] and from man [17].

Consideration of the equilibrium species in mixtures of Mg and PP_i as the PP_i substrate species in competition with a phosphonoester has apparently not been performed in any previous study using mixed substrates. That free PP_i^{4-} and not $MgPP_i^{2-}$ was the PP_i-substrate species became apparent from the identical co-ordinates of the cross-over points in the experiments described in Figs 2 and 3. In the former the only PP_i species present would be free PP_i^{4-} because Mg was not added.

Independent evidence for free PP_i^{4-} as the real substrate was the result of the consideration of this species as an ordinary, competitive inhibitor of the hydrolysis of 4-nitrophenylphosphate. It was found, that the K_i values were similar to the K_m values for the hydrolysis of free PP_i^{4-}.

It appeared, that the roles of Mg^{2+} in the hydrolysis of PP_i and of 4-nitrophenyl phosphate were identical. Consistent with the non-catalytic role of free Mg^{2+} in the hydrolysis of free PP_i^{4-} previously described [8] it was found that Mg did not influence the kinetic parameters of phosphomonoester hydrolysis. In this connection should be noted, that with forms of alkaline orthophosphatase that are strongly activated by Mg [18], experiments like the present may be difficult to perform. The high affinity of PP_i for Mg [19] will prevent the concomitant existence of saturating levels of the real substrate, free PP_i^{4-}, and the potential activator, free Mg^{2+}.

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