Kinetic Model for the Action of the Inorganic Pyrophosphatase from *Streptococcus faecalis*

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ABSTRACT: Kinetic studies of the less active form of *Streptococcus faecalis* inorganic pyrophosphatase (EC 3.6.1.1), together with computational analysis, indicated that cooperativity in ligand binding contributes in a significant way to the behavior of this enzyme. The simplest model applicable to our data was a Monod–Wyman–Changeux-type, allosteric model, in which the enzyme is proposed to exist in two states, referred to as R and T states, respectively. In the absence of ligands, 94% of the enzyme was in the T state. $MgPP_i^{2-}$ was the only substrate for the enzyme in the R form. This substrate was bound equally well by both enzyme forms, but it was hydrolyzed 5 times more efficiently by the R form than it was by the T form. Mg_2PP_i was bound exclusively to the T state of the enzyme, and it was hydrolyzed 25% as rapidly as $MgPP_i^{2-}$ by the T form. Mg_2PP_i inhibited the hydrolysis of the more efficient substrate, $MgPP_i^{2-}$, by competing with $MgPP_i^{2-}$ for the enzyme in the T form and by shifting the R $\rightleftharpoons$ T equilibrium in favor of the T form. Mg^{2+} stabilized the R state, thus activating the hydrolysis of $MgPP_i^{2-}$ and inhibiting that of Mg_2PP_i.

Inorganic pyrophosphatase (EC 3.6.1.1; hereafter referred to as PPase) is a ubiquitous enzyme that catalyzes the hydrolysis of inorganic pyrophosphate to orthophosphate. It is highly efficient as a catalyst, enhancing the reaction rate by about 10^{10}-fold (Cooperman, 1982). The structural and kinetic properties of various microbial PPases have been actively studied (Josse & Wong, 1971; Unemoto et al., 1973; Schreier & Höhne, 1978; Klemme, 1976; Randahl, 1979; Cooperman, 1982). We have recently shown that inorganic pyrophosphatase of *Streptococcus faecalis* is regulated primarily at the activity level (Lahti & Heinonen, 1981a). The intracellular ratio of reduced glutathione to oxidized glutathione modulates in vivo activity (Lahti & Suonpää, 1982). The enzyme is a tetramer (Lahti & Niemi, 1981). It exists in two interconvertible forms that differ in activity. During the exponential phase of growth almost all of the enzyme is in a high-activity form, whereas during the stationary phase there are about equal amounts of a highly and a less active form (Lahti & Heinonen, 1981a). In vitro the highly active enzyme form is spontaneously converted to the less active form, but this change can be prevented and reversed by compounds containing thiol groups (Lahti & Heinonen, 1981b; Lahti, 1983).

The kinetics of *S. faecalis* PPase has not been previously studied. Because the simple kinetic models presented for PPases so far failed to describe our results with the less active form of *S. faecalis* PPase, we derived rate equations for allosteric PPases. The simplest model compatible with our results assumes that the enzyme exists in two different conformations. In this model, both $MgPP_i^{2-}$ and Mg_2PP_i are substrates, the latter being less reactive than the former. We propose that Mg^{2+} is an activator for $MgPP_i^{2-}$ hydrolysis and inhibits Mg_2PP_i hydrolysis.

Materials and Methods

Streptococcus faecalis ATCC 8043 was grown and the inorganic pyrophosphatase purified to homogeneity as described by Lahti & Niemi (1981). *S. faecalis* PPase is converted to the stable, less active form by incubation at 37 °C (Lahti & Heinonen, 1981b). Before each experiment the enzyme was preincubated for 2.5 h at 37 °C. Activity was measured at 25 °C in 0.05 M piperazine/HCl buffer, pH 9.1, containing 0.05 M tetramethylammonium chloride. The activity was determined by measuring the liberation of labeled phosphate from [^{32}P]pyrophosphate as described by Heinonen (1970). After stopping the reaction with trichloroacetic acid, "nonradioactive" pyrophosphate was added as a carrier to the assay mixture (final concentration 6 mM) to ensure efficient precipitation of unreacted pyrophosphate. Each assay was performed in duplicate. In no case was more than 15% of the starting PP_i hydrolyzed during the reaction. The stock solution of $MgCl_2$ was titrated with 8-hydroxyquinoline (Guibault, 1973) to establish the exact concentration.

In a solution of Mg^{2+} and pyrophosphate, the following molecular species occur at pH 9.1: Mg^{2+}, PP_i^{4-}, $MgPP_i^{2-}$, and Mg_2PP_i. The concentrations of these species were calculated for each assay mixture as described by Rapoport et al. (1972) on a DEC system-20 computer.

In the computational studies each kinetic model was handled by a program that calculated the reaction rates predicted by each model and its gradient at a given point. The equation was fitted to our experimental data by numerically minimizing the residual square (abbreviated as RSQ). The method of Davidson, Fletcher, and Powell [referred to as DFP method; see Rao (1979)] for determining the minimum value of RSQ was modified to accomodate those cases in which the Hess matrix was not positive definite. When the iteration had to be interrupted in a narrow valley before the RSQ minimum was found, we used, after the DFP iteration, the steepest descent method [see Rao (1979)], because under these conditions the modified DFP method did not in every step decrease to the bottom of the valley. The programs were written with Simula 67, and they were run in the DEC system-20.

Results

Free pyrophosphate (PP_i^{4-}) is a strong inhibitor of several microbial PPases (Josse, 1966; Klemme & Gest, 1971; Klemme 1976; Lahti & Raudaskoski, 1983). At 3 mM $[PP_i]_{tot}$ the activity of the less active form of *S. faecalis* PPase (Lahti & Heinonen, 1981a) was still about 91% of that observed at 0.5 mM $[PP_i]_{tot}$ when $[Mg]_{tot}$ was 0.5 mM. Furthermore, there was no activity in the absence of divalent cations (Lahti & Niemi, 1981). Hence, it is obvious that PP_i^{4-} is a very weak inhibitor that cannot serve as a subsrate by *S. faecalis* PPase.

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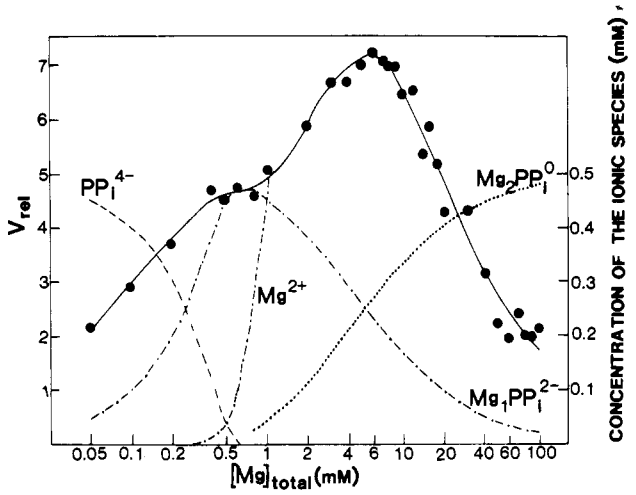


FIGURE 1: Dependence of the activity of the less active form of *S. faecalis* PPase on the concentrations of ionic species in the reaction mixture as a function of $[Mg]_{tot}$ at constant (0.5 mM) $[PP_i]_{tot}$: (●) rate measurements; (dashed lines) variation in the concentrations of different ionic species; (solid line) theoretical curve calculated according to the model 1F (Table I) with constants shown in Table III.

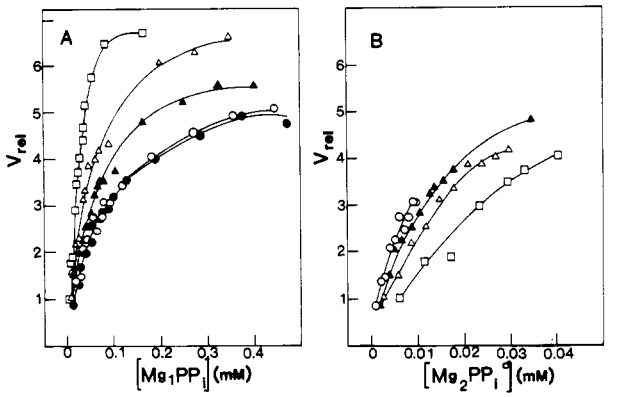


FIGURE 2: Activity of the less active form of *S. faecalis* PPase as a function of $[MgPP_i^{2-}]$ (A) and $[Mg_2PP_i]$ (B) at various concentrations of free Mg^{2+}. $[Mg^{2+}]$ (mM): (●) 0.15; (○) 0.5; (▲) 1.0; (△) 2.0; (□) 6.5.

The measured enzyme activity and the calculated concentrations of Mg^{2+}, PP_i^{4-}, $MgPP_i^{2-}$, and Mg_2PP_i at pH 9.1 are shown in Figure 1 as a function of $[Mg]_{tot}$ at constant $[PP_i]_{tot}$. Significant activities (up to about 60% of maximal activity) were observed under conditions where no free Mg^{2+} (and Mg_2PP_i) was present ($[Mg]_{tot} \leq 0.5$ mM).[1] In this case it is clear that activation by free Mg^{2+} is not essential for the reaction to occur, in contrast to inorganic pyrophosphatases of yeast (Moe & Butler, 1972; Rapoport et al., 1972) and *Vibrio alginolyticus* (Unemoto et al., 1973).

Maximal activity was obtained when the $[Mg]_{tot}/[PP_i]_{tot}$ ratio was about 13 (Figure 1), indicating that the enzyme is probably activated by free Mg^{2+}. Further increases in $[Mg]_{tot}$ sharply reduced the activity; at the same time, the concentration of $MgPP_i^{2-}$ decreased, and that of Mg_2PP_i increased (Figure 1), suggesting that Mg_2PP_i might be a less reactive substrate than $MgPP_i^{2-}$.[2]

Table I: Models and Submodels Tested for *S. faecalis* Inorganic Pyrophosphatase by Computational Experiments[a]

Models

(1) a model for tetrameric protein; a total of four equivalent binding sites for free Mg^{2+} ion per protein
(2) same as no. 1 except there is a total of eight equivalent binding sites for free Mg^{2+} ion per protein
(3) a model for monomeric protein; one free Mg^{2+} ion is bound per protein
(4) same as no. 3 except there are two equivalent binding sites for free Mg^{2+} ion per protein
(5) same as no. 3 except there are three equivalent binding sites for free Mg^{2+} ion per protein
(6) same as no. 3 except there are four equivalent binding sites for free Mg^{2+} ion per protein
(7) same as no. 4 but the two binding sites for free Mg^{2+} ion are nonequivalent
(8) same as no. 5 but the three Mg^{2+} ion binding sites are nonequivalent
(9) a model for monomeric protein; one free Mg^{2+} ion is bound per monomeric protein; a conformational change is induced by the binding of free Mg^{2+} ion to the protein
(10) same as no. 9 except there are two equivalent binding sites for free Mg^{2+} ion per monomeric protein; conformational transition was supposed to occur only after the binding of the last Mg^{2+} ion
(11) same as no. 10 except there are three equivalent binding sites for free Mg^{2+} ion per monomeric protein

Submodels[b]

(A) general model, no constraints
(B) Mg^{2+} ion is not bound to the enzyme in conformation T
(C) Mg^{2+} ion is bound neither to R nor T form of the enzyme
(D) $MgPP_i^{2-}$ complex is not bound by the enzyme in R state
(E) $MgPP_i^{2-}$ complex is not bound by the enzyme in T state
(F) Mg_2PP_i complex is not bound by the enzyme in R state
(G) Mg_2PP_i complex is not bound by the enzyme in T state
(H) $MgPP_i^{2-}$ complex is bound by the enzyme in R state in a nonreactive way
(I) $MgPP_i^{2-}$ complex is bound by the enzyme in T state in a nonreactive way
(J) Mg_2PP_i complex is bound by the enzyme in R state in a nonreactive way
(K) Mg_2PP_i complex is bound by the enzyme in T state in a nonreactive way
(L) $MgPP_i^{2-}$ complex is bound by each enzyme form in a nonreactive way
(M) $MgPP_i^{2-}$ complex is not bound by either enzyme form
(N) Mg_2PP_i complex is bound by each enzyme form in a nonreactive way
(O) Mg_2PP_i complex is not bound by either enzyme form

[a] In all the models (1–11) the protein exists in two conformational states, referred to as R and T states, respectively. By definition, the affinity of free Mg^{2+} ion for the enzyme in the R form is equal to or greater than that of the T state. In models 1–8 conformational change can occur without binding of free Mg^{2+} ion to the enzyme. Models 9–11 assume that the conformational change is induced only when all the binding sites for free Mg^{2+} ion become occupied. [b] All the submodels from B to O are submodels of A. In addition, some of the models are submodels of the other constrained models; e.g., M is a submodel of D and E.

[1] That this relatively high activity at low $[Mg]_{tot}$ was not due to Mg^{2+} present as an impurity in our homogeneous enzyme preparation was checked as follows: 0.2 M EDTA was added to the highly purified enzyme preparation, and the suspension was incubated in an ice–water bath for 1 h; EDTA was then removed from the enzyme solution by five successive $(NH_4)_2SO_4$ treatments (final concentration 85% saturation) followed by centrifugations (30000*g* for 15 min at 2 °C). That the $(NH_4)_2SO_4$ powder (Merck AG, Darmstadt, FRG) did not contain Mg^{2+} as an impurity was checked by the method described by Guibault (1973). The enzyme precipitate was suspended in 0.05 M piperazine/HCl buffer (pH 9.1) containing 0.05 M tetramethylammonium chloride, as a relatively dilute solution (about 0.1 mg of protein/mL), and then it was concentrated by aquacide to a concentration of about 5 mg protein/mL. The purpose of this step was to remove $(NH_4)_2SO_4$ from the enzyme preparation. The kinetics of *S. faecalis* PPase after EDTA treatment was similar to that presented in Figure 1 even at low magnesium concentrations ($[Mg]_{tot}/[PP_i]_{tot} < 1$), indicating that our enzyme preparation was free of magnesium.

[2] The sharp decrease in activity at high $MgCl_2$ concentrations was due to changes in the concentrations of the reaction components and not caused by general ionic effects because the results were practically identical, when the ionic strength was varied from 0.1 to 0.35 at $[MgCl_2]$ = 0–100 mM or when the ionic strength was kept constant (I = 0.35, $[PP_i]_{tot}$ = 0.5 mM) by tetramethylammonium chloride.

Table II: Residual Squares for the Models Described in Table I[a]

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	1.30	1.38	1.44	13.05	1.62	1.30	8.38	1.38	1.37	1.30	1.62	31.49	28.19	1.52	8.38
2	1.30	1.35		7.11	1.63	1.30	8.29	1.37	1.36	1.40	1.67	30.15	28.83	1.53	8.29
3	1.57	1.57	34.40	1.57	1.84	2.11	1.62	1.57	1.57	1.58	1.57	26.93	35.84	1.74	11.21
4	1.49	1.52		1.52	4.40	1.50	1.62	1.52	1.51	1.49	1.49	26.64	35.56	1.59	8.73
5	1.49	1.52		1.52	3.18	1.50	1.63	1.52	1.53	1.50	1.49	26.80	35.53	1.59	8.37
6	1.50	1.52		1.52	2.75	1.50	1.63	1.52	1.51	1.50	1.50	26.87	35.52	1.59	8.30
7	1.49	1.50		1.52	5.12	1.50	1.60	1.49	1.50	1.50	1.49	2.11	10.24	1.59	4.23
8	1.43	1.52		1.44	3.18	1.82	1.57	1.44	1.50	1.50	1.47	2.10	7.48	1.57	8.73
9	1.57		34.40	1.57	26.93	11.21	1.57	1.57	26.93	1.74	1.57	37.23	37.23	1.74	11.21
10	1.50			1.60	18.39	1.57	1.72	1.52	18.39	1.58	1.73	35.86	29.71	1.73	8.73
11	1.56			1.97	24.76	1.57	2.38	1.86	24.76	1.57	2.38	31.09	31.09	2.38	8.42

[a] The numbers 1–11 and the letters A–O refer to the models and their submodels described in Table I, respectively.

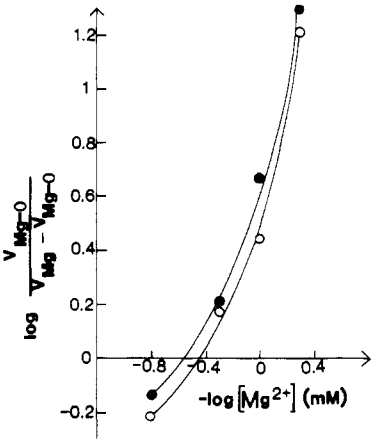


FIGURE 3: Hill plot presentation of the cooperative effects Mg^{2+} exerts on the activity of the less active form of *S. faecalis* PPase at various concentrations of $MgPP_i^{2-}$. $[MgPP_i^{2-}]$ (mM): (●) 0.02; (○) 0.04.

The shoulder observed in Figure 1 at 0.5–1 mM $[Mg]_{tot}$ is due to Mg^{2+} activation; when $[Mg]_{tot}$ is increased up to 0.5 mM the increase in activity is due to an increase in $[MgPP_i^{2-}]$, whereas the increment of activity observed when $[Mg]_{tot}$ is further increased is primarily due to Mg^{2+} activation.

The measured enzyme activity as a function of $[MgPP_i^{2-}]$ and $[Mg_2PP_i]$ at different constant concentrations of Mg^{2+} is shown in Figure 2. Mg^{2+} seems to activate the hydrolysis of $MgPP_i^{2-}$ but inhibits the reaction of Mg_2PP_i. The Hill coefficient for Mg^{2+} activation estimated from Figure 3 is about 2.2, suggesting that Mg^{2+} exerts cooperative effects on the enzyme.

The complex composition of the reaction mixture makes a critical examination of kinetic results very difficult. It is not possible to change the concentration of only one of the reaction components (see Figure 1). The intuitive deductions presented above are therefore not totally unambiguous. Computational experiments were performed to get a more quantitative and probably more reliable kinetic model of *S. faecalis* PPase. The rate equations used in the computational analysis are shown in the Appendix. A brief description of the models tested is presented in Table I. Table II shows the residual square values (abbreviated as RSQ, see Materials and Methods) obtained repeatedly for each model after several computational runs. It is clear from Table II that the simple, nonallosteric models analogous to that presented for *E. coli* PPase (Josse, 1966), in which Mg^{2+} does not activate the enzyme, fitted our results poorly (RSQ = 34.40). Furthermore, the simple models with three to five enzyme species presented by Rapoport et al. (1972) and Moe & Butler (1972) for yeast PPase did not provide a good fit with our data; the RSQ value for the best of these simple models was 1.57 (Table II), indicating the kinetic model of *S. faecalis* PPase to be more complex. Of

Table III: Parameters Estimated for the Simplest Kinetic Model (Model 1F in Tables I and II) with Good Fit with Our Experimental Data

parameter	value estimated by computer model fitting	parameter	value estimated by computer model fitting
$[R]/[T]$	6.3×10^{-2}	$[TMg_2PP_i]/[T]$	8.4
$[Mg^{2+}R]/R$	6.0×10^{-1}	$v(RMgPP_i^{2-})$	5.6×10^3
$[Mg^{2+}T]/[T]$	1.1×10^{-1}	$v(TMgPP_i^{2-})$	1.1×10^3
$[RMgPP_i^{2-}]/[R]$	1.2×10^1	$v(RMg_2PP_i)$	0
$[TMgPP_i^{2-}]/[T]$	1.2×10^1	$v(TMg_2PP_i)$	3.9×10^2
$[RMg_2PP_i]/[R]$	0		

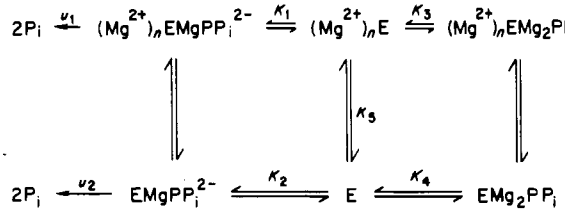
the 165 different models presented in Table I, model 1F was the simplest with a good fit with our experimental data (Table II); comparison of the experimental data points with the theoretical curve calculated according to model 1F with the constants shown in Table III is presented in Figure 1. Model 1F is a simple, Monod–Wyman–Changeux- (MWC; Monod et al., 1965) type, allosteric model, in which the enzyme is postulated to exist in two different conformations, referred to as R and T. The kinetic parameters of this model are shown in Table III. These values should not be taken as real, absolute values but rather as relative ones. In the absence of ligands 94% of the enzyme was in the T state. Mg^{2+} ions were bound to the R form 5.5 times as tightly as to the T form. $MgPP_i^{2-}$ was bound equally well to both enzyme forms, whereas Mg_2PP_i did not bind to the R form significantly (Table III). The R form of *S. faecalis* PPase was 5-fold as efficient as the T form in catalyzing the hydrolysis of $MgPP_i^{2-}$. $MgPP_i^{2-}$ was the only substrate of the enzyme in R state, whereas both $MgPP_i^{2-}$ and Mg_2PP_i were the substrates for the T state, the latter being hydrolyzed about 25% as rapidly as the former (Table III). As Mg^{2+} stabilized the enzyme in R form, it acts as an activator for $MgPP_i^{2-}$ hydrolysis and an inhibitor for Mg_2PP_i reaction (Figure 2).

It is not possible on the basis of the results presented in Table II to conclude whether one or two Mg^{2+} ions are bound per subunit. Ligand binding studies are now in progress to solve this problem.

Discussion

Nearly all of the PPases whose subunit structure are known are oligomeric proteins (Wong et al., 1970; Hachimori et al., 1975; Tominaga & Mor, 1977; Cohen et al., 1978; Morita & Yasui, 1978; Lahti & Niemi, 1981). In spite of this, in the kinetic studies no attention has been paid to oligomerism. Simplification to monomers has been done in part because even monomer treatment has led to a fairly good fit with experimental data. However, enzymes are generally flexible in structure, responding rapidly to environmental changes (Citri,

Scheme I



1973). For this reason it should not be surprising if alternative conformations existed in some multisubunit PPases.

The enzyme activity curve in Figure 1 (v_{rel} vs. $[Mg]_{tot}$) was clearly more complicated than that presented for yeast PPase (Moe & Butler, 1972). Thus, a more complex mechanism was to be expected. It was also shown by computational analysis that the simple kinetic models presented before do not fit well with the data of *S. faecalis* PPase (Table II). Furthermore, the Hill plot presentation in Figure 3 suggested that Mg^{2+} exerts cooperative effects on the enzyme. For these reasons, in this paper the tetrameric structure with two different conformations was taken into consideration in a detailed kinetic analysis.

In the simplest model with good fit to our experimental data the enzyme is presumed to exist in two states, R and T, respectively. $MgPP_i^{2-}$ was the only substrate for the R form of the enzyme, and it was hydrolyzed 5-fold more efficiently by the R form than by the T form. Mg_2PP_i was hydrolyzed 25% as rapidly as $MgPP_i^{2-}$ by the T state of the enzyme. In the absence of ligands the equilibrium between the two enzyme forms was in favor of T, so that only 6% of the protein was in the R form. Mg^{2+} ion stabilized the enzyme in R form, activating the hydrolysis of $MgPP_i^{2-}$ and inhibiting Mg_2PP_i hydrolysis. Mg_2PP_i inhibited the hydrolysis of the more efficient substrate, $MgPP_i^{2-}$, in two ways. First, it competed with $MgPP_i^{2-}$ for the enzyme in T form. Furthermore, Mg_2PP_i bound exclusively to the T state of the enzyme and shifted the R $\rightleftharpoons$ T equilibrium in favor of the T form, which was the less active enzyme form in catalyzing the hydrolysis of $MgPP_i^{2-}$.

For the sake of simplification, the role of PP_i^{4-} was disregarded in the computational studies, because we found that PP_i^{4-} does not act as a substrate, and only a weak inhibition was observed with excess of PP_i^{4-}. High excesses of PP_i^{4-} were excluded from the computational modeling studies. For several PPases, PP_i^{4-} is a potent inhibitor that cannot serve as a substrate (Josse, 1966; Klemme & Gest, 1971; Klemme, 1976; Lahti & Raudaskoski, 1983). However, in the case of human serum PPase PP_i^{4-} is the actual substrate. With that enzyme, Mg^{2+} is a strong inhibitor because it turns the active substrate into inactive $MgPP_i^{2-}$ and Mg_2PP_i complexes (Hörder, 1973). Furthermore, McLaughlin et al. (1978) observed that Mg^{2+} has no effect on the activity of PPase from *Entamoeba histolytica*. This could be so only if PP_i^{4-}, $MgPP_i^{2-}$, and Mg_2PP_i were equally efficient as substrates.

There were four other models out of the 165 models tested with the same RSQ value as that of model 1F. However, most significant was the fact that models showing a good fit with the experimental data were derivatives of each other (Table II). Hence, it is evident that the simplest model with lowest RSQ values best described the essential features of the kinetic model. Analogously, Josse (1966), Moe & Butler (1972), Rapoport et al. (1972), and Schreier & Höhne (1978) selected the simplest model with good fit to describe the kinetics of *Escherichia coli*, yeast, and *Bacillus stearothermophilus* PPase, respectively.

It was not possible to tell unambiguously whether one or two Mg^{2+} ions, in addition to the one complexed with pyrophosphate, were bound by each subunit. The more complicated model with two Mg^{2+} ions bound per subunit did not provide any better fit with the results than that with one Mg^{2+}, and so the binding of the second Mg^{2+} ion was not considered to be essential for the reaction. Ligand binding studies are in progress to solve this problem. In the case of yeast PPase three divalent cations per active site are required for catalysis; two of these metal ions bind directly to the enzyme while the third cofactor coordinates with PP_i (Springs et al., 1981; Ting & Dunaway-Mariano, 1984).

The model presented in this paper is more complicated than any other presented for PPases so far because it includes cooperative effects in the tetrameric structure of the protein. However, in other aspects our model closely resembles those presented for inorganic pyrophosphatases of yeast (Moe & Butler, 1972; Rapoport et al., 1972), *Vibrio alginolyticus* (Unemoto et al., 1973), *Bacillus stearothermophilus* (Schreier & Höhne, 1978), and *Rhodospirillum rubrum* (Randahl, 1979) with the exception that for *S. faecalis* PPase activation by free Mg^{2+} is not essential for the reaction to occur.

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APPENDIX

Equations 1 and 2 were used for the computational analysis

$$v = \frac{4[E]_{tot}(A_T^3 B_T^n D_T + bA_R^3 B_R^n D_R)}{A_T^4 B_T^n + bA_R^4 B_R^n} \quad (1)$$

$$v = \frac{[E]_{tot}(B_T^n D_T + bB_R^n D_R)}{A_T B_T^n + bA_R B_R^n} \quad (2)$$

in the case of models 1 and 2 and 3–8 (Table I), respectively, where $A_c = 1 + h_{c,MgPP_i^{2-}} + h_{c,Mg_2PP_i}$, $B_c = 1 + K_{c,Mg^{2+}}[Mg^{2+}]$, $D_c = u_{c,MgPP_i^{2-}} h_{c,MgPP_i^{2-}} + u_{c,Mg_2PP_i} h_{c,Mg_2PP_i}$, $h_{cL} = K_{cL}[L]$, c refers to the conformations R and T, respectively, L refers to the ligands (Mg^{2+}, $MgPP_i^{2-}$, and Mg_2PP_i), K_{cL} is the association constant between the ligand L and the enzyme in conformation c, u_{cL} is the rate constant describing the rate by which the ligand L ($MgPP_i^{2-}$ or Mg_2PP_i) bound to the enzyme in conformation c is converted to the product, $b = [R]/[T]$ in the absence of ligands, i.e., the equilibrium constant between R and T states of the enzyme when no ligands are present, and n is the number of binding sites for free Mg^{2+} ion per protein.

Models 9–11 in Table I are described by Scheme I and eq 3, where u_1–u_4 are rate constants and K_1–K_5 are association constants.

$$v = [E]_{tot}(u_1 K_5 K_1 [Mg^{2+}]^n [MgPP_i^{2-}] + u_2 K_2 [MgPP_i^{2-}] + u_3 K_5 K_3 [Mg^{2+}]^n [Mg_2PP_i] + u_4 K_4 [Mg_2PP_i])/(1 + K_5[Mg^{2+}]^n + K_5 K_1 [Mg^{2+}]^n [MgPP_i^{2-}] + K_2 [MgPP_i^{2-}] + K_5 K_3 [Mg^{2+}]^n [Mg_2PP_i] + K_4 [Mg_2PP_i]) \quad (3)$$

It should be noted that $[Mg_2PP_i]$ is a constant multiple of $[Mg^{2+}][MgPP_i^{2-}]$, and so some of these terms can be combined. Hence, the kinetic parameters cannot be uniquely determined by kinetic studies even in an ideal case. However, we found it so complicated to combine the terms of models 1–8 (Table I) that we rather used the original kinetic terms. For the sake of clarity we used the same type of presentation

also for the simple models described by eq 3.

Registry No. Mg, 7439-95-4; PP_i, 9024-82-2; EC 3.6.1.1, 19262-94-3.

REFERENCES

Citri, N. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 37*, 397-648.

Cohen, S. A., Sterner, R., Keim, P. S., & Heinrikson, R. L. (1978) *J. Biol. Chem. 253*, 889-897.

Cooperman, B. S. (1982) *Methods Enzymol. 87*, 526-548.

Guibault, G. G. (1973) *Practical Fluorescence, Theory, Methods and Techniques*, p 250, Marcel Dekker, New York.

Hachimori, A., Takeda, A., Kaibuchi, M., Ohrawara, N., & Sameijima, T. (1975) *J. Biochem. (Tokyo) 77*, 1177-1183.

Heinonen, J. (1970) *Anal. Biochem. 37*, 32-43.

Hörder, M. (1973) *Biochim. Biophys. Acta 321*, 329-335.

Josse, J. (1966) *J. Biol. Chem. 241*, 1948-1957.

Josse, J., & Wong, S. C. K. (1971) *Enzymes (3rd Ed.) 4*, 499-527.

Klemme, J. H. (1976) *Z. Naturforsch., C: Biosci. 31*, 544-550.

Klemme, J. H., & Gest, H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 721-725.

Lahti, R. (1983) *Microbiol. Rev. 47*, 169-179.

Lahti, R., & Heinonen, J. (1981a) *J. Gen. Microbiol. 125*, 185-188.

Lahti, R., & Heinonen, J. (1981b) *Acta Chem. Scand., Ser. B B35*, 33-38.

Lahti, R., & Niemi, T. (1981) *J. Biochem. (Tokyo) 90*, 79-85.

Lahti, R., & Suonpää, M. (1982) *J. Gen. Microbiol. 128*, 1023-1026.

Lahti, R., & Raudaskoski, M. (1983) *Folia Microbiol. (Prague) 28*, 371-378.

McLaughlin, J., Lindmark, D. G., & Müller, M. (1978) *Biochem. Biophys. Res. Commun. 82*, 913-920.

Moe, O. A., & Butler, L. G. (1972) *J. Biol. Chem. 247*, 7308-7314.

Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88-118.

Morita, J., & Yasui, T. (1978) *J. Biochem. (Tokyo) 83*, 719-726.

Randahl, H. (1979) *Eur. J. Biochem. 102*, 251-256.

Rao, S. S. (1979) *Optimization: Theory and Application*, Wiley Eastern Limited, New Delhi.

Rapoport, T. A., Höhne, W. E., Reich, J. G., Heitmann, P., & Rapoport, S. M. (1972) *Eur. J. Biochem. 26*, 237-246.

Schreier, E., & Höhne, W. E. (1978) *FEBS Lett. 90*, 93-96.

Springs, B., Welsh, K. M., & Cooperman, B. S. (1981) *Biochemistry 20*, 6384-6391.

Ting, S.J., & Dunaway-Mariano, D. (1984) *FEBS Lett. 165*, 251-253.

Tominaga, N., & Mori, T. (1977) *J. Biochem. (Tokyo) 81*, 477-483.

Unemoto, T., Tanaka, M., & Hyashi, M. (1973) *Biochim. Biophys. Acta 327*, 490-500.

Wong, S. C. K., Hall, D. C., & Josse, J. (1970) *J. Biol. Chem. 245*, 4335-4345.

Reversible Inhibitors of β-Glucosidase†

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ABSTRACT: A variety of reversible inhibitors of sweet almond β-glucosidase were examined. These included simple sugars and sugar derivatives, amines and phenols. With respect to the sugar inhibitors and, indeed, the various glycoside substrates, the enzyme has what can be considered a "relaxed specificity". No single substituent on glucose, for example, is essential for binding. Replacement of a hydroxyl group with an anionic substituent reduces the affinity while substitution with a cationic (amine) substituent enhances the affinity. Amines, in general, are good inhibitors, binding more tightly than the corresponding alcohols: $pK_i^{RNH_3^+} = 0.645pK_i^{ROH} + 1.77$ ($n = 9$, $r = 0.97$). The affinity of a series of 10 primary amines was found to be strongly influenced by substituent hydrophobicity: $pK_i = 0.52\pi + 1.32$ ($r = 0.95$). The major binding determinant of the glycoside substrates is the aglycon moiety. Thus, the K_i values of phenols are similar in magnitude to the K_s values of the corresponding aryl β-glucoside. The pH dependence for the inhibition by various phenols indicates that it is the un-ionized phenol which binds to the enzyme when an enzymic group of $pK_a = 6.8$ (±0.1) is protonated. The affinity of the phenol inhibitor is dependent on its basicity with a Brønsted coefficient for binding of $\beta = -0.26$ ($n = 14$, $r = 0.98$). The pH dependence of the binding of two particularly potent β-glucosidase inhibitors was also examlned. 1-Deoxynojirimycin (1;5-dideoxy-1,5-imino-D-glucitol) has a pH-corrected $K_i = 6.5$ μM, and D-glucono-1,5-lactam has a pH-corrected $K_i = 29$ μM. Although these are two of the most effective reversible inhibitors of the enzyme, they are not transition-state analogues. The evidence for this is that their affinities for β-glucosidase do not change with changing pH in a way that completely resembles the influence of pH on k_{cat}/K_m for normal substrates as might be expected for an ideal transition-state analogue inhibitor.

Although almond β-glucosidase (EC 3.2.1.21) was one of the first enzymes studied (Wohler & Liebig, 1837; Fischer, 1898), relatively little is known about its structure or catalytic mechanism (Legler, 1975). One striking feature of the enzyme, however, is its remarkably broad specificity. This broad specificity refers not only to the aglycon portion of the substrate but also to the glycon moiety as well. Thus, in addition to

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