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Microbial Inorganic Pyrophosphatases

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INTRODUCTION

Inorganic pyrophosphate (PP_i) is produced in various reversible nucleoside 5'-triphosphate-dependent reactions, which presumably are pulled in the biosynthetic direction in vivo by enzymatic hydrolysis of PP_i catalyzed by inorganic pyrophosphatase (EC 3.6.1.1; hereafter referred to as PPase) (58, 89). In addition, PP_i is formed, like ATP, by photophosphorylation (2, 27, 45), oxidative phosphorylation (1, 60, 75), and glycolysis (74).

PP_i serves as a source of energy for several reactions in pro-and eucaryotes (3, 11, 44, 46, 47, 71, 72, 98, 126, 127). It also regulates many enzymes without actually participating in the reactions. Almost invariably the effect of PP_i is then inhibitory (49, 50, 56, 90, 92, 102, 105, 122). Contrary to common opinion, it has been suggested that the ratio PP_i/P_i rather than the ratio ATP/ADP might control certain of the key reactions in carbohydrate metabolism (69, 115). The intracellular PP_i concentration depends mainly on the activity of PPase in the cells. Hence factors which affect the intracellular location or total activity of PPase influence secondarily all reactions in which PP_i has a role as a substrate or regulator. Changes in the state of PPase thus have a wide effect on the general metabolism. In addition, recent discoveries have suggested that PPase might have important roles not only in the regulation of macromolecular synthesis and growth (20, 30, 31, 48, 61), but also in evolutionary events by affecting the accuracy by which DNA molecules are copied during chromosome duplication (32).

In spite of much work on PPases during the last few years, no general review article has been published on the subject. Josse and Wong (43) reviewed the properties of PPase from *Escherichia coli* and Butler (10) reviewed those of PPase from *Saccharomyces cerevisiae*. This review deals primarily with the regulatory and kinetic properties of microbial PPases. In the kinetic survey I will mainly deal with those aspects needed to understand the regulation at the activity level on the basis of kinetic results.

INTRACELLULAR LOCALIZATION

Generally more than 90% of PPase total activity is observed in soluble cytoplasmic proteins (34, 41, 55, 108, 111, 120). Neujahr et al. (85) found PPase activity in membrane fractions of *Streptococcus faecalis*. Deficiency of niacin decreased the membrane-bound activity by 80%. Klemme et al. (55) noticed that there are two groups of nonsulfur purple bacteria in regard to intracellular distribution of PPase: one represented by *Rhodopseudomonas gelatinosa*, *R. spheroides*, and *R. capsulata*, containing the soluble enzyme only; and the other represented by *R. palustris* and *Rhodospirillum rubrum*, with both soluble and membrane-bound activities.

The membrane-bound PPase of *Rhodospirillum rubrum* is coupled to the electron transport chain in chromatophores and is unique in that it catalyzes not only the hydrolysis of PP_i, but also the synthesis of PP_i in light (4). In this case PP_i hydrolysis is significantly depressed in light (87). It was suggested that this depression results from an alteration in the oxidation-reduction state of the electron transport system (87). Membrane-bound PPase in *Rhodospirillum rubrum* is

also able, like H^+-ATPase, to generate the gradient of electrochemical potential of H^+ ions in the membrane (57). Comparative studies on the kinetics of PP_i hydrolysis have revealed that cytoplasmic PPase has a much greater affinity for the substrate and thus can work much more efficiently than the membrane-bound pyrophosphatase (53, 93). The solubilized enzyme has been shown to require phospholipids for its PP_i hydrolyzing activity (94).

Also in eucaryotes, membrane-bound PPase is responsible for PP_i synthesis coupled to the mitochrondrial respiratory chain (76). Volk et al. (116) studied the kinetics of PP_i hydrolysis with the soluble and membrane-bound mitochondrial PPases and found no qualitative differences in their catalytic properties. Shakhov et al. (104) observed that the soluble mitochondrial pyrophosphatase, in contrast to the membrane form, is activated by phospholipids. Furthermore, it was shown that preincubation with phosphatidylcholine converts the soluble enzyme into the membrane form, which carries out energy-dependent synthesis of PP_i (104).

REGULATION OF PPase SYNTHESIS

Effects of Culture Conditions

Although PP_i is used as a source of energy and phosphorus in various phosphorylating reactions (98, 126, 127), these reactions probably consume only a minor proportion of intracellular PP_i. If PPase were not continually present in the cells, PP_i concentration would quickly become so high that it would inhibit growth. (From the rates of PP_i formation given by Klemme [52] it can be calculated that in 1 h intracellular PP_i concentration would be about 3 M in *E. coli* [when generation time is 40 min] if there were no PP_i hydrolyzing activity. This is of course merely a hypothetical situation in which it is thought that nothing would restrict PP_i formation. According to Blumenthal et al. [8], even 2 mM PP_i inhibits growth of some bacteria.) Therefore, it seems reasonable to suppose that PPases are among the enzymes whose continual synthesis is essential for growth; i.e., they are constitutive enzymes (107).

Josse (41) found that the specific activity of *E. coli* PPase is not influenced by (i) composition of the medium (carbon or phosphorus source, pH, salts), (ii) phase or rate of growth of the cells, (iii) bacteriophage infection, or (iv) condition of protein synthesis in the cell. Josse and Wong (43) noticed that in a double mutant of *E. coli*, which had no alkaline phosphatase and contained only 2% of the PPase activity found in the wild-type cells, PPase was not induced in a medium with PP_i as the sole source of phosphorus. Based on all of these results, it was concluded that *E. coli* PPase is synthesized constitutively (41, 43).

Starr and Oginsky (108) found that the specific activity of PPase was the same in extracts of *Streptococcus faecium* grown in different P_i and PP_i concentrations. Likewise, Klemme et al. (55) observed that P_i limitation exerted no specific effects on PPases in *Rhodospirillum rubrum* and *R. capsulata*. Blumenthal et al. (8) cultured several bacteria in a medium containing no PP_i. They termed PPases constitutive and inductive on the basis of the effects that PP_i exerted on the synthesis. PPases of *Staphylococcus aureus* and *Micrococcus lysodeikticus* were inductive and were synthesized only in a medium containing PP_i. The molecular mechanism of PPase induction by PP_i in these two bacteria was not studied in detail. On the other hand, that kind of induction has not been observed in any other bacterium.

The specific activity of PPase decreased with increasing glucose concentration as assayed with stationary-phase extracts of *Streptococcus faecium* (108). The decrease was inversely related to the growth yield. Starr and Oginsky (108) made similar observations by changing the concentration of lipoic acid. Furthermore, they found that the specific activity of PPase from *Streptococcus faecium* has its maxima in the exponential phase of growth. They thought that activation in the exponential phase or inhibition in the stationary phase was unlikely since additive values were obtained on assaying mixtures of crude sonicates from different phases of growth (108). On the other hand, we have recently shown that PPase of *Streptococcus faecalis* is synthesized constitutively; the amount of the enzyme was constant, whereas its activity was changed during the batch culture (65).

Addition of Co^{2+} to the culture of *Streptococcus faecalis* increased the specific activity of PPase sevenfold (88). PPase activity was significantly increased also in spores of *Bacillus subtilis* grown in excess Mn^{2+} (112). The mechanisms by which the metals exert their effects are not known.

To sum up, with the exceptions mentioned above, changes in the composition of the culture medium generally exert no specific effects on PPase.

PPase Mutants

To find out whether a cell devoid of PPase activity is able to grow or survive, Josse and Wong (43) prepared temperature-sensitive mutants of *E. coli* which grew at a low (15 to 25°C) but not at a higher (35 to 40°C) temperature. None of the isolated mutants (>250) contained temperature-sensitive PPase. By contrast, mutants with different levels of PPase can be pre-

pared quite easily (43, 62). However, as far as I know, nobody has managed to isolate a mutant containing no PPase at all.

Josse and Wong (43) found that mutants with low PPase activity grow like a wild-type cell. On the other hand, a mutant containing higher PPase activity than the wild-type cell grew more slowly than the parental cell (J. Heinonen, unpublished data). This would be explained, for example, if some of the PP_i-utilizing reactions (98, 126, 127) became growth limiting as intracellular PP_i concentration decreased too low. However, it must be emphasized that interpretation of the results obtained with the mutants is arbitrary since the intracellular PP_i concentrations of the mutants were not measured.

Inhibition of Cell Division and Macromolecular Synthesis

It is a biochemical dogma that biosynthetic reactions producing PP_i are subject to a "thermodynamic pull" by PPase (58, 89). Therefore, it might be anticipated that a disturbance in some of the PP_i-producing reactions would be reflected in PPase and vice versa. Indeed, as a result of partial inhibition of DNA synthesis PPase production is stimulated 1.5- to 3-fold in *E. coli* (30, 31). DNA synthesis is inhibited immediately after the addition of nalidixic acid to the culture, whereas PPase production is not stimulated until after 3 to 4 h of growth. When the inhibitor of DNA synthesis is removed, the ratio DNA/cell mass returns to the normal value within 1 h, but PPase activity reaches the control level only after 5 to 6 h (31). The delay suggests that the inhibition of DNA synthesis affects PPase synthesis indirectly, by blocking cell division, for example. This explanation is supported by the fact that PPase production can also be stimulated by inhibiting cell division with penicillin (61). Furthermore, with a mutant of *E. coli* temperature sensitive to cell division it has been found that only at a permissive (but not at a restrictive) temperature is PPase production stimulated by partial inhibition of DNA synthesis (J. Heinonen and E. Kukko, unpublished data).

In *Streptococcus faecalis* partial inhibition of DNA synthesis produced by hydroxyurea has no effect on the synthesis or activity level of PPase. Moreover, inhibition of RNA (by rifampin) and protein (by chloramphenicol) synthesis exerts no specific effects on PPase production in *E. coli* (J. Heinonen, E. Kukko, and R. Lahti, unpublished data). Instead, it has been shown that PPase acts as a stimulator for protein synthesis by removing PP_i, the potent inhibitor of aminoacyl-tRNA synthetases (20). Furthermore, Kent and Guterman (48) have recently introduced a novel mechanism by which PP_i, and thus also PPase, might regulate ρ-termination activity of transcription in *E. coli*.

POLYMORPHIC FORMS OF PPase

Multiple forms of PPase have been reported by several authors (19, 53, 64, 117, 118). Earlier the names "alkaline" and "acid" PPases were commonly used (88), but they turned out to be different expressions for a single enzyme (108). The outer charge of an enzyme is changed with pH, and this is reflected in the kinetic properties (53, 64).

Chromatographically separable forms of PPase were observed in various eucaryotes (25, 38, 77, 84). The proportions of the different enzyme forms vary during differentiation (6). Furthermore, certain forms of enzyme are located in certain cell organelles (51, 76) or tissues (23).

In addition to the soluble and membrane-bound enzyme forms mentioned before, polymorphic microbial PPases were observed only in *Pseudomonas aeruginosa*, *Staphylococcus albus* (80), and *B. megaterium* (113). Meloni et al. (80) fractionated crude extracts of nine different bacteria on a DEAE-cellulose column. They observed two separate peaks of PPase from *P. aeruginosa* and *Staphylococcus albus*, but could not tell whether the two enzyme peaks were products of different gene loci or different states of a single protein (80). Tono and Kornberg (113) observed in spores of *B. megaterium* two forms of PPase which could be separated by disc gel electrophoresis. The minor enzyme could be transformed to the major with 1 mM Mn^{2+}.

REGULATION AT THE ACTIVITY LEVEL

Problems Encountered in Kinetic Studies

Intrinsic to the enzymatic hydrolysis of pyrophosphate is the fact that the substrate ($MgPP_i^{2-}$) is a complex of effectors. In this kind of system the substrate concentration in the reaction mixture is not linearly related to the amount of added ligands. In solutions containing Mg^{2+} and PP_i the following molecular and ionic species occur: Mg^{2+}, $MgPP_i^{2-}$, $MgHPP_i^{1-}$, MgH_2PP_i, Mg_2PP_i, PP_i^{4-}, HPP_i^{3-}, $H_2PP_i^{2-}$, $H_3PP_i^{1-}$, and H_4PP_i. At pH 9 protonated species are virtually nonexistent and can be neglected (42, 53, 54). However, it must be noted that there are protonated species in vivo. Moe and Butler (81) and Rapoport et al. (97) studied the kinetics at pH 7.2, and the kinetic equations became extremely complicated. The concentration of each molecular species can be calculated if pH, total concentrations of Mg and PP_i, and the stability constants for each $Mg_xH_yPP_i^{z-}$ complex are known (42, 54).

Hörder (37) emphasized that the stability con-

stants should be measured under the same conditions as PPase activity. He observed that the stability constant of the $MgPP_i^{2-}$ complex increases with temperature (twofold increase if the temperature rises from 15 to 37°C) and decreases when ionic strength increases (from $2 \times 10^5\ M^{-1}$ at 10 mM to $0.4 \times 10^5\ M^{-1}$ at 250 mM). On the other hand Rapoport et al. (97) found that threefold changes in the magnitude of the constants have no significant effect on the results. Most remarkable was the fact that the same reaction model fit best with the results in all values of the stability constants. In spite of Rapoport's (97) view, it is clear that substantial misinterpretation could arise if the concentrations of the molecular species in the reaction mixture were significantly altered.

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 just one of the reaction components. Thus, when the ratio $[Mg]_{tot}/[PP_i]_{tot} < 1$ and the total concentration of PP_i is constant, increase in the total concentration of Mg increases substrate ($MgPP_i^{2-}$) and activator (Mg^{2+}) concentrations. At the same time the concentration of inhibitor (PP_i^{4-}) decreases (81). As a result of these changes the substrate saturation curve (v versus $MgPP_i^{2-}$) is sigmoidal. Moreover, when the total PP_i concentration is changed, then at low PP_i concentration, $[PP_i]_{tot} < [Mg]_{tot}$, the rate of reaction increases linearly with increasing PP_i concentration. As $[PP_i]_{tot}$ increases further, the concentration of inhibitor becomes gradually significant and simultaneously the activator concentration decreases. As a result of these events the substrate saturation curve is a flattened hyperbola similar to that observed in the case of negative cooperativity defined by Levitzki and Koshland (70).

Kinetic Survey

In the initial studies on PPase, attention was paid to the fact that divalent cations are essential for enzymatic hydrolysis of PP_i. It was observed that the $MgPP_i^{2-}$ complex is the substrate and free pyrophosphate (not combined with metal = PP_i^{4-}) is a strong inhibitor (7, 63, 101). Subsequent kinetic studies have confirmed these observations (10, 43). However, conflicting reports have been published about the role of Mg^{2+} and the Mg_2PP_i complex in the reaction catalyzed by PPase. Making use of the theoretical treatment put forward by London and Steck (73), Rapoport et al. (97) developed a general model for the enzymatic hydrolysis of PP_i (Fig. 1). In their model Mg^{2+} is essential for the reaction. Whereas this is true in almost every system studied so far, Hörder (36) found free pyrophosphate to be the substrate of PPase from human serum. In this case Mg^{2+} is a strong inhibitor because it turns the active substrate into inactive $MgPP_i^{2-}$ and Mg_2PP_i complexes (36). Furthermore, McLaughlin et al. (78) 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.

The model in Fig. 1 is far too complex for practical use. For this reason Josse (42), Moe and Butler (81), and Rapoport et al. (97) analyzed their results in terms of simple submodels of the general model. They found the kinetic results to have a good fit with several models. Hence, it is not possible from kinetic studies to say which of the models is right. Most significant was the fact that models showing a good fit with the experimental data were derivatives of each other. In other words, the best model with four molecular species was an extension of the best model with three species and the best model with five species was an extension of the best model with four molecular species (97).

Josse (42), Moe and Butler (81), and Rapoport et al. (97) selected the simplest model with good fit to represent their views on the kinetics of PPase. In doing this, however, they did not intend to exclude the more complicated models. These simple kinetic models will now be briefly examined.

In the model of *E. coli* PPase, PP_i^{4-} is a competitive inhibitor, $MgPP_i^{2-}$ is substrate, and Mg_2PP_i is bound (as a competitive inhibitor) relatively weakly if at all (Fig. 2A). PP_i^{4-} is bound to the active site roughly 50 times more tightly than the substrate (42). In his model Josse (42) disregarded the possibility that free Mg^{2+} could activate the enzyme because it would have led to too complicated state equations. Josse (42) stated that the activating role of Mg^{2+} cannot be ruled out, although he thought that there was no evidence to suggest it.

The model proposed by Moe and Butler (81) for *S. cerevisiae* PPase involves activation by free Mg^{2+} before the enzyme can bind the substrate or the inhibitor. Uncomplexed PP_i is a strong competitive inhibitor. Both $MgPP_i^{2-}$ and Mg_2PP_i are substrates; at pH 7.4 the latter is hydrolyzed 22% as rapidly as the former (Fig. 2B). Moe and Butler (81) established that the model in which free Mg^{2+} does not activate the enzyme shows poor agreement with the results.

Activation by free Mg^{2+} is also necessary for reaction in the model developed by Rapoport et al. (97) for *S. cerevisiae* PPase (Fig. 2C). However, in their model, binding of free Mg^{2+} to the enzyme is not essential for substrate binding. Moreover, Mg_2PP_i does not bind to the enzyme.

If routes 1 and 2, which I added to the model of Rapoport et al. (97) (Fig. 2C), were of differ-

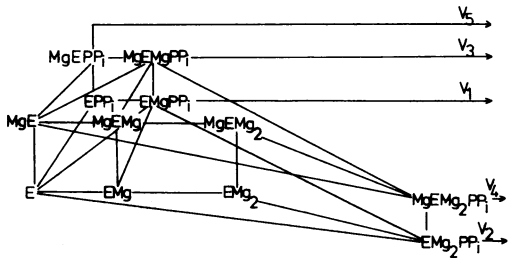

FIG. 1. General model for enzymatic hydrolysis of PP_i, a modification of that presented by Rapoport et al. (97).

ent efficiency, then changes in the ratio of $[Mg]_{tot}/[PP_i]_{tot}$ would alter the relative proportion of PPase functioning via each route, with the result of a nonhyperbolic substrate saturation curve. This situation is analogous to that described by Ferdinand (22) for two-substrate enzymes. Such events might have some role in the regulation of PPase activity in vivo.

The kinetic models presented for PPases of *Vibrio alginolyticus* (114), *B. stearothermophilus* (103), and *Rhodospirillum rubrum* (53, 93) closely resemble those described for *S. cerevisiae* PPase (81, 97).

All in all, owing to the difficulties encountered in the kinetic studies, it is not possible to say unambiguously which of the best-fitting models is the right one. However, the results obtained with most microbial PPases fit well with the model in which $Mg_1PP_i^{2-}$ is the primary substrate, Mg^{2+} is an activator, and PP_i^{4-} is an inhibitor.

Allosteric PPases

Nearly all of the PPases whose subunit structures are known are oligomeric proteins (14, 28, 67, 84, 111, 125). In spite of this, in the kinetic studies no attention has been paid to oligomerism. Josse (42), Moe and Butler (81), and Rapoport et al. (97), for example, treat PPases like monomers (Fig. 1 and 2). So their models are suitable only for proteins with no subunit interactions. Naturally, this has been done for the sake of simplification because even monomer treatment leads to extremely complicated models (Fig. 1). However, enzymes are generally flexible in structure, responding rapidly to environmental changes (13). For this reason the simplification to monomers appears questionable and, as I show below, is, in fact, totally unfounded.

Deviation from Michaelis-Menten kinetics (21) is observed for PPases without subunit interactions (see above). It is therefore very difficult to demonstrate by kinetic studies the existence of homotropic and heterotropic interactions in the enzymatic hydrolysis of PP_i. Many authors have measured substrate saturation curves in conditions where only the changes in $MgPP_i^{2-}$ concentration are significant for the reaction. Then hyperbolic v versus $MgPP_i^{2-}$ curves have been found, and this has been thought to argue against subunit interactions (7, 81, 91, 114). In all of these studies, however, the concentration of Mg^{2+} activator was high, $[Mg]_{tot} > [PP_i]_{tot}$. Under such conditions even "allosteric PPases" behave according to Michaelis-Menten kinetics (see below).

Horn et al. (33) observed sigmoidal v versus $MgPP_i^{2-}$ curves with mouse liver cytoplasmic PPase when the ratio of $[Mg]_{tot}/[PP_i]_{tot}$ was constant. They noticed that under the influence of the substrate the cooperativity of the activator decreases whereas that of the inhibitor increases. On the other hand, the cooperativity of the substrate decreases with increasing activator concentration (33). These effects are typical of allosteric proteins (59, 83), although they are also seen with nonallosteric PPases (see above). Horn et al. (33) apparently presumed that the cooperation of inhibitor and activator is nearly constant at a constant ratio of $[Mg]_{tot}/[PP_i]_{tot}$. This assumption seems doubtful. Klemme and Gest (53, 54) have shown that at least the inhibition caused by PP_i is not constant under such conditions.

Klemme and Gest (53, 54) found at a constant $[Mg]_{tot}/[PP_i]_{tot}$ ratio with *Rhodospirillum rubrum* PPase that correction of the actual assay data for inhibition by free PP_i does not completely elimi-

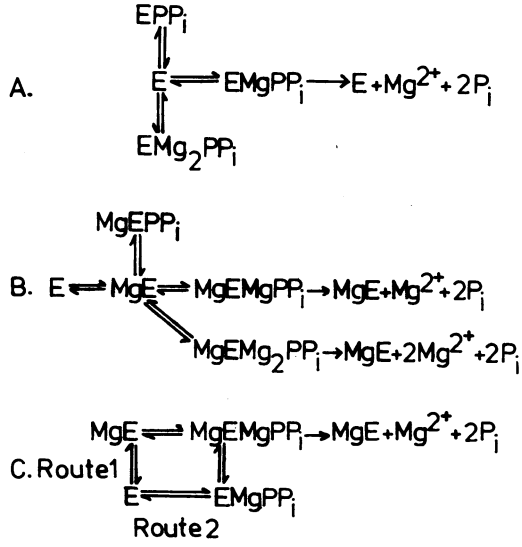

FIG. 2. Simple kinetic models for PPases from *E. coli* (A) and *S. cerevisiae* (B, C) according to Josse (42), Moe and Butler (81), and Rapoport et al. (97), respectively.

nate the sigmoidicity of the substrate saturation curve. Furthermore, they noticed that increase in the ratio of $[Mg]_{tot}/[PP_i]_{tot}$ decreases the sigmoidicity. At a ratio of 4, the concentration of free Mg^{2+} is so high that the enzyme is completely activated. This is seen in the hyperbolic v versus $MgPP_i^{2-}$ curve (53, 54). Klemme et al. (55) obtained similar results with the PPases of *R. palustris* and *R. gelatinosa*, whereas *R. capsulata* and *R. spheroides* PPases exhibited simple Michaelis-Menten kinetics even at a low ratio of $[Mg]_{tot}/[PP_i]_{tot}$.

Horn et al. (33) and Klemme and Gest (53, 54) established by kinetic studies that Mg^{2+} ions not only are necessary for the formation of the substrate, but also act as an allosteric activator. Analyzing the binding of Mg^{2+} and $MgPP_i$ analogs to *S. cerevisiae* PPase, Cooperman and Chiu (16) noticed that free Mg^{2+} and the substrate bind to different sites. This is supported by the observation (17) that inhibitors of *S. cerevisiae* PPase, namely, phenylglyoxal and 1-ethyl-(dimethylaminopropyl)-carbodiimidehydrochloride, prevent completely the binding of $MgPP_i$ analogs, but have no effect on the binding of free Mg^{2+}. Different type of binding studies with *S. cerevisiae* PPase have further shown that both subunits of the dimeric enzyme (14) contain three divalent metal binding sites, one or two of which are the same as the proposed allosteric activator sites (15, 18, 96, 106), two P_i sites (29; P_i is the product of PP_i hydrolysis), and one substrate (PP_i) site (99), in which at least one of the three metal binding sites is included. Binding of the allosteric Mg^{2+} ion results in a reversible change in conformation which is seen in the kinetic and spectroscopic properties of the enzyme (96, 97). In the presence of free pyrophosphate, conformational transition takes several minutes (35). So the effect of Mg^{2+} on *S. cerevisiae* PPase can be regarded as a hysteretic-heterotropic allosterism as defined by Frieden (24).

In the studies of PPase kinetics it has almost always been assumed that allosterism can be demonstrated "simply" by showing that the sigmoidicity of the substrate saturation curve does not totally result from the cooperation of effectors in the reaction mixture (see above). However, there are several cases, not mentioned above, which result in nonhyperbolic kinetics without allosterism. I will enumerate these briefly because it is important to be aware of them when the existence of allosterism is being judged by kinetic results. (i) Interaction between catalytic and regulatory sites is based on direct ligand-ligand interactions (82). (ii) The enzyme contains an impurity which combines with the substrate (121). (iii) There are two enzymes with one substrate (121). (iv) The enzyme has several noninteracting active sites with different dissociation constants (123). (v) The substrate is polymerized (86). (vi) An enzyme with one active site exists in two forms of different activity (119). (vii) There are two substrate systems with two or more alternative reaction pathways (22, 26, 109).

Case v is evidently out of the question for PPases. The possible role of a two-substrate-like system in the regulation of PPase has been mentioned above. Cases ii and iii might have been relevant in the studies of Horn et al. (33) and Klemme et al. (53–55), in which PPase was only partially purified. Elimination of cases i, iv, and vi as irrelevant requires detailed studies on the structure of PPases.

There are uncertainties in the reports of Horn et al. (33) and Klemme et al. (53–55) (see above), the significance of which is difficult to evaluate. Hence, it has not been shown reliably with any PPase that the sigmoidicity of the substrate saturation curve is due to homotropic interactions in the substrate binding. However, homotropic interactions are not essential for allosteric proteins. Intrinsic to allosteric proteins are two or more separated binding sites with either heterotropic or homotropic interactions (39). For the present, heterotropic effects have been established reliably only with *S. cerevisiae* PPase (16, 17). That allosterism has been demonstrated only with *S. cerevisiae* PPase does not imply that allosteric regulation is uncommon for PPases. It is more a reflection of the difficulties encountered in the kinetic studies. Most studies on allosteric PPases have ceased when investigators have incorrectly assumed that a hyperbolic v versus $MgPP_i$ curve means no subunit interactions when $[Mg]_{tot} > [PP_i]_{tot}$ (see above).

Regulation of PPase Activity by Adenylate Nucleotides

As described before, Mg^{2+} is involved in the catalytic activity of PPase in two ways: as a component of the substrate, $MgPP_i^{2-}$, and as an activator. Comparing the intracellular concentrations of PP_i and Mg^{2+}, Klemme (52) concluded that in certain conditions the intracellular concentration of Mg^{2+} available for PPase is so low that it limits the enzymatic hydrolysis of PP_i. Under such conditions adenylate nucleotides (and especially ATP) might regulate PPase activity by competing with PP_i for Mg^{2+} (54, 103, 110). The effects of competition could be quite dramatic in *E. coli* because inhibitory PP_i^{4-} binds to the enzyme much more tightly than the substrate (42). The intracellular concentrations of adenylate nucleotides are determined by the balance between endergonic and exergonic metabolism, i.e., between biosynthetic processes on the one hand and electron transport-linked phosphorylation on the other (12). The

activity of PPase in vivo might be adjusted to these functions via the relation between the adenylate charge and Mg^{2+}.

In addition to Mg^{2+} taking part in the reaction, several PPases require still another specific divalent metal to stabilize the structure (Table 1). These PPases are inactivated by chelators in vitro (53, 54, 100). Accordingly, the energy charge of the adenylate system (12) might also exert some effects on the state of PPases stabilized by divalent metals. The physiological significance of stabilizing divalent metals in the regulation of PPase activity has not been systematically studied. However, Oginsky and Rumbaugh (88) observed that addition of Co^{2+} to a growing culture of *Streptococcus faecalis* increases the specific activity of PPase sevenfold. Similarly, Heinonen (unpublished data) noticed a two- to threefold increase in the specific activity of PPase after addition of 0.1 mM Mn^{2+} to a batch culture of *B. subtilis*.

TABLE 1. Microbial PPases stabilized by specific divalent cations

Organism	Stabilizer	Reference(s)
Rhodospirillum rubrum	Zn^{2+}	53, 54
Rhodopseudomonas palustris	Zn^{2+}	55
Rhodopseudomonas gelatinosa	Zn^{2+}	55
Rhodopseudomonas capsulata	Co^{2+}	55
Rhodopseudomonas spheroides	Co^{2+}	55
Streptococcus faecalis	Co^{2+}	88
Bacillus subtilis	Mn^{2+}	112
Bacillus megaterium	Mn^{2+}	113
Saccharomyces cerevisiae	Mg^{2+}	100

Regulation of PPase Activity by Glutathione

Several bacterial PPases are stabilized by reductants in vitro (19, 53, 64, 117, 118). In the case of *Streptococcus faecalis*, we have shown that this stabilization reflects regulation at the activity level (65). *Streptococcus faecalis* PPase exists in two interconvertible forms which differ in activity (64). Reduced glutathione and other thiol compounds tend to turn the enzyme into the high-activity form, whereas oxidized glutathione and thiol inhibitors have an opposite effect (64). During the early exponential phase of growth almost all of the enzyme is in the high-activity form, whereas during the stationary phase the highly active and the less active form exist in equal amounts (65). These activity changes correspond well to the cell's demand for PPase activity since during the exponential phase the PP_i-producing, biosynthetic reactions are also highly active. The equilibrium between these two enzyme forms is regulated in vivo by the intracellular ratio of reduced glutathione to oxidized glutathione (68).

PPases of *Streptococcus cremoris*, *Streptococcus lactis*, *Lactobacillus lactis*, *L. helveticus*, *M. flavus*, and especially *Beneckea harveyi* and *Photobacterium mandapamensis* resemble the enzyme from *Streptococcus faecalis* when studied in vitro (66). It remains to be shown whether PPases from these bacteria are regulated in vivo similarly to that of *Streptococcus faecalis*. Rapoport and Scheuch (95) have suggested that glutathione might regulate PPase of rabbit reticulocytes.

The described model of regulation is probably not specific for PPase. Several authors have suggested that glutathione regulates enzymes by maintaining their essential thiol groups in a reduced state (5, 9, 40). However, there is so far very little in vivo evidence to verify this idea (79, 124, 128, 129).

SUMMARY

Nearly all of the PPases studied so far are synthesized constitutively. PPases of *Staphylococcus aureus* and *M. lysodeikticus* are inductive, synthesized only in a medium containing PP_i. Recent discoveries have suggested that PPase might have an important role not only in the regulation of macromolecular synthesis and growth, but also in evolutionary events by affecting the accuracy by which DNA molecules are copied during chromosome duplication. The production of PPase is stimulated 1.5- to 3-fold in *E. coli* as a result of partial inhibition of DNA synthesis. Possibly inhibition of DNA synthesis affects PPase synthesis indirectly, by blocking cell division, for example. PPase has been shown to act as a stimulator for protein synthesis by removing PP_i, the potent inhibitor of aminoacyl-tRNA synthetases. Furthermore, a novel mechanism has been presented according to which PP_i, and thus also PPase, might regulate ρ-termination activity in the transcription of *E. coli*. Distribution of PPase to soluble and membrane fractions has a great importance in directing enzyme functions to specific reaction pathways.

Intrinsic to the enzymatic hydrolysis of PPase is the fact that the substrate ($MgPP_i^{2-}$) is a complex of effectors. Free pyrophosphate is a strong competitive inhibitor and Mg^{2+} is an activator. In a solution containing Mg^{2+} and PP_i several different molecular species occur, and their concentrations can be calculated if pH, total concentrations of Mg^{2+} and PP_i, and the stability constants for each molecular species are known. The complexity of the reaction mixture makes the critical examination of kinetic results very difficult, because it is not possible to change the concentration of only one of the reaction components at a time. Hence, it has not been convincingly shown with any PPases that the sigmoidicity of the substrate saturation

curve is due to homotropic interactions, and heterotropic effects have been established with certainty only for PPase from *S. cerevisiae*.

Adenylate nucleotides regulate PPase activity by competing with PP_i for Mg^{2+}, so the activity of PPases might be adjusted to anabolic and catabolic reactions via the relation between the adenylate charge and Mg^{2+}. Several bacterial PPases are stabilized by reductants in vitro. In the case of *Streptococcus faecalis* it has been shown that this stabilization reflects regulation at the activity level, with the ratio of reduced glutathione to oxidized glutathione as an effector in vivo.

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