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Yeast Inorganic Pyrophosphatase. III. Active-Site Mapping by Electrophilic Reagents and Binding Measurements†

Barry S. Cooperman* and Ning Yu Chiu

ABSTRACT: The effects of electrophilic reagents on the enzymatic activity of inorganic pyrophosphatase are studied. Phenylglyoxal incubation results in complete inactivation, while incubation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride leads to a retention of 7% activity. Trinitrobenzenesulfonate, *O*-methylisourea, and iodoacetic acid are ineffective as inactivating agents. The rates of both inactivation processes are virtually unaffected by added Mg^{2+}, but Mg^{2+}–hydroxymethanebisphosphonate complex, an inhibitor of enzymatic activity, slows the rate considerably. This protective effect is used to measure inhibitor binding and the dissociation constant so obtained is found comparable to a K_i value found previously from steady-state kinetic measurements (Cooperman, B. S., and Chiu, N. Y. (1973), *Biochemistry 12*, 1670). Magnetic resonance measurements show that incubation of enzyme with both inactivating reagents has only a minor effect of Mn^{2+} binding, but that binding of hydroxymethanebisphosphonate to the phenylglyoxal-inactivated enzyme has been abolished. These results and those of related studies are used to construct a plausible enzymatic mechanism.

In the first two papers of this series we presented methods for measuring three functions of yeast inorganic pyrophosphatase: enzymatic activity, divalent metal ion binding, and pyrophosphate analog binding. In this paper we begin using these methods to construct a preliminary structure–function map of the active site. Our strategy has been to (1) determine the sensitivity of the enzyme to a wide range of "group specific" electrophilic reagents, and for those reagents which inactivate the enzyme; (2) test whether inactivation is inhibited by Mg^{2+} or pyrophosphate analog added either separately or together; and (3) determine whether inactivation can be

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received August 28, 1972.* This work was supported by Research Grant AM-13202 from the National Institutes of Health.

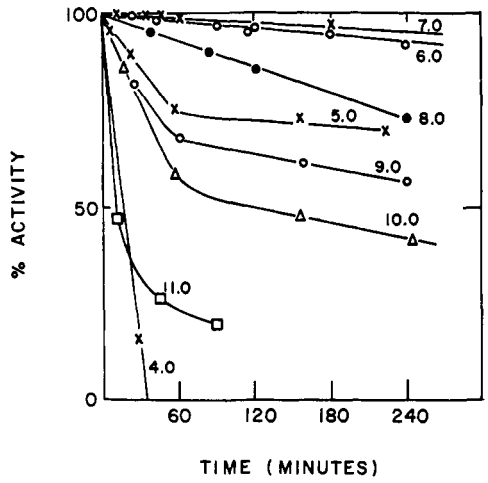

FIGURE 1: Enzyme stability at various pH values. Enzyme (2–5 units/ml) was incubated at a series of pH values, and aliquots were assayed at various times in the standard assay medium. Buffers were: pH 4.0, 0.05 M acetate; pH 5.0, 0.05 M *N,N'*-dimethylpiperazine; pH 6.0, 0.003 M 2-(*N*-morpholinoethanesulfonate); pH's 7.0 and 8.0, 0.2 M *N*-ethylmorpholine; pH's 9.0, 10.0, and 11.0, 0.05 M borate.

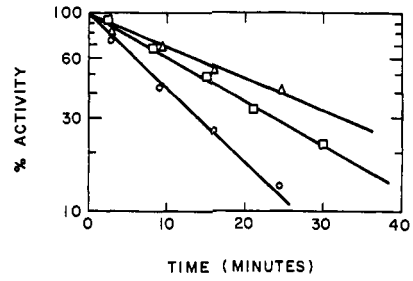

FIGURE 2: Mg^{2+}–PCHOHP protection of enzyme inactivation by 0.08 M PhGx in 0.2 M *N*-ethylmorpholine, pH 7.4 (1.0 N in KCl). No protection (○); 1.2 mM Mg^{2+}, 7×10^{-5} M PCHOHP (□); 1.2 mM Mg^{2+}, 2×10^{-4} M PCHOHP (△).

accounted for by a blockage of either the metal ion or pyrophosphate binding site.

Our results offer evidence for the presence of at least one essential arginine at the active site which is necessary for pyrophosphate binding. By contrast, there appear to be no essential aspartates, glutamates, lysines, or cysteines at the active site.

Experimental Section

Materials

Phenylglyoxal monohydrate (PhGx)[1] (Aldrich), was twice recrystallized from water before use. *N*-Ethylmorpholine and *N,N'*-dimethylpiperazine (Aldrich) were distilled before use. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (R(NCN)Et) was obtained from Sigma.

Yeast inorganic pyrophosphatase (35–45 Kunitz units/mg) was prepared as described elsewhere (Cooperman *et al.*, 1973; Cooperman and Chiu, 1973).

Methods

Enzyme solutions (2–5 Kunitz units/ml) of varying composition were incubated at 25° and aliquots were withdrawn at various times and assayed for enzymatic activity using the pH-Stat assay described elsewhere (Cooperman *et al.*, 1973). The aliquots were diluted between 100- and 1000-fold on transfer to the assay medium, the dilution serving as a quench procedure. In all cases the large dilution led to an immediate loss in activity of 15–20%, which may be a surface inactivation effect. All activity measurements are corrected for this loss.

For all experiments measuring inactivation by added electrophile, a control run of enzyme in buffer was performed, and the measured residual activities were corrected for losses in enzyme activity on standing in buffer. As can be seen in Figure 1, such corrections were minor in the pH range 6.0–8.0. Runs measuring PhGx inactivation of enzyme were begun by addition of solid PhGx. Solution was complete within ten seconds. These studies could not be done in Tris buffer because of a side reaction between PhGx and Tris. Runs measuring R(NCN)Et inactivation of enzyme were begun by addition of an aliquot of a freshly prepared (daily) R(NCN)Et solution.

T_1 (measurement). The proton relaxation rate of water was measured at 24.3 MHz at 30° by a pulsed nuclear magnetic resonance (nmr) technique described previously (Cohn and Leigh, 1962). Values of the observed enhancement (Eisinger *et al.*, 1962), ϵ_{obsd}, were calculated from

$$\epsilon_{obsd} = \frac{1/T_1^* - 1/T_{1,0}^*}{1/T_1 - 1/T_{1,0}} \quad (1)$$

where T_1 and $T_{1,0}$ are the observed relaxation times in the presence and absence of Mn^{2+}, respectively. The terms with asterisks represent the same parameters in the presence of added complexing agent. The significance of ϵ_{obsd} in binary (Mn^{2+} and enzyme) and ternary (Mn^{2+}, enzyme, and PCHOHP) solutions has been discussed previously (Cooperman and Chiu, 1973).

Results

Stability of Enzyme. The stability of the enzyme was tested over a wide range of pH values in order to determine what the suitable pH range would be for studies on the effects of electrophilic reagents. The results are summarized in Figure 1 and are qualitatively similar to those obtained previously by Kunitz (1952).

Effect of PhGx. Enzyme incubated with PhGx showed an apparent first-order loss in activity (Figure 2) over at least three to four half-lives. The reaction was much faster at pH 8 than at pH 7, which parallels similar studies on ribonuclease (Takahashi, 1968). The pseudo-first-order constant at pH 7, 25°, 0.1 M PhGx is 0.082 min^{-1}, so that the calculated second-order rate constant is 0.82 M^{-1} min^{-1}. This is considerably (5.5 times) faster than the second-order rate constant of 0.15 M^{-1} min^{-1}, which can be calculated from Takahashi's data, obtained under identical experimental conditions, for reaction with both ribonuclease and free arginine. PhGx treatment appears to fully inactivate the enzyme. Enzyme incubated with 0.08 M PhGx at pH 7.85 for 75 min (>15 half-lives) showed no (<0.1%) residual activity.

[1] Abbreviations used are: PhGx, phenylglyoxal; R(NCN)Et, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PCHOHP, hydroxymethanebisphosphonate; PCH_2P, methanebisphosphonate; PCH_2CH_2P, 1-hydroxyethane-1,1-bisphosphonate; $PC(CH_3)OHP$, ethane-1-hydroxy-1,1-bisphosphonate; PNHP, imidobisphosphonate.

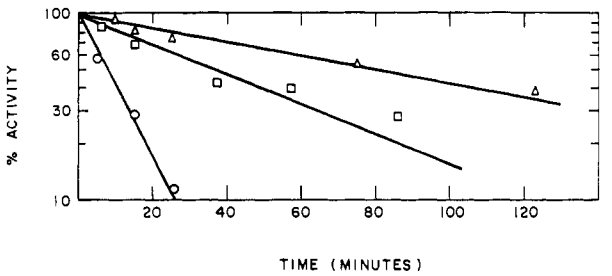

FIGURE 3: Mg^{2+}–PCHOHP protection of enzyme inactivation by 0.05 M R(NCN)Et in 0.003 M 2-(*N*-morpholinoethanesulfonate) (pH 6.0). No protection (○); 1 mM Mg^{2+}, 3×10^{-5} M PCHOHP (□); 1 mM Mg^{2+}, 1.25×10^{-4} M PCHOHP (△).

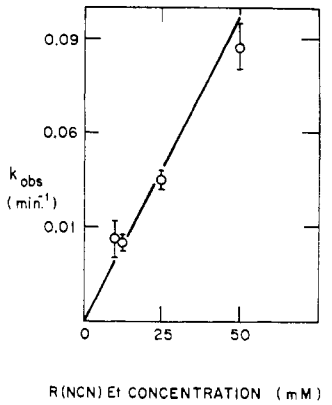

FIGURE 4: Dependence of the observed first-order rate constant for inactivation on R(NCN)Et concentration in 0.003 M 2-(*N*-morpholinoethanesulfonate) (pH 6.0).

Sample data illustrating the protective effects of Mg^{2+} and PCHOHP at pH 7.4, are also shown in Figure 2. The observed rate constants for these and other runs are listed in Table I.

Effect of R(NCN)Et. Enzyme incubated with R(NCN)Et showed an apparent first-order loss in activity (Figure 3) over two to three half-lives. The pseudo-first-order rate constants were proportional to reagent concentration (Figure 4). The 2nd order rate constant at pH 6.0 and 25° was 1.74 M^{-1} min^{-1}. This may be compared with a value of 0.9 M^{-1} min^{-1} for the reaction with *m*-nitrobenzoic acid at pH 4.75 and 25° (Hoare and Koshland, 1967). Addition of glycine methyl ester did not alter the rate of inactivation.

Enzyme incubated at 0.05 M reagent for long periods (>90 min, which is in excess of ten half-lives) retains 7 ± 1% residual activity. This is not due to hydrolysis of reagent since it is completely stable in the reaction medium for periods of 100 min (it is about half hydrolyzed in 3 days (N. Y. Chiu, unpublished experiments.)). Furthermore, addition of fresh reagent after a 100-min incubation leads to no further loss in activity. We thus believe the retention of activity to be real.

Protection experiments, similar to those performed with PhGx, are also shown in Figure 3. Measured rate constants are listed in Table II.

Effects of Other Reagents. Incubation of enzyme with 1 mM trinitrobenzenesulfonate (pH 8.0 (phosphate), 28°, 2.5 hr) led to the trinitrobenzylation of approximately 50 lysine residues/mol of enzyme, measured spectrophotometrically at 367 nm (Goldfarb, 1966), with only a 13% loss in activity. This corresponds to virtually complete derivatization of lysine residues (Negi and Irie, 1971). Incubation with 0.4 M *O*-methylisourea (pH 9.9, 0°) led to apparent first-order loss in activity with a half-life of 22 hr (measured over one half-life). At pH 9.0, 0°, a 3-day incubation with 0.32 M *O*-methylisourea led to a 13% loss in activity. Incubation with 0.1 M iodoacetate at pH 6.0 (phosphate) or pH 8.0 (phosphate) for 1 day at 26° led to activity losses of 8 and 24%, respectively.

TABLE I: Pseudo-First-Order Rates of PhGx Inactivation at 25°.[a]

No.	[Mg^{2+}], mM	[PP_i or PP_i Analog], mM	k_{obsd} (min^{-1})
1			0.076
2	1.2	PCHOHP, 0.01	0.076
3	1.2	PCHOHP, 0.05	0.059
4	1.2	PCHOHP, 0.07	0.048
5	1.2	PCHOHP, 0.10	0.046
6	1.2	PCHOHP, 0.20	0.041
7	1.2	PCHOHP, 0.30	0.041
8			0.085
9	1.0		0.071
10	0.30	PCHOHP, 0.01	0.050
11	0.30	PCHOHP, 0.03	0.030
12	0.30	PCHOHP, 0.10	0.031
13	0.30	PCHOHP, 0.30	0.024
14		PCHOHP, 1.0	0.035
15		PCHOHP, 20.0	0.022
16		PCH_2P, 1.0	0.084
17		PCH_2P, 40.0	0.066
18		PNHP, 1.0	0.062
19		PNHP, 20.0	0.068
20		PP_i, 10.0	0.043
21		$PC(CH_3)OHP$, 1.0	0.068
22		PCH_2CH_2P, 1.0	0.077

[a] Runs 1–7: 0.2 M *N*-ethylmorpholine·HCl (pH 7.4–1.0 M KCl); PhGx concentration, 0.08 M. Rate constants accurate to ±10%. Runs 8–22: 0.2 M *N*-ethylmorpholine·HCl (pH 7.0); PhGx concentration, 0.10 M. Rate constants accurate to ±15%.

TABLE II: Psuedo-First-Order Rate Constants for R(NCN)Et Inactivation at 25°.[a]

No.	[PCHOHP], mM	k_{obsd},[b] min^{-1}
1		0.087[c]
2		0.071
3	0.005	0.050
4	0.010	0.044
5	0.030	0.026
6	0.050	0.021
7	0.125	0.010
8	0.50	0.010
9	1.0	0.007

[a] Reaction medium contained 1.0 mM Mg^{2+} in 0.003 M 2-(*N*-morpholinoethanesulfonate) (pH 6.0). [b] Rate constants are accurate to ±10%. [c] No Mg^{2+} present.

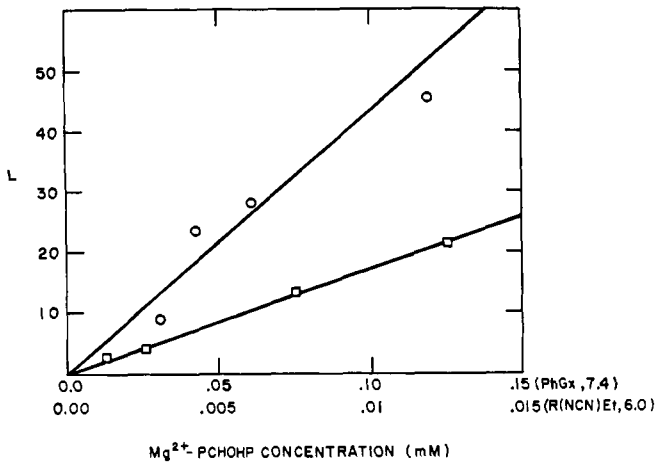

FIGURE 5: Plot of L *vs.* Mg^{2+}–PCHOHP concentration (eq 7) for PhGx inactivation at pH 7.4 (○) and R(NCN)Et inactivation at pH 6.0 (□).

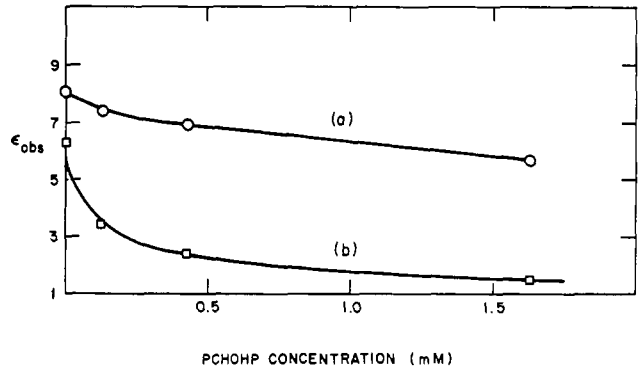

FIGURE 6: Effect of PhGx incubation on ϵ_{obsd} in solutions of enzyme, Mn^{2+}, and PCHOHP. Small aliquots of stock PCHOHP solutions were added to: (a) a solution of enzyme (0.053 mN), $MnCl_2$ (0.027 mM) in 0.2 M *N*-ethylmorpholine (pH 7.0) (○); (b) an identical solution in which enzyme had been preincubated with 0.11 M PhGx for 1 hr at room temperature (23 ± 1°) (□). The total dilution caused by PCHOHP addition was 25%.

Interpretation of Protection Results. Binding studies (Cooperman and Chiu, 1973) on ternary solutions of enzyme Mn^{2+}, and PCHOHP, have shown that the following four equilibria are needed to account for the data: K_D = (E) (M)/(EM) (2a), K_1 = (I) (M)/(MI) (2b), K_2 = (E) (MI)/(EMI) (2c), and K_I = (E) (I)/(EI) (2d), where (I) represents PCHOHP, (M) represents metal ion, and the constants are apparent equilibrium constants at fixed pH. On the basis of kinetic and magnetic resonance experiments we have shown that analogous equilibria are also important with Mg^{2+} in place of Mn^{2+} (Cooperman and Chiu, 1973). Since in the protection studies, enzyme concentration is very low compared to either I or Mg^{2+}, the conservation equations are

$$(E)_T = (E) + (EM) + (EMI) + (EI) \quad (3)$$

$$(M)_T = (M) + (MI) \quad (4)$$

$$(I)_T = (I) + (MI) \quad (5)$$

In principle each of the four enzyme species will react at a different rate with the electrophilic reagent

$$\text{observed rate of inactivation} = k_1(E) + k_2(EM) + k_3(EMI) + k_4(EI) \quad (6)$$

where k_1, k_2, k_3, and k_4 are observed pseudo-first-order rate constants at a given concentration of electrophile.

From eq 2 to 6 one obtains

$$\left[1 + \frac{(M)}{K_D}\frac{k_2 - k_{obsd}}{k_1 - k_{obsd}} + \frac{(I)}{K_I}\frac{k_4 - k_{obsd}}{k_1 - k_{obsd}}\right] \times \left[\frac{k_1 - k_{obsd}}{k_{obsd} - k_3}\right] = \frac{(MI)}{K_2} \quad (7)$$

where

$$(M) = \frac{(M)_T - (I)_T - K_1 \pm \sqrt{[(M)_T - (I)_T - K_1]^2 + 4K_1(M)_T}}{2}$$

Similar derivations for inhibition studies on other enzymes have been presented previously (Scrutton and Utter, 1965; O'Sullivan and Cohn, 1966).

In our experiments, the free PCHOHP concentration was small enough that $(I)/K_I$ could be set equal to zero. The left-hand side of eq 7, L, when plotted (Figure 5) against the calculated concentration of Mg^{2+}–PCHOHP complex yields a value for K_2 for both the PhGx and R(NCN)Et experiments. For the former experiment, k_3 was obtained by extrapolating k_{obsd} to infinite MI concentration. For the latter experiment, k_3 was obtained directly from Table II. Values for the relevant rate and equilibrium constants are summarized in Table III.

T_1 Experiments on Modified Enzyme. As previously demonstrated (Cooperman and Chiu, 1973), ϵ_{obsd} can be used as a measure of Mn^{2+} binding. Incubation of PhGx (0.056 M) with a solution of enzyme (0.03 mN) and Mn^{2+} (0.03 mM) for 150 min has little or no effect on ϵ_{obsd}. Under the conditions of this experiment, the half-life for inactivation of the enzyme is approximately 15 min. This lack of effect is not due to protection by Mn^{2+}. If, separately, enzyme and PhGx are in-

TABLE III: Equilibrium and Rate Constants.

	K_D (mM)	K_1 (mM)	K_2 (μM)	k_1, min^{-1}	k_2, min^{-1}	k_3, min^{-1}
0.08 M PhGx at pH 7.4	0.083[a]	0.75[c]	2.3	0.076	0.076	0.030
0.05 M R(NCN)Et at pH 6.0	0.29[b]	3.00[c]	0.58	0.087	0.071	0.010

[a] Measured by Cooperman and Chiu (1973) under similar conditions (pH 7.2). [b] Calculated assuming that the pH dependence of Mg^{2+} binding is the same as that measured for Mn^{2+} binding (Cooperman and Chiu, 1973). [c] K apparent values calculated from published values for proton and Mg^{2+} binding to PCHOHP, PCH_2P, and $PC(CH_3)OHP$ (Carroll and Irani, 1967; Grabenstetter *et al.*, 1967; Carroll and Irani, 1968).

cubated for 160 min and then Mn^{2+} is added, the same value of ϵ_{obsd} is obtained as without PhGx incubation.

A similar set of experiments were performed for enzyme incubated with R(NCN)Et with similar results. There was a small drop in ϵ_{obsd} after a long incubation, but this proceeded at a much slower rate than enzyme inactivation. These experiments are strong evidence that inactivation of enzyme by either reagent is not linked to inhibition of metal ion binding.

By contrast, there is a very marked effect of PhGx incubation on the value of ϵ_{obsd} in ternary solutions of enzyme, Mn^{2+}, and PCHOHP (Figure 6). Curve a is similar to what has been previously obtained (Cooperman and Chiu, 1973) and need not be discussed further. In terms of the four equilibria (2a–2d) which describe the ternary native enzyme–Mn^{2+}–PCHOHP solution, the simplest explanation for curve b is that the effect of PhGx incubation is to block equilibrium 2c, leaving 2a (as indicated above), 2b, and perhaps 2d. In this case, the effect of adding PCHOHP to a solution of enzyme and Mn^{2+} would be to pull Mn^{2+} off the enzyme through formation of a Mn^{2+}–PCHOHP complex, and, in addition, if 2d is retained, to push Mn^{2+} off the enzyme through formation of an E–I complex. ϵ_{obsd} would thus drop to the characteristic value of the Mn^{2+}–PCHOHP complex (~1.2) (Cooperman and Mark, 1971). This experiment is thus strong evidence that PhGx inactivation of enzyme is due to a blockage of the inorganic pyrophosphate binding site in the ternary enzyme–metal ion–PP_i complex.

Discussion

The major ambiguity in attempting to construct a structure function map of the active site using "group specific" reagents is that changes in any of the functions we measure–activity, metal ion binding, or pyrophosphate analog binding–may be due to changes in active site structure resulting from the modification of amino acids removed from the active site, rather than at the active site itself (Vallee and Riordan, 1969).

However, it is important to recognize that despite this inherent ambiguity, the group specific reagent approach has in fact been remarkably successful in identifying amino acid residues at the active site of a number of enzymes.[2] Moreover, in the present case, the probability that we are seeing modifications of the active site is increased by the demonstrated protective effects of substrate analogs. In any event, by indicating the types of electrophilic reagents toward which the enzymatic activity is sensitive, these studies provide a good starting point for the design of less ambiguous experiments, as, for example, the study of the effects of an affinity-labeling reagent (Singer, 1967) containing an electrophilic center toward which enzymatic activity is sensitive.

PhGx Inactivation. Experiments on the effects of PhGx incubation on inorganic pyrophosphatase suggest the presence of least one arginine at the active site of the enzyme which is vital to PP_i binding and hence to the activity of the enzyme. The evidence for this is as follows. (1) PhGx has been found to be a very specific reagent for arginine. Takahashi (1968) found that on incubation of ribonuclease with the reagent only arginine residues and the amino-terminal residue (lysine) are modified. Since we have shown that incubation of inorganic pyrophosphatase with either trinitrobenzenesulfonate or *O*-methylisourea has little effect on enzymatic activity, the evidence that inactivation is a result of arginine modification is strong. Moreover, our inactivation studies were performed at pH's 7.0 and 7.4, whereas the ribonuclease work was done at pH 8.0 and lower pH increases the relative reactivity of arginines over amino-terminal groups. (2) Inorganic pyrophosphatase has five to six arginine residues per protomer (Negi and Irie, 1971) (assuming two protomers per molecule), but the observations that (a) the rate of enzyme inactivation is significantly faster than the rate of free arginine modification, and (b) inactivation follows smooth pseudo-first-order kinetics for three half-lives, suggest that inactivation is due to modification of one or a small number of arginine residues of similar, abnormally high, reactivity. This suggestion should be readily testable through use of isotopically labeled PhGx, and such experiments are planned for the future. (3) Mg^{2+}–PCHOHP protects against inactivation. The apparent dissociation constant for Mg^{2+}–PCHOHP measured in the protection studies is equal to the product of K_2 and $(1 + [Mg^{2+}]/K_d)$ (Table III), or approximately 40 μM. Under nearly identical experimental conditions, the effect of Mg–PCHOHP on pyrophosphatase activity is consistent with it being a competitive inhibitor, with an apparent K_i of 120 μM (Cooperman and Chiu, 1973). As both of these values are only approximations, this agreement within a factor of three is consistent with the idea that the modification responsible for inactivation is occurring at the active site. The data in Table I show that both uncomplexed PP_i and PCHOHP also protect, and thus presumably bind to the active site, which is in accord with other binding studies (Cooperman and Chiu, 1973), and with recent analyses of steady-state kinetics (Rapaport *et al.*, 1972; Baykov *et al.*, 1972). (4) Table I also shows that Mg^{2+} alone does not protect against inactivation and a reciprocal observation from the T_1 studies is that PhGx treatment has no appreciable effect on Mn^{2+} binding. It does, however, block PCHOHP (and thus, presumably, PP_i) binding, so that the arginine we have placed at the active site is implicated as forming a vital part of the PP_i binding site in the ternary complex, which is of course perfectly reasonable on electrostatic grounds.

R(NCN)Et Inactivation. Our studies with R(NCN)Et are similar in approach to those discussed above with PhGx. This reagent is, at pH 6.0, almost totally specific for carboxyl groups (Hoare and Koshland, 1967). A lysine of abnormally low pK could, in principle, react, but this would probably not lead to inactivation based on our studies with *O*-methylisourea and trinitrobenzenesulfonate.

The important results of this work are as follows. (1) R(NCN)Et treatment does not lead to total inactivation of the enzyme, which leads to the conclusion that either there are no essential carboxylates or, less likely, that such carboxylates are inaccessible to solvent. (2) Mg^{2+} affords only slight protection against inactivation and, as with PhGx treatment, Mn^{2+} binding is not appreciably affected by R(NCN)Et treatment. This is evidence that carboxylate groups do not form an important part of the metal ion binding site. (3) Mg^{2+}–PCHOHP protects against inactivation which implicates a carboxyl group somewhere near the active site. The retention of 7–8% activity can be taken as evidence that this carboxyl group aids in the catalytic process but is not indispensable to it, but could equally as well be due to disruption of active site structure on modification of a neighboring residue with what is, after all, a rather bulky reagent. (4) The rate of the inactivation reaction is first order in reagent (Figure 4) and indepen-

[2] See, for example, ribonuclease A (Crestfield *et al.*, 1963a,b; Murdock *et al.*, 1966), carboxypeptidase A (Sokolovsky and Vallee, 1967), lysozyme (Parsons *et al.*, 1969; Lin and Koshland, 1969), and chymotrypsin (Jensen *et al.*, 1949).

dent of added glycine methyl ester, which is consistent with inactivation resulting from the initial covalent linkage between a carboxylate group and reagent, but does not exclude rapid internal amide formation *via* attack of, for example, a lysine side chain on the carboxylate-R(NCN)Et adduct.

Other Reagents. Both trinitrobenzenesulfonate and *O*-methylisourea are highly specific reagents for amino group modification (Vallee and Riordan, 1969). That virtually complete lysine modification by trinitrobenzenesulfonate is possible without major loss of activity is good evidence against the presence of an essential lysine at the active site of the enzyme. This argument is supported by the results with *O*-methylisourea, since the rate of inactivation found with this reagent is at least five to ten times slower than rates of lysine modification previously reported for other proteins (Kassell and Chow, 1966; Hughes *et al.*, 1949). A very rapid inactivation is observed when enzyme is incubated with 0.02 M maleic anhydride (pH 9.0, 0°) (N. Y. Chiu, unpublished observations), but this is probably due to a dissociation of the enzyme into subunits (Avaeva *et al.*, 1970a) rather than essential lysine modification. The ineffectiveness of iodoacetate in inactivating the enzyme supports the conclusion reached earlier by Negi *et al.* (1971) that the enzyme lacks essential cysteines.

There are few other published studies on active-site nucleophiles. There is a brief report of a reversible (on standing in buffer) inactivation of quite impure enzyme by diisopropyl fluorophosphate (Avaeva *et al.*, 1970b) which would be consistent with a phosphorylation of an essential serine, as with chymotrypsin (Jensen *et al.*, 1949) with subsequent slow hydrolysis to regenerate activity. However, Ridlington *et al.* (1972) find pyrophosphatase activity to be insensitive to incubation with phenylmethanesulfonyl fluoride, which argues against the presence of an essential serine. Nazarova *et al.* (1972) report isolation of phosphohistidine from enzyme incubated with inorganic phosphate at pH 5.0 and then subjected to alkaline hydrolysis, but go on to show that formation of the phosphohistidine is a result of phosphoryl migration during work-up so that these results cannot be taken as evidence for an active-site histidine.

Finally, Negi *et al.* (1972) have shown that *N*-bromosuccinimide oxidation of a single tryptophan leads to complete inactivation of enzyme, and that inactivation is blocked in the presence of Mg^{2+}–PCH_2P. Given the ionic character of Mg^{2+} and PP_i, a tryptophan is almost certainly not involved in binding, nor is it likely to participate in the catalytic process as a general acid, general base, or nucleophile. It could, however, form part of a hydrophobic pocket around bound PP_i which could lead to an acceleration of hydrolysis *via* a metaphosphate-type mechanism (Kirby and Varvoglis, 1967), and could also participate in hydrophobic bonding necessary for the maintenance of the correct tertiary or quaternary structure.

A Plausible Mechanism for Inorganic Pyrophosphatase. Our knowledge of the active site of inorganic pyrophosphatase is at present so incomplete and even primitive as compared with what is known about some other enzymes[3] that in a strict sense it is premature to entertain detailed mechanisms for inorganic pyrophosphatase action. Nevertheless, the presentation of a plausible mechanism which is consistent with what is known can serve as the basis for further discussion, as a guide to future work, and as a visual summary of previous work, and

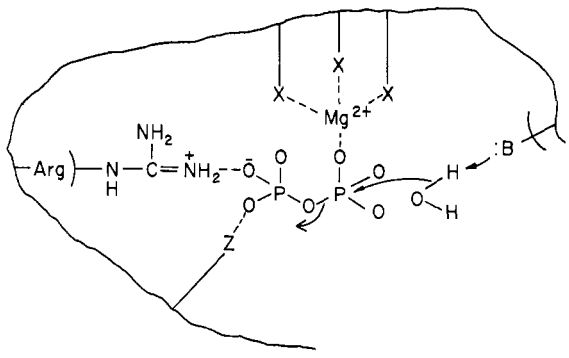

FIGURE 7: A plausible mechanism for inorganic pyrophosphatase.

it is for these reasons that we present the mechanism shown in Figure 7.

In this proposed mechanism substrate is shown as being the 1:1 Mg–PP_i complex, in accord with the results of kinetic and binding studies (Cooperman and Chiu, 1973; Moe and Butler, 1972; Rapaport *et al.*, 1972; Baykov *et al.*, 1972) although it should be mentioned that the Mg_2–PP_i complex is apparently a substrate as well, having a V_{max} four to five times slower than that for $MgPP_i$. Arginine is depicted as binding to the phosphoryl group being displaced because of results showing that the rate of nucleophilic attack on a phosphoryl dianion is strongly accelerated by increasing the conjugate acidity of the leaving group (Kirby and Younas, 1970). Similarly, it is important that metal ion is shown coordinated to the phosphoryl group undergoing nucleophilic attack by incipient hydroxide ion, since model system work indicates that without the charge shielding effect of such coordination, electrostatic repulsion causes anionic attack to be immeasurably slow (Lloyd and Cooperman, 1971).[4]

Figure 7 shows a direct activated water attack on PP_i without formation of a covalent phosphorylated enzyme intermediate (E–P) but this is only for reasons of simplicity since, despite a fair amount of work, this question remains unresolved. Cohn (1958) found that the enzyme catalyzes ^{18}O exchange between P_i and water 500 times faster than reversal of the overall reaction, which would be consistent with an E–P intermediate but which does not require it. More recently, Avaeva and Mevkh (1970) have claimed isolation of [^{32}P]P_i-labeled enzyme on incubation of enzyme with either [^{32}P]P_i or [^{32}P]PP_i at pH 5.0. Their results are most consistent with a serine as the phosphorylation site, and Figure 7 can readily be altered to fit an E–P mechanism by substituting a serine side chain for the attacking water. However, Butler reports no evidence for covalently bound ^{32}P on incubation of enzyme with [^{32}P]PP_i and in related work, also reports no evidence for an enzyme-catalyzed phosphoryl transfer to nucleophiles other than water (Butler, 1971). While the reasons for this apparent contradiction are unclear, it should be emphasized that the isolation of an E–P species, while interesting in itself, is no proof that the enzymatic reaction proceeds *via* an E–P intermediate. In this regard, it is perhaps significant that whereas both PP_i hydrolysis and ^{18}O exchange show absolute requirements for added divalent metal ion, Avaeva and Mevkh find that the extent of labeling actually increases in the absence of added divalent metal ion. Further-

[3] See, for example, carboxypeptidase A (Hartsuck and Lipscomb, 1971), ribonuclease A (Richards and Wyckoff, 1971), chymotrypsin (Blow, 1971; Hess, 1971), and lysozyme (Chipman and Sharon, 1969).

[4] The essential points of this model would be unchanged if the proposed roles of arginine and metal ion were reversed.

more, at pH 7.2, P_i up to a concentration of 0.025 M shows no inhibition of enzymatic activity (B. S. Cooperman, unpublished observations), whereas it might be expected to if E–P were a true intermediate.

We do not as yet know the identity of the ligands to metal ion, of the postulated general base for water activation, or of the postulated additional binding site for PP_i. Based on the amino acid modification studies summarized above, which seem to have eliminated lysines, cysteines, aspartates, and glutamates, the remaining residues which could be present in these roles are histidine, tyrosine, serine, and threonine. In an accompanying paper (Cooperman and Chiu, 1973) we presented a limited study on the pH dependence of Mn^{2+} binding, which would be consistent with at least one histidine side-chain ligand. Further work on the roles of these residues is currently underway in our laboratory and will be the subject of future publications.

Acknowledgment

We thank Dr. Mildred Cohn for use of her pulsed nmr equipment and Dr. Larry Butler for making his results available to us prior to publication.

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