# Glutamic Acid-149 Is Important for Enzymatic Activity of Yeast Inorganic Pyrophosphatase†

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ABSTRACT: Modification of *Saccharomyces cerevisiae* inorganic pyrophosphatase (PPase) with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide is known to lead to a loss of enzymatic activity, the rate of which is decreased in the presence of ligands binding to the active site [Cooperman, B. S., & Chiu, N. Y. (1973) *Biochemistry 12*, 1676–1682; Heitman, P., & Uhlig, H. J. (1974) *Acta Biol. Med. Ger. 32*, 565–594]. In this work we show that, when such inactivation is carried out in the presence of [$^{14}$C]glycine ethyl ester (GEE), (a) GEE is covalently incorporated into PPase, (b) incorporation into the most highly labeled tryptic peptide is site-specific, as evidenced by the reduction of such incorporation in the presence of the active site ligands $Zn^{2+}$ and $P_i$, (c) the extent of formation of this specifically labeled peptide correlates with the fractional loss of PPase activity, and (d) the specifically labeled peptide corresponds to residues 145–153 and the position of incorporation within this peptide is Glu-149. The significance of our findings for the location of the active site and for the catalytic mechanism of PPase is briefly considered in the light of the 3-Å X-ray crystallographic structure of Arutyunyun and his colleagues [Arutyunyun, E. G., et al. (1981) *Dokl. Akad. Nauk SSSR 258*, 1481–1485; Kuranova, I. P., et al. (1983) *Bioorg. Khim. 9*, 1611–1919; Terzyan, S. S., et al. (1984) *Bioorg. Khim. 10*, 1469–1482].

The *Saccharomyces cerevisiae* inorganic pyrophosphatase (EC 3.6.1.1; PPase)[1] is one of a limited number of phosphoryl-transfer enzymes about which sufficient is known or under active study that there is a very real prospect of being able to understand its catalytic mechanism in terms of its detailed structure (Cooperman, 1982). The enzyme is a dimer of identical monomers of $M_r$ 32 042. Its complete sequence of 285 amino acids has been determined (Cohen et al., 1978), and an X-ray crystallographic structure at 3-Å resolution has been published (Arutyunyun et al., 1981; Kuranova et al., 1983; Terzyan et al., 1984). PPase has been shown to require three divalent metal ions per subunit for activity (Moe & Butler, 1972; Springs et al., 1981; Welsh et al., 1983b; Knight et al., 1984), to catalyze inorganic pyrophosphate ($PP_i$) hydrolysis via direct phosphoryl transfer from $PP_i$ to water (Gonzalez et al., 1984), and to employ a common mechanism for catalysis of $PP_i$ hydrolysis, $H_2O$–inorganic phosphate ($P_i$) oxygen exchange and $PP_i$–$P_i$ exchange (Springs et al., 1981). In addition, NMR and EPR studies have shown a mutual proximity of at least three divalent metal ions on the enzyme surface, presumably clustered at the active site (Banerjee & Cooperman, 1983; Knight et al., 1984; Banerjee et al., 1986), as well as evidence for both inner and outer sphere binding of divalent metal ions to inorganic phosphate ($P_i$) bound at the active site (Hamm & Cooperman, 1978; Cooperman et al., 1981; Welsh et al., 1983a; Welsh & Cooperman, 1984). Experiments with Co(III), $Cr^{3+}$, and lanthanide ion complexes of $PP_i$ also provide evidence for inner sphere interaction between substrate and metal ion at the active site (Knight et al., 1981; Ting & Dunaway-Mariano, 1984).

Arutyunyun and his colleagues at the Institute of Crystallography (Moscow) have located a cavity within the 3-Å structure for PPase that contains binding sites for four divalent metal ions as well as a binding site for $CaPP_i$, a known competitive inhibitor of the enzyme (Moe & Butler, 1972b), and have proposed that the active site for PPase falls within this cavity. The amino acid residue Arg-77 is present within the cavity, a finding that agrees with our previous result (Bond et al., 1980) identifying Arg-77 as as probable active site residue on the basis of chemical modification studies with [$^{14}$C]phenylglyoxal.

Earlier work by ourselves and others (Cooperman & Chiu, 1973; Heitmann & Uhlig, 1974; Cooperman et al., 1981) demonstrated that PPase was inactivated by water-soluble carbodiimides and further that such inactivation is protected against by substrate or substrate inhibitors. These results led to the suggestion that an active site carboxylate was being modified, on the basis of the chemical selectivity of carbodiimides for carboxylate modification (Hoare & Koshland, 1967; Carraway & Koshland, 1972). In the present work we demonstrate that incubation of PPase with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) and [$^{14}$C]glycine ethyl ester (GEE) leads to site-specific labeling of Glu-149, the extent of which correlates quantitatively with PPase activity loss. Glu-149 is also present within the cavity described above, providing further evidence that this cavity contains the active site of PPase.

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[1] Abbreviations: EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GEE, glycine ethyl ester; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $P_i$, inorganic phosphate; PPase, inorganic pyrophosphatase; $PP_i$, inorganic pyrophosphate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TPCK, *N*-*p*-tosylphenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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## Experimental Procedures

### *Materials*

[$^{14}$C]Glycine ethyl ester (GEE) (50 mCi/mol) was obtained from New England Nuclear. Guanidine hydrochloride, TPCK-trypsin, nonradioactive GEE, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC), and 2-(*N*-morpholino)ethanesulfonic acid (Mes) were from Sigma. Ammonium acetate, acetic acid, trifluoroacetic acid (TFA) and acetonitrile, all HPLC grade, were from Fisher. All other reagents were reagent-grade unless otherwise specified.

PPase was prepared by a major modification of our previously (Cooperman et al. 1973; Bond, 1979) described procedure, the most important change being the addition of the protease inhibitor phenylmethanesulfonyl fluoride (Sigma) throughout the purification. A detailed description of this modified procedure will appear elsewhere (A. Banerjee et al. unpublished results). The specific activity of PPase used in most of this work was 480 $\mu$mol of $PP_i$ hydrolyzed min$^{-1}$ mg$^{-1}$. Enzyme having lower specific activity (320 $\mu$mol of $PP_i$ hydrolyzed min$^{-1}$ mg$^{-1}$) was used in the earlier part of this work dealing with the inactivation of PPase activity. PPase concentration was determined either by $A_{280}$ (1.45 for 0.1% solution; Kunitz, 1952) or by the Bradford (1976) assay with bovine serum albumin (Polysciences) as a standard. Application of both assays to the same samples gave results in agreement to ±5%. PPase concentration is expressed in terms of subunits. Concentrated (4 mM) solutions of PPase in 100 mM Tris-HCl (pH 7.05) were prepared in a Centricon microconcentrator (Amicon) with a 10000 molecular weight cutoff.

### *Methods*

*Inactivation of PPase Activity.* All incubations were carried out in a constant-temperature water bath at 25 °C. Incubation mixtures were made up by combination of three separate solutions: first, a PPase (0.2 mM) solution in Mes buffer (75 mM, pH 6.0) preincubated as appropriate with $ZnCl_2$ (6.6 mM) and/or inorganic phosphate ($P_i$) (40 mM); second, an aqueous solution of GEE (200 mM), adjusted to pH 6.0 with NaOH; third, an aqueous solution of EDAC (500 mM), made up just prior to use and added last. These solutions were combined in a volume ratio of 1.0:2.2:0.8. For incubations not including GEE, the GEE solution was replaced by water.

Aliquots, containing 0.15–0.9 nmol of PPase were assayed for activity as a function of incubation time, with the standard titrimetric assay (Cooperman et al., 1973). The large dilution (300–2000-fold) on addition of the aliquot to the assay buffer served as an effective quench. All assays were measured in triplicate.

*Covalent Modification of PPase by [$^{14}$C]GEE Incorporation.* Incubations leading to covalent incorporation of [$^{14}$C]GEE into PPase were carried out in a very similar fashion to that described above for PPase inactivation, except that a higher concentration was used. A solution (15 $\mu$L) containing PPase (2.6 mM) and $ZnCl_2$ (6.6 mM) in 100 mM Mes (pH 6.0) was preincubated for 90 min at 25 °C; 2 $\mu$L of $P_i$ (200 mM, pH 6.0) was added, followed by the addition of 25 $\mu$L of [$^{14}$C]GEE (200 mM, pH 6.0, 2.0–2.5 Ci/mol) and 10 $\mu$L of EDAC (500 mM). After a 5-min incubation, corresponding to an enzyme activity loss of 50 ± 5%, the modification reaction was quenched by addition of glacial acetic acid (15 $\mu$L) and quick freezing in a dry ice/ethanol slurry.

*Purification and Trypsin Digestion of Covalently Modified PPase.* [$^{14}$C]GEE–PPase and PPase were purified away from the other components of the modification reaction mixture by HPLC, on a Perkin-Elmer Series 4 liquid chromatograph equipped with a 3600 Model data station, a 15S-100 autosampler, an LC-15B detector, and a Synchropak RP-P silica column [6.5-$\mu$m silica; 300-Å pore; 250 × 4.1 mm (inner diameter)] obtained from SynChrom. Fractions were collected at 1-min intervals and their radioactivities determined by making up aliquots in a standard Triton–toluene cocktail (Springs et al., 1981) and counting in a Beckman LS-1801 liquid scintillation counter. The collected PPase fractions were lyophilized, dissolved in 1 mL of 5 M guanidine hydrochloride, and dialyzed overnight against 100 mM ammonium bicarbonate, pH 8.3. TPCK-trypsin was added in a weight ratio of 1:50 to a PPase (1 mg/mL) solution, and digestion was allowed to proceed for 10 h at 37 °C. A second addition of TPCK-trypsin in the same amount was then made, and following an additional 10-h incubation, the digestion was quenched by addition of glacial acetic acid. Tryptic peptides were separated and analyzed for radioactivity by the same HPLC and counting procedures described above.

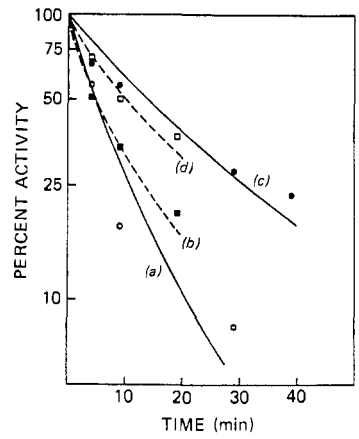



FIGURE 1: Inactivation of PPase by EDAC. Residual enzymatic activity in the presence or absence of either GEE or $Zn^{2+}P_i$: (a) –GEE, $-Zn^{2+}P_i$ (○); (b) +GEE, $-Zn^{2+}P_i$ (■); (c) –GEE, $+Zn^{2+}P_i$ (●); (d) +GEE, $+Zn^{2+}P_i$ (□). The final concentrations used in these studies were as follows: EDAC, 100 mM; GEE, 110 mM; $Zn^{2+}$, 1.65 mM; $P_i$, 10 mM. The pseudo-first-order rate constant for activity loss in curve a is 0.17 min$^{-1}$.

*Amino Acid and Peptide Sequence Analyses.* Peptides (1–3 nmol) were hydrolyzed in distilled 6 N HCl (500 $\mu$L) (Fisher) in sealed tubes at 110 °C for 24 h. Following evaporation, the hydrolysate was dissolved in Citrate Buffer (pH 2.2) for Amino Acid Analysis (Beckman) and analyzed on a Beckman 6300 amino acid analyzer.

Automated Edman peptide sequencing was carried out on a 470A gas-phase sequencer from Applied Biosystems. The PTH-amino acids were identified on a Hewlett-Packard 1090A HPLC system equipped with a Zorbax (Du Pont) ODS column. Elution was carried out with a sodium acetate (pH 5.0)–acetonitrile system at 50 °C. The average repetitive yield, on the basis of the absorbance of the PTH-amino acids, was 77 ± 4%.

## Results

*Inactivation of PPase with EDAC and Incorporation of [$^{14}$C]GEE.* As previously described (Chiu & Cooperman, 1973; Heitmann & Uhlig, 1974; Springs et al., 1981), incubation of PPase with EDAC leads to an essentially complete loss of enzymatic activity, in a second-order process characterized by a rate constant of 1.74 M$^{-1}$ min$^{-1}$ (pH 6.0, 25 °C). In the results presented in Figure 1, we first duplicate this result (curve a) and demonstrate further that the rate of inactivation is reduced 2-fold on addition of $Zn^{2+}P_i$ (curve c)

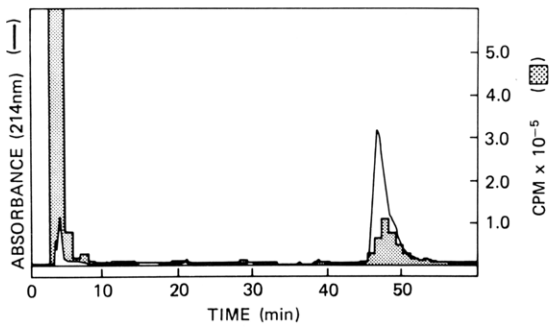



FIGURE 2: RP-HPLC purification of [$^{14}$C]GEE-modified PPase and PPase from modification reaction mixture. A sample of quenched reaction mixture containing PPase (1.0–1.5 mg), [$^{14}$C]GEE, and EDAC was applied to a Synchropak RP-P column (see Methods) and eluted with a linear gradient of 0%–60% acetonitrile in 60 min. TFA (0.1%) was present throughout the gradient. The flow rate was 0.7 mL/min. The shaded areas represent radioactivity from [$^{14}$C]GEE.

Table I: Relative Yields of Peak X[a]

| added ligands | relative yield[b] |
|---|---|
| none | 1.00 |
| $Zn^{2+}$ (1.65 mM); $P_i$ (10 mM) | 0.49 ± 0.04 (5) |
| $Zn^{2+}$ (2.00 mM) | 0.57 ± 0.03 (2) |
| $P_i$ (10 mM) | 0.68 ± 0.03 (2) |

[a] Measured by radioactivity in peak X. [b] Values in parentheses correspond to number of determinations.

and that similar results are obtained in the presence or absence of [$^{14}$C]GEE (compare curves a and c and curves b and d).

When EDAC inactivation of PPase is carried out in the presence of [$^{14}$C]GEE, it is accompanied by covalent incorporation of radioactivity into PPase. The extent of such incorporation can be estimated by RP-HPLC analysis of a quenched reaction mixture as shown in Figure 2. For the experiment shown, a sample of PPase inactivated to the extent of 50 ± 5% in the absence of $Zn^{2+}P_i$ (unprotected) incorporated 2.3 GEE/PPase subunit. A strictly comparable sample prepared in the presence of $Zn^{2+}P_i$ (protected) incorporated 1.7 GEE/PPase subunit. In these experiments, the recovery of PPase was 75 ± 5%.

*Isolation of a Tryptic Peptide Specifically Labeled with [$^{14}$C]GEE.* Mixtures of PPase and [$^{14}$C]GEE-modified PPase, purified as described in Figure 2, were subjected to tryptic digestion, and the resulting peptides were separated by RP-HPLC, as shown in Figure 3. Two major radioactive peaks are seen in the unprotected sample (Figure 3A), the larger at fraction 70 corresponding to 25% of total eluted radioactivity and the smaller at fraction 60 corresponding to 15%. The same two peaks are seen when modification is carried out in the presence of $Zn^{2+}P_i$ (protected sample) (Figure 3B). When the radioactivity in the protected sample is subtracted from that of the unprotected sample (Figure 3C), it is clear that the major radioactive peak, eluting at fraction 70 (peak X), is the only one showing specific labeling. Twice as much as of peak X is formed in the unprotected (Figure 3A) as compared with the protected sample (Figure 3B), as can be seen by examination of both the radioactivity (Table I) and the $A_{214}$ profiles. For an unprotected sample inactivated to the extent of 50 ± 5%, the amount of peak X formed, as calculated by its radioactivity and the specific radioactivity of GEE, corresponds to 0.54 per PPase subunit, thus demonstrating that the coupled EDAC–GEE reaction leading to the formation of peak X fully accounts for the loss of PPase activity. As indicated in Table I, $Zn^{2+}$ and $P_i$ added alone also decrease the level of peak X formation, but to a lesser extent compared with $Zn^{2+}$ and $P_i$ added together.

Table II: Amino Acid Analysis of Peptide X″[a]

| amino acid | amount measured | mol/mol of peptide |
|---|---|---|
| Asp | 1.75 | 2.24 (2) |
| Thr | 0.74 | 0.95 (1) |
| Ser | 0.166 | 0.21 (0) |
| Glu | 1.77 | 2.26 (2) |
| Gly | 1.92 | 2.46 (2) |
| Ala | 0.144 | 0.18 (0) |
| Ile | 0.115 | 0.15 (0) |
| Leu | 0.74 | 0.95 (1) |
| Tyr | 0.09 | 0.11 (0) |
| Phe | 0.08 | 0.10 (0) |
| His | 0.03 | 0.04 (0) |
| Lys | 0.78 | 1.00 (1) |

[a] The amount of each amino acid measured was normalized to lysine. The composition of the peptide is $Asp_2ThrGlu_2Gly_2LeuLys$. Trp is not determined.

Table III: Automated Edman Peptide Sequence Analysis of Peptide X″

| cycle | major amino acids released[a] | expected for 145–153 | cpm/nmol ($\times 10^{-3}$) |
|---|---|---|---|
| 1 | Leu (1.00), Tyr (0.05), Gly (0.05) | Leu | |
| 2 | Asp (1.00), Ala (0.11), Leu (0.07), Ile (0.06) | Asp | |
| 3 | Glu (1.00), Asp (0.2) | Glu | |
| 4 | Gly (1.00), A[b] (0.21), Lys (0.2), Glu (0.16), Asp (0.08), Ile (0.05) | Gly | |
| 5 | Tyr[c] (1.00), B[b] (0.50), Glu (0.14), Gly (0.10) | Glu | 4.2 |
| 6[d] | Thr (1.00), C[b] (0.47), Tyr (0.32), Ala (0.70), Asn (0.28), D[b] (0.22), Gly (0.08) | Thr | 0.56 |
| 7 | Asp (1.00), Val (0.10), Asn (0.09), Thr (0.06) | Asp | <0.1 |
| 8 | Trp (1.00), Asp (0.3), E[b] (0.14), Tyr (0.07) | Trp | |
| 9 | Lys (1.00), Trp (0.05) | Lys | |

[a] Relative amounts of amino acids released are shown in parentheses. [b] Peaks A–E do not correspond to any of the standard PTH-amino acids obtained from Pierce. [c] This material, though migrating close to the position of PTH-Tyr, probably corresponds to the PTH derivative of γ-glycine ethyl ester amide of Glu. [d] Thr is usually found in low yield on Edman analysis, accounting for the high relative yields of other amino acids (Chang, 1979).

*Identification of Glu-149 as the Position of Specific Labeling.* The material in peak X (Figure 3) was pooled and subjected to two further RP-HPLC purification steps (Figure 4). As is clear from its high $A_{280}$ (Figure 4D), the resulting highly purified peptide (X″) is tryptophan-containing. This peptide was next subjected to both amino acid analysis (Table II) and automated Edman peptide sequence analysis (Table III). For each cycle of the latter, an aliquot was removed prior to the injection of the PTH-amino acid for HPLC analysis, and the radioactivity in the aliquot was determined. The results of these analyses clearly show that peptide X″ is a nonapeptide corresponding to residues 145–153 (Cohen et al., 1978) having the sequence

•••Leu AspGluGlyGlu*ThrAspTrpLys•••
145 149 153

in which Glu-149 has been derivatized with [$^{14}$C]glycine ethyl ester. Specifically: (1) as noted above, the peptide is Trp-containing; (2) the amino acid analysis is consistent with this assignment, and the presence of a second glycine indicates that a derivative has been formed; (3) the sequence also supports this assignment, and the release of virtually all of the radioactivity in cycle 5 indicates that Glu-149 is the site of derivatization.

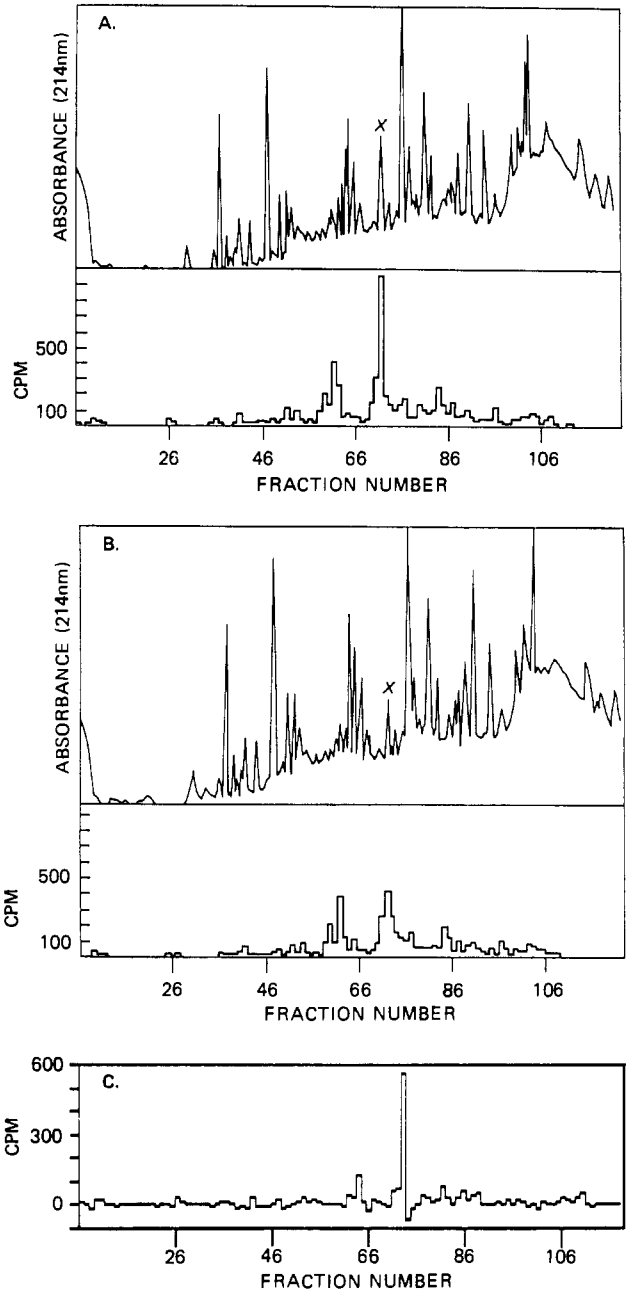



FIGURE 3: RP-HPLC purification of peptides from TPCK-trypsin digestion of a mixture of PPase and [$^{14}$C]GEE-modified PPase. TPCK-trypsin-digested mixtures of PPase and [$^{14}$C]GEE-modified PPase (1 nmol) were applied to a Synchropak RP-P column (see Methods) and eluted with the following gradient: 0–10 min, 0% acetonitrile; 10–85 min, 0%–25% acetonitrile; 85–110 min, 25%–75% acetonitrile; 110–120 min, 75%–80% acetonitrile. TFA (0.1%) was present throughout the gradient. The flow rate was 0.7 mL/min. (A) Sample prepared from PPase modified in the absence of $Zn^{2+}P_i$; (B) sample prepared from PPase modified in the presence of $Zn^{2+}P_i$; (C) calculated cpm profile for sample A minus sample B.

To further characterize the material released in cycle 5 (Table III), an aliquot was hydrolyzed in 6 N HCl at 110 °C, and the hydrolysate was subjected to amino acid analysis. The results (Figure 5) show the presence of glycine, formed to the extent of 100% of expected (calculated on the basis of the specific radioactivity of [$^{14}$C]glycine ethyl ester), and glutamate, formed to the extent of 20% of expected, as well as an unknown material formed as a side product of either the sequencing or the hydrolysis step. Separately, an authentic sample of PTH-glutamate was acid-hydrolyzed and the hydrolysate subjected to amino analysis as above, giving gluta-

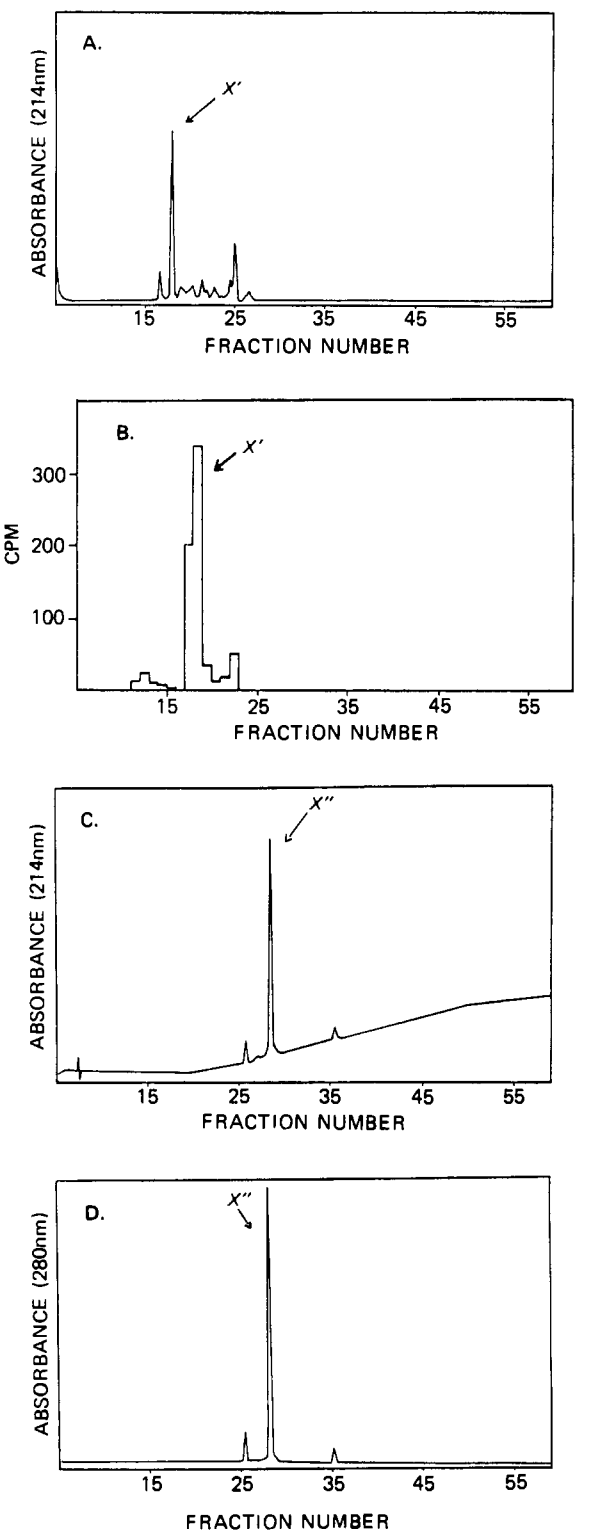



FIGURE 4: RP-HPLC purification of peak X. Peak X (Figure 3A) was pooled, lyophilized, redissolved in 5 M guanidine hydrochloride, applied to a Synchropak RP-P column (see Methods), and eluted with a linear gradient of ammonium acetate–water–acetonitrile going from 25 mM:100%:0% to 20 mM:40%:60% in 60 min. The flow rate was 1.0 mL/min. The results are shown in panels A and B. Peak X′ (fractions 17–19) was pooled, lyophilized, redissolved in 5 M guanidine hydrochloride, applied to a Synchropak RP-P column, and eluted with a linear gradient of 0%–50% acetonitrile in 50 min. TFA (0.1%) was present throughout the gradient. The flow rate was 1 mL/min. The results are shown in panels C and D. From a total of 32 nmol of PPase containing 2.3 [$^{14}$C]GEE/PPase and subjected to trypsin digestion, we isolated 5 ± 1 nmol (estimated by the recovered radioactivity and the known specific radioactivity of GEE) of peak X″ as a result of the three HPLC steps described in Figure 3 and this figure.

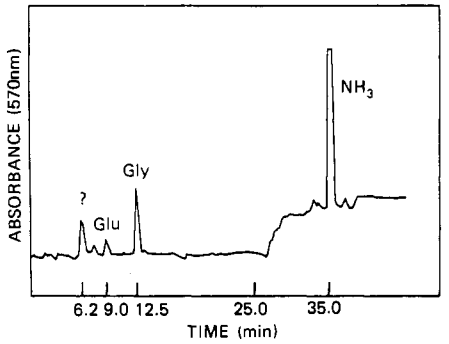



FIGURE 5: Amino acid analysis of acid hydrolysate of PTH derivative released in the fifth cycle of automated Edman analysis of peak X''. Note the peaks at 12.5 (glycine) and 9.0 min (glutamate) as well as the unidentified peak at 6.2 min. The large peak at 35.2 min corresponds to ammonia.

mate, but in only 20% yield (based on the absorbances of the PTH-Glu starting material and the ninhydrin–Glu hydrolysis product). These results further support the notion that the radioactive material released in cycle 5 of the automated Edman analysis is the PTH-derivative of the $\gamma$-glycine ethyl ester amide of a glutamate and that Glu-149 is the site of specific labeling in the coupled EDAC–GEE reaction.

It is worth noting that formation of the nonpeptide 145–153 requires TPCK-trypsin cleavage of the Leu-144–Leu-145 peptide bond. Although there is ample precedent for cleavage after residues other than Arg or Lys when TPCK-trypsin is employed, presumably reflecting either contamination of the trypsin with other protease activities or the presence of a family of partially proteolyzed trypsins having different specificities (Keil, 1982; Gilles et al., 1984), to our knowledge this is the first reported example of a Leu–Leu cleavage.

## Discussion

The major conclusion that may be drawn from this work is that Glu-149 is either important or essential for PPase activity. Three experimental results obtained herein support this conclusion: first, for the inactivation conditions chosen, the fraction of Glu-149 derivatized corresponds quantitatively to the fraction of enzyme activity lost; second, in the presence of a substrate for the enzyme, $ZnP_i$, the rate of both Glu-149 modification and activity loss is significantly reduced [some decrease in rate is also seen in the presence of $Zn^{2+}$ and $P_i$ added separately (Table I), and both of these are active site ligands (Cooperman, 1982)]; third, the rate of Glu-149 derivatization is much more rapid than that of any other Glu or Asp residue.

PPase catalyzes oxygen exchange between $H_2O$ and $P_i$. In previous work we have shown that such exchange proceeds via $P_i$ binding to two sites per PPase subunit and have measured both binding and kinetic parameters for $P_i$ in the presence of several divalent metal ions that can serve as cofactors for this reaction (Springs et al., 1981; Cooperman et al., 1981; Welsh et al., 1983b). It would thus be straightforward to interpret the $P_i$- (or $M^{2+}P_i$ complex) induced decrease in the rate of PPase modification as resulting from $P_i$ (or $M^{2+}P_i$) binding to the enzyme and reducing access by EDAC to the active site. However, because the rate of EDAC inactivation of PPase is first order in EDAC (Cooperman & Chiu, 1973), a less interesting possibility that must be considered is that the $P_i$-induced rate decrease arises from $P_i$ reacting with EDAC to reduce EDAC concentration [see Metz and Brown (1969) and George and Borders (1979)]. Among divalent metal ion cofactors, $Zn^{2+}$ is second only to $Mg^{2+}$ in the enzymatic activity it confers on PPase. We have chosen to use $Zn^{2+}$ in the current study since $P_i$ binding to PPase is considerably tighter in the presence of $Zn^{2+}$ than in the presence of $Mg^{2+}$. As a result, we can achieve significant occupancy of the active site at a concentration of $P_i$ (10 mM) that is well below the EDAC concentration (100 mM) used to carry out modification, thus eliminating reduction of EDAC concentration as a possible explanation for observed $P_i$ protection. However, it should be noted that, in the opposite sense, reaction with EDAC could significantly reduce the $P_i$ concentration, so that the observed protection effect might reflect less than full occupancy of the two $P_i$ binding sites. In addition, although at least three divalent metal ions per subunit are required for the tightest binding of $P_i$, we were forced by solubility considerations not to exceed a $Zn^{2+}$:$PP_i$ ratio of 2.5 (see Results). These considerations lead us to conclude that the protection observed in the presence of $ZnP_i$ represents a true active site effect but that the 2-fold effect (Table I) we observe must be considered a lower limit since we are not certain that both $P_i$ sites are fully saturated during EDAC inactivation of the protected sample.

Reaction of carbodiimides with carboxylic acids follows the formal rate law shown in eq 1 (Ibrahim & Andrews, 1980),

$$\text{rate} = k[\text{carbodiimide}][RCO_2H] \quad (1)$$

and there are many cases known in which the rate of inactivation of an enzyme with a carbodiimide decreases as a function of pH [e.g., Pho et al. (1977), Ariki and Fukiu (1978), Matsuo et al. (1980), Fleer et al. (1983), and Takata et al. (1985)], presumably reflecting formation of the carboxylate ion. With respect to the inactivation of PPase with EDAC, two points are worth noting: first, the rate of reaction is essentially independent of pH over the pH range 5.5–7.0 (Cooperman & Chiu, 1973; Cooperman et al., 1981; M. A. Gonzalez, unpublished experiments); second, the rate of Glu-149 modification is quite rapid compared to that of other carboxylic acid side chains in the enzyme. While there are a total of 42 such side chains in PPase (19 Glu and 23 Asp), in the modification experiment we report fully 23% (0.54/2.3) of the [$^{14}$C]GEE incorporated into PPase is found in Glu-149. Both of these points are well accounted for by invoking the presence of a general acid that is strategically placed to selectively catalyze EDAC addition to Glu-149 by analogy with the known general acid catalysis of the reaction of acetate ion with EDAC (Ibrahim & Andrews, 1980). Thus, as the pH is raised above pH 5 and the reactivity of most carboxylic side chains decreases, reaction at Glu-149 as described by eq 2

$$\text{rate} = k_{HA}[\text{carbodiimide}][RCO_2^-][HA] \quad (2)$$

would, up to the $pK_a$ of the general acid, be pH-independent. Studies of the pH dependence of $V$ and $V/K_m$ for PPase catalysis of $PP_i$ hydrolysis (Knight et al., 1981) have yielded evidence for the presence of a general acid at the active site, having a $pK_a$ of 7.6. It would thus be of interest to extend the EDAC inactivation studies to higher pH, to test whether the putative general acid catalyst of EDAC addition has the same $pK_a$.

In the present work we identify Glu-149 as important or essential for PPase activity, and earlier work from this group led to the identification of Arg-77 as another probable active site residue (Bond et al., 1980). A representation of the active site cavity, putatively identified by the Moscow crystallogaphy group (Kuranova et al., 1983; Terzyan et al., 1984), is presented in Figure 6 and shows the locations of the four metal ions and of the Arg-77 and Glu-149 side chains. Although it would be premature to engage in model building for the exact placement of $PP_i$ and $H_2O$ (or of two $P_i$s) within this cavity, it is worth noting that a mechanism in which Glu-149

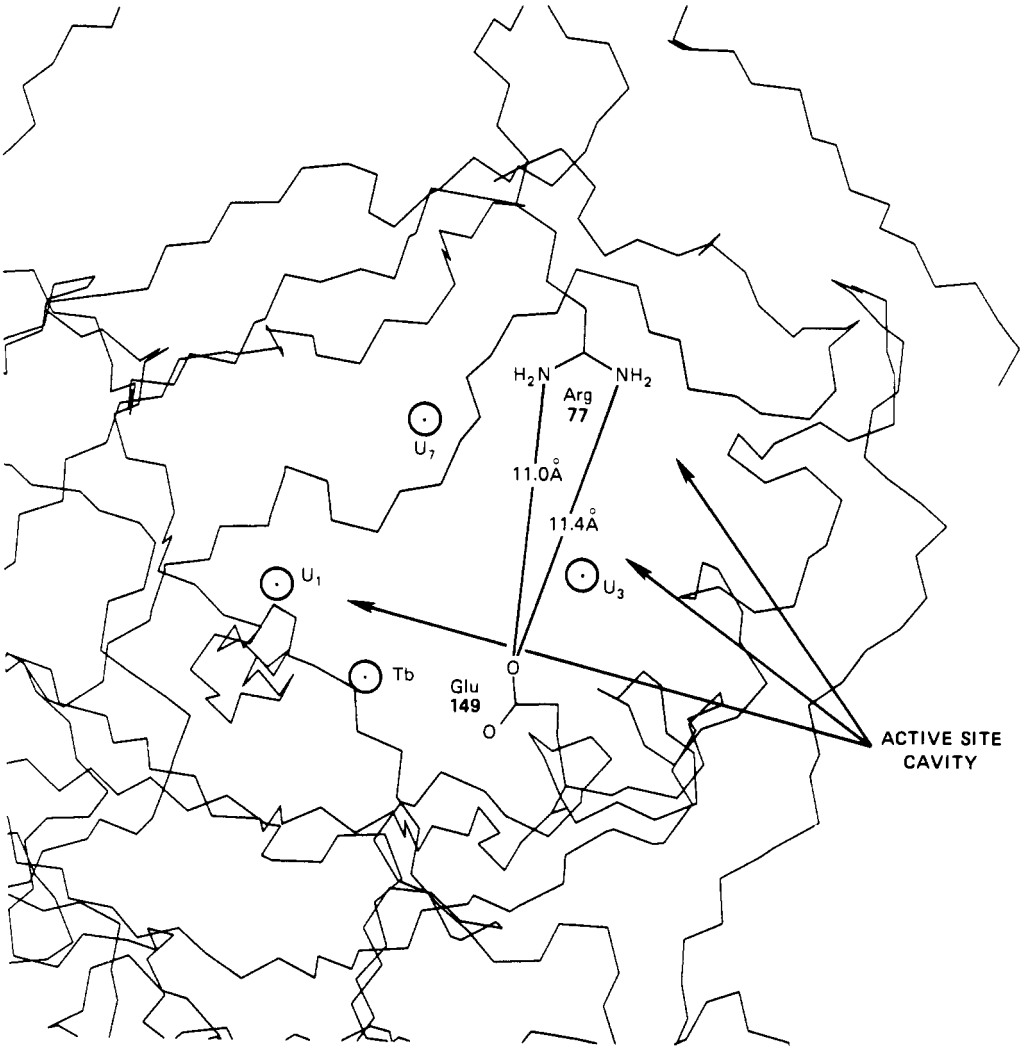
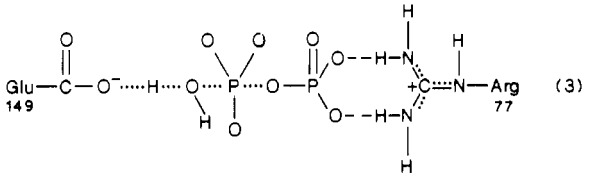



FIGURE 6: Detail of putative active site cavity of PPase. Only the peptide backbone is shown, except for the Arg-77 and Glu-149 side chains. The $U_1$, $U_3$, $U_7$, and Tb circles represent the positions of the three uranyl ions and one $Tb^{3+}$ ion bound per subunit (Terzyan et al., 1984). The figure is generated with the DOCK program (Wood, 1982) and the coordinates deposited by the Moscow crystallography group at the Brookhaven Protein Data Bank at the Brookhaven National Laboratory.

acts as a general base catalyst for nucleophilic attack of a water molecule on $PP_i$ while, simultaneously, Arg-77 coordinates to the leaving phosphoryl group (eq 3) is not only consistent with




(3)

the results of many previous studies of PPase (as summarized in Welsh and Cooperman (1984)] but also fits well with the distances indicated in Figure 6 between the terminal nitrogens of Arg-77 and one of the carboxylate oxygens of Glu-149.

Other results also support the assigning of the active site of PPase to the cavity shown in Figure 6. In particular, (1) the enzyme is known to require three divalent metal ions per subunit for activity, with a fourth divalent metal ion capable of being bound but playing an inhibitory role (Moe & Butler, 1972a; Springs et al., 1981; Volk et al., 1981; Welsh et al., 1983b; Knight et al., 1984), which fits very well with the finding of four metal ion binding sites in the cavity. ESR results on both the $Mn^{2+}$ (Banerjee & Cooperman, 1983; Knight et al., 1984) and Cu(II) Banerjee et al., 1986) enzymes also give good evidence for the mutual proximity of at least three divalent metal ions on a PPase subunit. (2) Chemical modification (Cooperman, 1982), $^{113}Cd$ NMR (Welsh et al., 1983b), and Cu(II) ESR (Banerjee et al., 1986) results are all consistent in showing a heavy involvement at the active site amino acid of residues with functional oxygens but little if any involvement of amino acid side residues with functional nitrogens, with the exception of arginine. The functional residues identified as potential active site participants in the crystallographic structure include four glutamates (48, 58, 147, 149), four aspartates (114, 119, 146, 151), three tyrosines (88, 92, 191), and only two lysines (56, 153). (3) A partial sequence of *Escherichia coli* PPase has been determined including approximately 115 amino acid residues out of a total length that can be estimated at 160–170 residues (Cohen, 1978). The known sequences show considerably homology (~25%) with the yeast PPase sequence corresponding (with some gaps) to residues 28–157 (Cohen, 1978). As will be noted, by comparison with point 2, this region of yeast PPase includes most of the residues implicated as potential active site participants. Moreover, examination of the entire structure of yeast PPase reveals that few, if any, of the residues 1–25 or 201–285 (with the exception of residues 231–238) come into close contact with the active site cavity.

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**Registry No.** EDAC, 1892-57-5; GEE, 459-73-4; PPase, 9024-82-2; L-Glu, 56-86-0.

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