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A Site-Directed Mutagenesis Study on *Escherichia coli* Inorganic Pyrophosphatase. Glutamic Acid-98 and Lysine-104 Are Important for Structural Integrity, whereas Aspartic Acids-97 and -102 Are Essential for Catalytic Activity†

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ABSTRACT: Analysis of the conservation of functional residues between yeast and *Escherichia coli* inorganic pyrophosphatases (PPases) suggested that Asp-97, Glu-98, Asp-102, and Lys-104 are important for the action of *E. coli* PPase [Lahti, R., Kolakowski, L. F., Heinonen, J., Vihinen, M., Pohjanoksa, K., & Cooperman, B. S. (1990) *Biochim. Biophys. Acta 1038*, 338–345]. We replaced these four residues by oligonucleotide-directed mutagenesis, giving variant PPases DV97, DE97, EV98, DV102, DE102, KI104, and KR104. PPase variants DV97, DV102, and KI104 had no enzyme activity, whereas PPase variants DE97, EV98, DE102, and KR104 had 22%, 33%, 3%, and 3% of the wild-type PPase activity, respectively. This suggests that Asp-97, Asp-102, and Lys-104 are essential for the catalytic activity of *E. coli* PPase. PPase variants DV98 and KR104 also had an increased sensitivity to heat denaturation; incubation of these mutant PPases at 75 °C for 15 min in the presence of 5 mM magnesium ion decreased the activity to 20% and 1%, respectively, of the initial value while 74% of the activity was observed with wild-type PPase. Furthermore, these thermolabile mutant PPases displayed the most profound conformational changes of the PPase variants examined, as demonstrated by the binding of the fluorescent dye Nile red that monitors the hydrophobicity of protein surfaces. Accordingly, Glu-98 and Lys-104 seem to be important for the structural integrity of *E. coli* PPase.

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) catalyzes specifically the hydrolysis of inorganic pyrophosphate (PP_i) to two orthophosphates. This reaction plays an important role in energy metabolism, providing a thermodynamic pull for biosynthetic reactions, such as protein, RNA, and DNA synthesis (Kornberg, 1962; Peller, 1976; Lahti, 1983). PPases have been purified from a variety of sources primarily for kinetic (Josse, 1966a; Moe & Butler, 1972; Randahl, 1979;

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Schreier, 1980; Springs et al., 1981; Welsh et al., 1983; Lahti & Jokinen, 1985; Lahti et al., 1989) and structural (Burton & Josse, 1970; Bond et al., 1980; Terzyan et al., 1984; Gonzales & Cooperman, 1986; Samejima et al., 1988) studies.

We have recently cloned and characterized the *ppa* gene encoding *Escherichia coli* PPase (Lahti et al., 1988). This, together with the analysis of the conservation of functional residues between yeast and *E. coli* PPases (Lahti et al., 1990), has made it possible to start detailed studies on the structure, function, and mechanism of *E. coli* PPase. In this paper, we describe the preparation and characterization of two types of amino acid replacements for four residues thought to be essential for the catalytic activity of *E. coli* PPase.

Materials and Methods

Chemicals. Restriction endonucleases, T4 DNA ligase, and alkaline phosphatase from calf intestine were bought from Boehringer Mannheim, Mannheim, West Germany. Antibiotics were obtained from Sigma Chemical Co., St. Louis, MO. DNA polymerase I (Klenow fragment) and [^{35}S]dATP (500 Ci/mmol) were purchased from Du Pont, Boston, MA. T4 polynucleotide kinase was from Pharmacia, Uppsala, Sweden, and Nile red, 9-(diethylamino)-5*H*-benzo[*a*]phenoxazine-5-one, was from Eastman-Kodak.

Mutagenesis. Site-specific mutagenesis was performed by the method of Kunkel (1985). This mutagenesis procedure uses a single (mutant) primer at a time, and primed synthesis goes all the way around the template followed by blunt-end ligation of the newly synthesized strand. Selection against wild type is achieved by use of a uracil-containing template which cannot be repaired by the normal cell machinery of *E. coli* and thus will not survive upon transformation into *E. coli dut*$^+$ *ung*$^+$ cells such as JM103, for example (Kunkel, 1985). Oligonucleotides for mutagenesis (and for use as specific sequencing primers, as well) were synthesized by using the phosphoramidite method in an automated DNA synthesizer (Cyclone, Bioresearch Inc.). Oligonucleotides used as sequencing primers were not purified. Oligonucleotides used for mutagenesis were purified by HPLC in a reverse-phase column (Spherisorb ODS-2, 5 μm, 4 × 10 mm, reverse-phase cartridge). Mutagenic oligonucleotides were phosphorylated at their 5′ ends by T4 polynucleotide kinase.

For mutagenesis, the 0.8-kb *Acc*I–*Bgl*I fragment encoding the entire *ppa* gene was transferred from plasmid pTP1 (Lahti et al., 1988) into the *Sma*I site of the replicative form of M13mp18 (Messing, 1983) in the opposite orientation to *lac*p. The orientation of the insert was checked by sequencing by Sanger's dideoxynucleotide method (Sanger et al., 1977). Prior to each mutagenesis, fresh *E. coli* RZ1032 (*dut ung*) strain (Tye et al., 1978; Kunkel, 1985) was grown on minimal glucose plates (Maniatis et al., 1982) containing tetracycline (12.5 μg/mL). RZ1032 cells grown in 2×YT medium (Rodriguez & Tait, 1983) containing tetracycline (12.5 μg/mL) were made competent by the calcium chloride procedure, and single-stranded M13mp18 DNA containing the insert was transformed into RZ1032 as described by Maniatis et al. (1982). The uracil-containing template was prepared as follows: (a) RZ1032 was grown overnight in 2×YT containing tetracycline. (b) In the morning, 50 mL of 2×YT containing tetracycline was inoculated with 0.5 mL of an overnight culture, and grown to mid log phase. (c) One plaque, obtained from transformation of RZ1032 with single-stranded M13mp18 DNA containing the insert, was inoculated into 1 mL of 2×YT, heat-treated at 60 °C for 5 min, and spun for 5 min in an Eppendorf centrifuge to pellet agar and cells. (d) One hundred microliters of this clear supernatant and 10 mL of a mid log culture of RZ1032 were added to 100 mL of 2×YT supplemented with tetracycline (12.5 μg/mL) and uridine (0.25 μg/mL). (e) Phage were amplified in RZ1032 at 37 °C for 8 h with vigorous shaking, and single-stranded, uridinylated template was isolated by standard methods (Messing, 1983). After 0.2 pmol of uridinylated template was annealed with 5 pmol of 5′-kinased, mutagenic oligonucleotide (incubation at 70 °C for 5 min, slow cooling to room temperature in the heating block), the mixture was allowed to stand 5 min at room temperature, and the extension reaction catalyzed by Klenow fragment was performed simultaneously with ligation by T4 DNA ligase for 1 h at room temperature. Reactions were stopped by the addition of EDTA to a final concentration of 5 mM. Aliquots of the reaction mixture were transformed into competent *E. coli* JM103 cells, plated on H plates (Maniatis et al., 1982), and incubated overnight at 37 °C.

The mutation frequency was rather high (40–70%), permitting mutations to be screened directly by sequencing. Hence, after mutagenesis, single-stranded DNA was isolated from the plaques, and to confirm the mutation and to ensure that no other mutations had arisen, we redetermined the sequence of the whole *Acc*I–*Bgl*I fragment by the method of Sanger et al. (1977) using in addition to the universal primer the following four *ppa*-specific primers designed for this purpose [numbering of the oligonucleotides is according to Lahti et al. (1988)]: 5′ 65-GATCTGCCGGAAGACATCT-83 3′, 5′ 227-CTGGACGGTGACCCGGTTG-246 3′, 5′ 401-GACGTTAACGATCTGCCTG-419 3′, and 5′ 564-AAGTTCTTCTGGCGTAATA-582 3′.

Production, Purification, and Assay of PPase. For the production of PPase, mutated *Acc*I–*Bgl*I fragments were transferred as *Eco*RI–*Hin*dIII fragments from M13mp18 to pUC19 (Norrander et al., 1983), which had been double-digested with *Eco*RI and *Hin*dIII. Plasmids (Table I) were transformed into *E. coli* HB101 (Maniatis et al., 1982) and plated on LA plates containing ampicillin (100 μg/mL). Transformants were grown with vigorous shaking at 37 °C to an A_{550} of 1.5 in 250 mL of 2×YT medium containing ampicillin (100 μg/mL). Cells were pelleted by centrifugation and disrupted by sonic oscillation in 20 mL of 50 mM Tris-HCl buffer (pH 8.0). Cell debris was removed by centrifugation, and nucleic acids were precipitated from the supernatant by a slow addition of one-third volume of 5% streptomycin sulfate. After incubation for 30 min in an ice–water

Table I: Specific Activities of PPase Purified to Homogeneity from the Strains Used in This Work

strain[a,b]	sp act.[c]	strain[a,b]	sp act.[c]
HB101/pWT	100	HB101/pDV102	0
HB101/pDV97	0	HB101/pDE102	3
HB101/pDE97	22	HB101/pKI104	0
HB101/pEV98	33	HB101/pKR104	3

[a] These strains contain both chromosomal- and plasmid-encoded PPases. The plasmid-encoded PPase was about 70-fold amplified over the chromosomal PPase. The chromosome-encoded PPase was taken into account as a blank value in each experiment. [b] The plasmid pWT contains the functional, wild-type *E. coli ppa* gene [*Acc*I–*Bgl*I fragment shown in the paper of Lahti et al. (1988)] inserted into the *Sma*I site of pUC19 in the same orientation with *lac*p. The plasmids pDV97–pKR104 are in vitro mutated derivatives of pWT encoding mutant PPases with the amino acid replacements indicated; i.e., HB101/pDV97 produces the mutant PPase with Asp-97 replaced by Val-97. [c] Specific activity given in relative units. The reactions were carried out as described by Heinonen and Lahti (1981) in 50 mM Tris-HCl buffer (pH 8.0) at 30 °C. The relative unit of 100 corresponds to the specific activity of 700 mkat/kg, whereas the subtracted background value (see footnote *a*) was 10 mkat/kg.

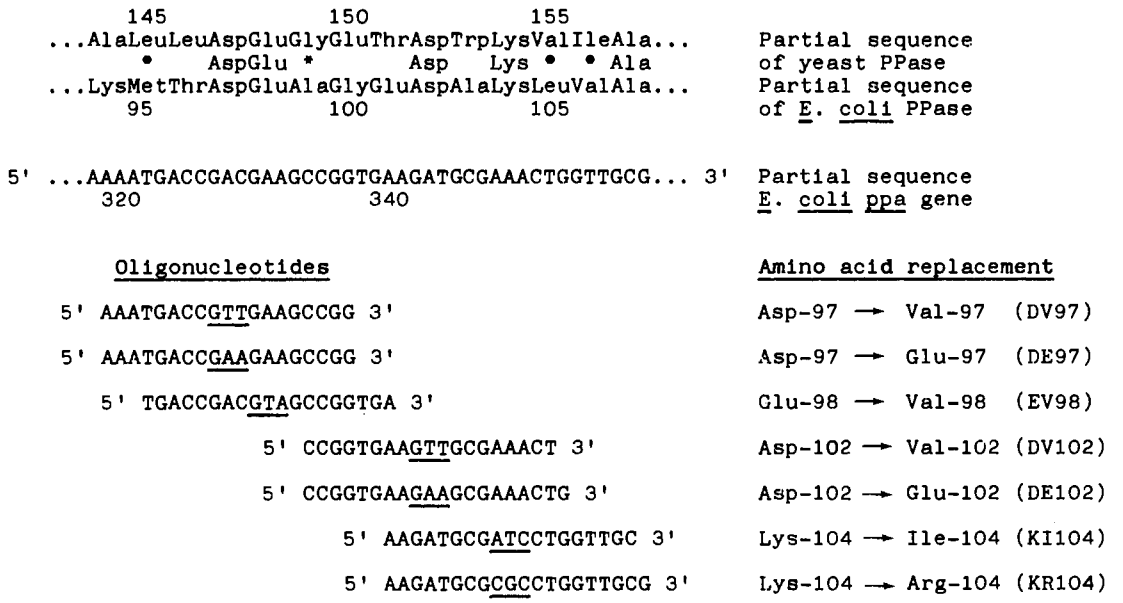

FIGURE 1: Oligonucleotides used for the site-directed mutagenesis of *E. coli* PPase. The partial sequences of *E. coli ppa* and PPase are taken from the paper of Lahti et al. (1988), whereas that of yeast PPase is from Kolakowski et al. (1988). Partial alignment of *E. coli* and yeast PPases is from Lahti et al. (1990). The amino acids replaced by similar ones in this alignment are shown by asterisks.

bath, the precipitate was pelleted (10000*g*, for 30 min at 4 °C) and discarded, and the supernatant with 20 mM magnesium ion was heat-treated for 15 min at 70 °C. Denaturated impurities were pelleted (10000*g*, for 30 min at 4 °C), and PPase was precipitated by 70% saturation of $(NH_4)_2SO_4$ (30 min at room temperature). The precipitate was pelleted (10000*g* for 30 min at 20 °C), resuspended in 5 mL of 50 mM Tris-HCl, pH 8.0, and dialyzed overnight at 4 °C against 50 mM Tris-HCl buffer (pH 8.0).

The purity of enzyme preparations was checked by native and SDS–PAGE using PhastSystem gradient gels (from 10% to 15%), and the gels were stained with Coomassie Brilliant Blue as specified by the supplier (Pharmacia, Uppsala, Sweden). PPase activity of the samples was assayed by the method of Heinonen and Lahti (1981), and protein concentration was determined as described by Bradford (1976) with bovine serum albumin as a standard protein.

Fluorescence Spectroscopy. Nile red, 9-(diethylamino)-5*H*-benzo[*a*]phenoxazin-5-one (Eastman-Kodak), was used to monitor the surface hydrophobicity (Sackett & Wolff, 1987) of the enzymes. These studies were performed at room temperature on a Perkin-Elmer LS-5 luminescence spectrometer equipped with CLS Data Station LS-X. Excitation was at 550 nm. Excitation and emission slits were set at 5 nm. Protein samples diluted with 0.05 M Tris-HCl (pH 8.0) were used at a concentration of 0.5 mg/mL in a volume of 2 mL. Nile red (final concentration of 1 μM) was added to the protein solution and mixed, and spectra were recorded after standing for 15 min. The fluorescent signal was stable for at least 1 h at room temperature.

RESULTS

Preparation of Mutant PPases. Because our recent analysis of the conservation of functional residues between yeast and *E. coli* PPases predicted that the region from Asp-97 to Lys-104, and in particular residues Asp-97, Glu-98, Asp-102, and Lys-104, would be essential for the catalytic activity of *E. coli* PPase (Lahti et al., 1990; Figure 1), studies on the relationship between structure and function were started in this region. Missense mutations in *E. coli* PPase (Figure 2) were produced by site-specific mutagenesis as described under Materials and Methods. The plasmids containing the mutated *ppa* genes were transformed into *E. coli* HB101. In these transformants, PPase production was amplified about 70-fold compared to the wild-type *E. coli* strain. Hence, owing to its high amount and thermostability, PPase was readily purified from these transformants to homogeneity as described under Materials and Methods (Figure 3).

Characterization of Mutant PPases. (*A*) *Activity.* Two types of amino acid replacements were made for the four conserved residues in the region from Asp-97 to Lys-104 (Figure 1). First, each of them was replaced by another amino acid of about the same size but lacking the functional polar group; i.e., aspartic and glutamic acids were replaced by valine, and lysine was replaced by isoleucine. These replacements resulted in total loss of PPase activity except for mutant EV98 which had 33% of the wild-type PPase activity (Table I). Next, the most conservative replacements were made, i.e., aspartic acids-97 and -102 were changed to glutamic acid, and lysine-104 was replaced by arginine. These PPase variants (DE97, DE102, and KR104) had 22%, 3%, and 3% of the wild-type activity, respectively (Table I).

(*B*) *Thermostability.* Mutant PPases with Glu-98 replaced by valine (EV98) and Lys-104 replaced by arginine (KR104) were more thermolabile than wild-type enzyme (Figure 4). The other five PPase variants produced in this work showed the same thermostability as wild PPase. In the case of PPase variant KR104, the difference in thermostability was more pronounced when magnesium ions were present. The opposite effect was observed for PPase EV98 (Figure 4).

(*C*) *Surface Hydrophobicity.* Nile red is an uncharged hydrophobic molecule whose fluorescence is strongly influenced by the polarity of its environment. When Nile red binds to a protein, a change in its emission spectrum is observed. The extent of the emission shift and enhancement of intensity in the emission of Nile red increases with increasing hydrophobicity of the target protein (Sackett & Wolf, 1987). This point is illustrated for albumin, PPase, and lysozyme in Figure 5. In accordance with the observation of Sackett and Wolff (1987), the surface hydrophobicity of albumin is rather high, whereas that of lysozyme is low (Figure 5). *E. coli* PPase shows only a slight hydrophobic character (Figure 5).

A conformational change that exposes or forms hydrophobic environments on a protein can be detected by Nile red (Sackett

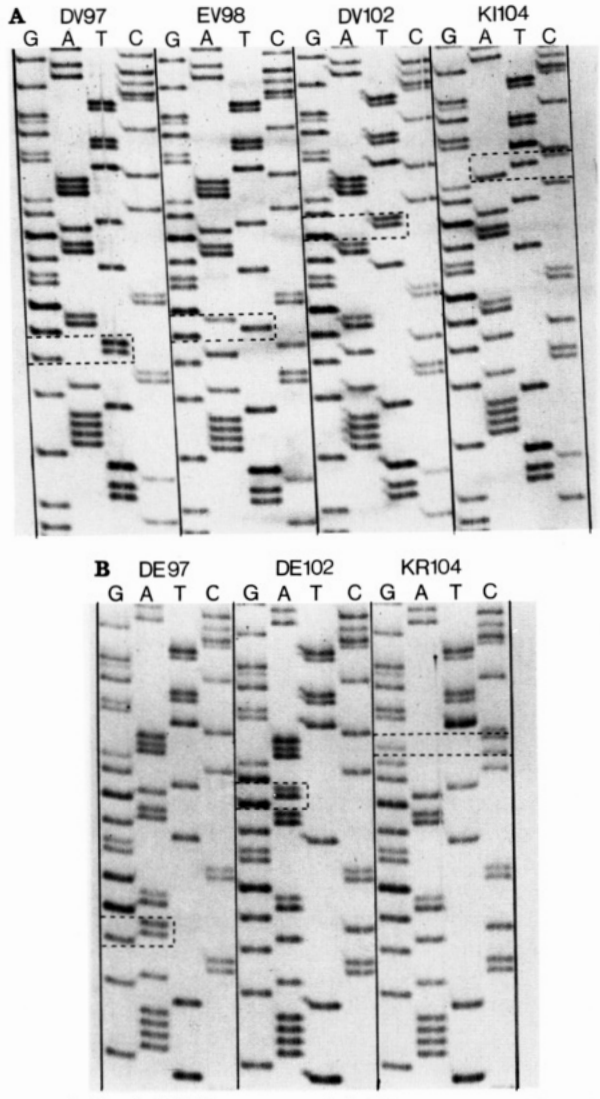

FIGURE 2: Partial DNA sequence of the PPase mutants. The codons produced by the site-directed mutagenesis are circled by dashed lines. DNA sequencing was carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977).

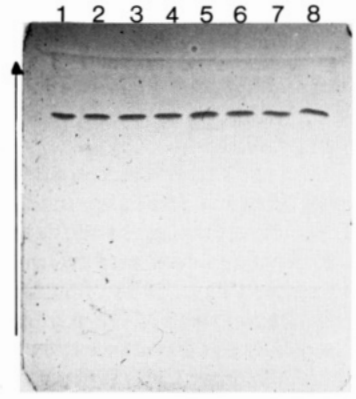

FIGURE 3: Purity of the native and mutant PPases as checked by SDS–PAGE using PhastSystem gradient gels (from 10% to 15%). The gels were stained with Coomassie Brilliant Blue as specified by the supplier (Pharmacia, Uppsala, Sweden). One microgram of protein was applied in each lane. Lane 1, native PPase; 2, DV97; 3, EV98; 4, DV102; 5, KI104; 6, DE97; 7, DE102; 8, KR104. The direction of the electrophoretic run is shown by an arrow.

& Wolf, 1987). The effects of the mutations on the fluorescence of Nile red are shown in Figure 6. Three of the four drastic mutations (EV98, DV102, and KI104) induced a profound change in the conformation of PPase, whereas two

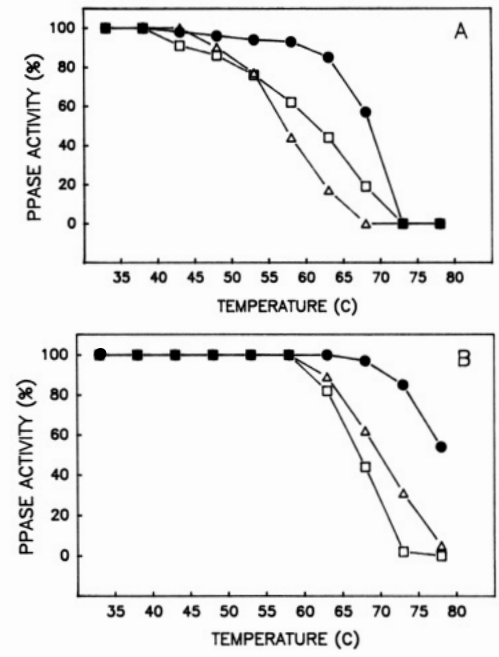

FIGURE 4: Thermal stability of native PPase and its variants EV98 and KR104 in the absence (A) and presence (B) of magnesium (5 mM). Residual PPase activity was measured following incubation of native and mutant PPases for 15 min at the temperatures indicated. (●) Native PPase; (△) EV98; (□) KR104.

of the three conservative mutations (DE97 and DE102) produced only small conformational changes (Figure 6). In addition, mutant PPase DV97 shows only a small change in surface hydrophobicity, whereas PPase variant KR104 shows a large conformational change. Both of the thermolabile mutant PPases, EV98 and KR104, had a very high surface hydrophobicity (Figure 6).

Some Considerations of PPase Measurements. Despite much time and effort, no PPase null mutants were produced by replacing the wild-type gene with a nonfunctional gene by the method described by Gutterson and Koshland (1983). Presumably a PPase null mutant is not viable. In this work, all the transformants shown in Table I contained both the wild-type and the mutant PPases. However, because of the high copy number of the plasmids, mutant PPase was greatly (about 70-fold) amplified over wild-type enzyme. In each experiment, chromosome-encoded PPase was taken into account as a blank value. As a control for 100% activity, we had the strain HB101/pWT containing the wild-type *ppa* gene inserted into the *Sma*I site of pUC19 (Table I).

The plasmids shown in Table I differed from each other only by a single missense mutation in the structural gene; so the 5′ flanking region responsible for the expression of PPase was identical with the wild type. Small (10–20%) variations observed in the yield of pure PPase between different samples had no effect on our results since the specific activities of homogeneous PPase preparations were measured. To minimize variation, all cell samples were grown to an A_{550} of 1.5.

Discussion

Josse and his collaborators studied the structural and kinetic properties of *E. coli* PPase in some detail [for a review, see Josse and Wong (1971)]. However, we do not have a clear insight into the structure and reaction mechanism of *E. coli* PPase. Yeast PPase is much better characterized than its *E. coli* counterpart. Kuranova, Terzyan, and their co-workers have determined the three-dimensional structure of yeast PPase by X-ray diffraction analysis (Kuranova et al., 1983; Terzyan

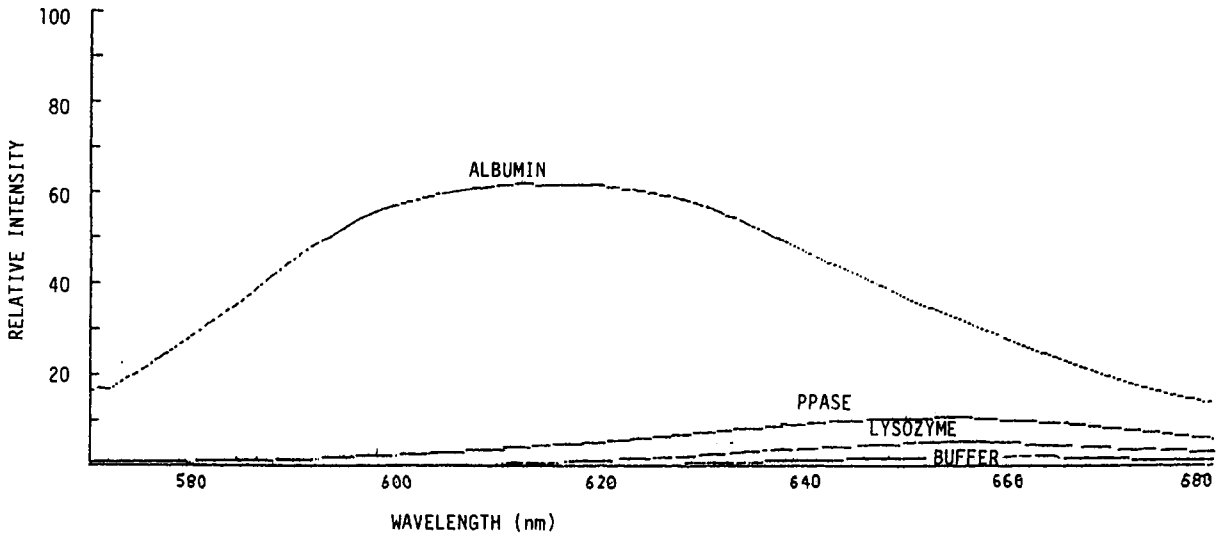

FIGURE 5: Fluorescence emission spectra of Nile red in the presence of albumin, native PPase, and lysozyme. The fluorescence emission spectrum in the absence of proteins is also shown (buffer: 0.05 M Tris-HCl, pH 8.0). In each case, the concentrations of protein and Nile red were 0.5 mg/mL and 1 μM, respectively.

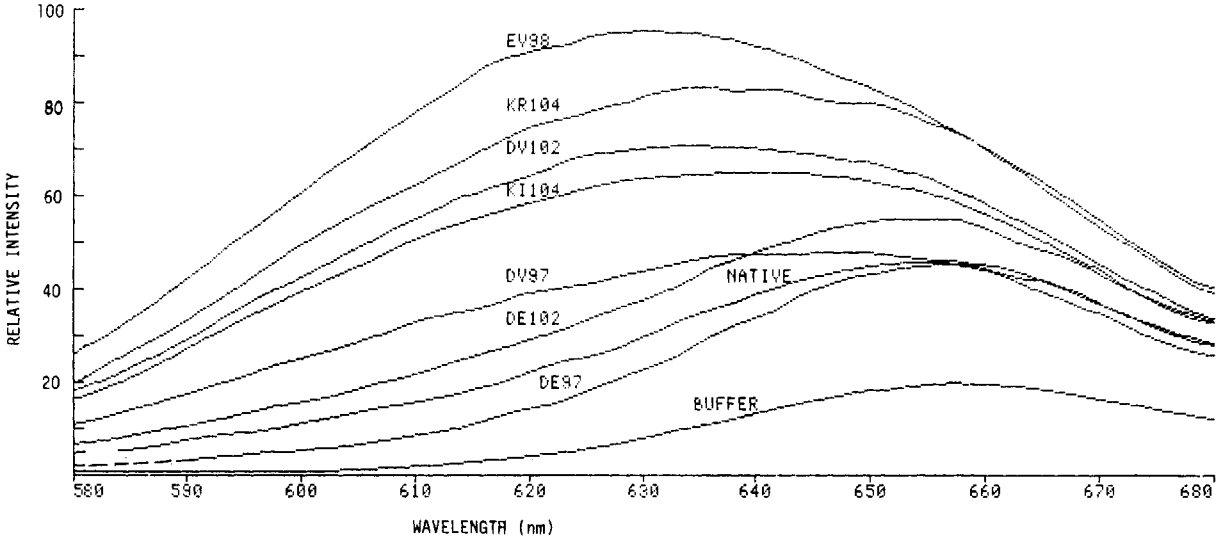

FIGURE 6: Fluorescence emission spectra of Nile red in the presence of native and mutant PPases. In each case, the concentrations of protein and Nile red were 0.5 mg/mL and 1 μM, respectively.

et al., 1984), and Cooperman with his collaborators has performed detailed studies on the structure and reaction mechanism of yeast PPase (Springs et al., 1981; Cooperman, 1982; Welsh et al., 1983; Gonzales & Cooperman, 1986).

The cloning and sequencing of the *E. coli ppa* gene (Lahti et al., 1988) made it possible to utilize techniques of molecular biology to study the structure and function of *E. coli* PPase. By comparative analysis of all the information available from various X-ray, chemical modification, and ligand binding studies, the amino acid residues that might be functionally important in *E. coli* PPase were predicted, and this suggested that aspartic and glutamic acids and lysine in the region from Asp-97 to Lys-104 are potential participants in the active site of *E. coli* PPase (Lahti et al., 1990). This region of eight amino acids contains four dicarboxylic acids, three of which are conserved in the yeast PPase sequence (Figure 1). In the present work, two (Asp-97 and Asp-102) out of these three acidic residues proved to be essential for the catalytic activity (Table I), whereas the third one (Glu-98) was shown to be important for the structural integrity of *E. coli* PPase (Figure 4).

Conformational changes induced by the mutations were monitored by Nile red, a fluorescent probe of protein surface hydrophobicity (Sackett & Wolff, 1987). There was no clear correlation between the extent of changes in conformation and activity (see Table I and Figure 6). However, as might be expected, the mutations that induced the most profound conformational changes also resulted in a drastic decrease in the thermostability of PPase (see Figures 4 and 6). The manner in which Glu-98 and Lys-104 stabilize the structure of *E. coli* PPase remains to be shown. Possibly they take part in salt bridges in the interior of PPase. Many thermostable enzymes including *E. coli* PPase are stabilized by bivalent cations (Mozhaev & Martinek, 1984; Josse, 1966b; Ichiba et al., 1988; Figure 4). The mechanism of stabilization is apparently through binding of cations to labile parts of the protein, in particular at the bends in the polypeptide chain, making the overall structure of the protein more compact and rigid (Mozhaev & Martinek, 1984). The difference in thermostability between the wild type and the mutant KR104 was more pronounced in the presence of magnesium than in its absence (Figure 4). The opposite was true with the PPase variant EV98. Hence, these missense mutations somehow affect the stabilization of PPase by magnesium. Glu-98 and Lys-104 of native *E. coli* PPase are conserved with Glu-148 and Lys-154 of yeast PPase (Figure 1) that are thought to be

involved in Mg^{2+} and PP_i binding, respectively (Lahti et al., 1990).

The fact that two of the null mutations (DV102 and KI 104; Table I) showed large changes in surface hydrophobicity (Figure 6) suggests that these mutations disrupted the structure of the enzyme. However, the more conservative mutation DE102 showed only a minor change in conformation but a drastic decrease in activity, indicating that Asp-102 is essential for the activity of *E. coli* PPase. Instead, in the case of the mutation KR104, there was a drastic change in thermostability (Figure 4), surface hydrophobicity (Figure 6), and activity (Table I). Therefore, the loss of activity that this mutation caused could result from the disruption of PPase structure rather than the residue being essential for activity. Accordingly, these experiments indicate that Lys-104 is important for the structural integrity but do not prove that Lys-104 is essential for the catalytic activity.

Large changes in hydrophobicity would also be expected if the mutations induced dissociation of the homohexameric *E. coli* PPase to trimers, dimers, and/or monomers. However, the wild-type enzyme and its variants shown in Table I all had the same mobility in the native PhastSystem PAGE gradient gels (see Materials and Methods) indicating that the mutations produced in this work did not alter the subunit association.

Inactivation of *E. coli* PPase by pyridoxal 5′-phosphate has been shown to result in the modification of a lysine residue located in a 23–27-residue tryptic peptide with the N-terminal sequence Asp-Leu-Pro-Glu (Komissarov et al., 1987). This tryptic peptide containing Lys-29 was identified in *E. coli* PPase (Lahti et al., 1990). In the present work, lysine-104 was shown to be essential for the native structure (Figure 4 and 6). Hence, at least two lysines seem to be important for the *E. coli* PPase. It remains to be shown whether the single "superreactive" lysine, whose modification by 2,4,6-trinitrobenzene leads to complete inactivation of *E. coli* PPase (Burton & Josse, 1970), is either Lys-29 or Lys-104.

In summary, studies on the structure and function of *E. coli* PPase using the site-directed mutagenesis technique were started in this work. The residues to be mutated were chosen according to the predictions obtained from the analysis of the conservation of functional residues between yeast and *E. coli* PPases (Lahti et al., 1990). The predictions proved to be correct, since all four putative active-site residues examined were shown to be important for the action of *E. coli* PPase. Glu-98 and Lys-104 proved to be important for structural integrity, whereas Asp-97 and Asp-102 were shown to be essential for catalytic activity of *E. coli* PPase.

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Registry No. Asp, 56-84-8; Glu, 56-86-0; Lys, 56-87-1.

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