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This paper is dedicated to Professor Włodzimierz Ostrowski

Covalent modification and site-directed mutagenesis of an active site tryptophan of human prostatic acid phosphatase*

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Because tryptophans are found as part of the phosphate binding sites in a number of proteins, human prostatic acid phosphatase (hPAP) was examined for the presence and the role of essential tryptophan residues. The pH dependence of the intrinsic fluorescence of hPAP resembled the kinetic pH dependence. Chemical modification by *N*-bromosuccinimide (NBS) resulted in an inactivation of the enzyme and produced a characteristic reduction of the protein absorbance at 280 nm. Two tryptophans per subunit were modified, and this was accompanied by an apparently complete loss of enzymatic activity. In the presence of the competitive inhibitor L-(+)-tartrate, the loss of enzyme activity was significantly reduced as compared to the rate of tryptophan modification. After labeling the protein with 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl), two tryptic peptides containing DNPS-labeled tryptophans were isolated and the sequences were identified by amino acid sequence analysis and mass spectroscopy. One peptide corresponded to residues 172–176, and included Trp174. The other corresponded to the C-terminal sequence, including Trp336. It was concluded that Trp174 was at the active site of the human enzyme because it was protected by the competitive inhibitor tartrate in the DNPS-Cl modification studies. This is also consistent with the location of a homologous residue in the structure of the rat enzyme. Using site-directed mutagenesis, Trp174 was replaced by Phe or Leu. Both mutants showed altered kinetic properties, including lower K_m values with several aromatic substrates, and also exhibited reduced stability towards urea denaturation.

Human prostatic acid phosphatase (hPAP) has been extensively studied. It is primarily present in prostate tissues and seminal fluid and has been widely utilized as a prostate cancer marker because patients with prostatic cancers frequently exhibit elevated level of prostatic acid phosphatase activity (Bodansky, 1972; Ostrowski, 1980). The en-

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Abbreviations: hPAP, human prostatic acid phosphatase; pNPP, *p*-nitrophenyl phosphate; NBS, *N*-bromosuccinimide; DNPS-Cl, 2,4-dinitrophenylsulfenyl chloride; TFA, trifluoroacetic acid.

zyme possesses a wide range of substrate specificities (Smith & Whitby, 1968), suggesting that the active site has a somewhat open structure. The protein consists of two identical subunits, with each subunit having 354 amino-acid residues and one active site. The active site residues, catalytic mechanism, protein sequence, cDNA sequence and effects of site-directed mutagenesis have been studied earlier in this laboratory (Van Etten, 1982; Van Etten *et al.*, 1991; Ostanin *et al.*, 1994). By analogy with the nomenclature of esterases, it has been proposed that this enzyme should be termed a histidine phosphatase because it utilizes histidine as a nucleophile involved in an $S_N2(P)$ mechanism (Van Etten, 1982; Buchwald *et al.*, 1984). The catalysis goes through a phosphoenzyme intermediate and the breakdown of the phosphoenzyme intermediate (dephosphorylation) is the rate-limiting step (Ostrowski & Barnard, 1973; McTigue & Van Etten, 1978; Ostrowski, 1978). This enzyme, together with the lysosomal type enzyme, numerous yeast enzymes and even the *Escherichia coli* acid phosphatase/phytase, all possess a characteristic RHGXRXP sequence motif (Van Etten *et al.*, 1991; Ostanin *et al.*, 1992). In addition to the nucleophilic histidine and several essential arginine residues, at least one essential acidic residue participates in the reaction (Saini & Van Etten, 1979; Ostanin *et al.*, 1994).

Recent research results have indicated that tryptophans may play many more important roles in protein structure stabilization and pK_a regulation of basic and acidic residues than was originally realized (Loewenthal *et al.*, 1992; Inoue *et al.*, 1992). Moreover, a number of recent papers also reveal the unexpected fact that proteins that bind phosphate-containing substrates often have a tryptophan at or near the binding site and that such tryptophans may be involved directly in exerting regulatory or stabilizing effects. Most directly relevant is the presence of a tryptophan at the active site of rat prostate acid phosphatase (Schneider *et al.*, 1993), but many other relevant examples can be cited. They include triosephosphate isomerase (Steczko *et al.*, 1983; Sampson & Knowles, 1992), myosin (Botts *et al.*, 1989), myosin light chain kinase (Knighton *et al.*, 1992), phosphoglycerate mutases (Fothergill-Gilmore, 1988), polyphosphate/ATP glucokinase (Hsieh *et al.*, 1993), mitochondrial F_1 adenosine 5'-triphosphatase (Divita *et al.*, 1993), and an analog of purple acid phosphatase from *Branchiostoma belcheri* (Chen *et al.*, 1993).

Important early studies by Rybarska & Ostrowski (1974) concluded that tryptophan residues are not directly involved in the catalytic function of hPAP, but that they might be involved in modifying the polarity of the active site environment. They also established that numerous tyrosine and tryptophan residues were present in the subunit-subunit contact region of the dimeric protein (Rybarska & Ostrowski, 1974). Understandably, these early covalent modification studies were made difficult by the lack of specificity of the reagents, the relatively insensitive protein analytical methodologies of the time, as well as the fact that quantitation was difficult because of problems in obtaining sufficient amounts of highly purified enzyme. Moreover, it is only recently that the complete sequence of the human protein has been established. In view of these considerations, we thought that it would be of interest to extend some of these pioneering investigations of Rybarska and Ostrowski with the aid of current enzymological approaches.

Consequently, we have examined the involvement of tryptophan at the active site of hPAP by examining the pH dependence of tryptophan fluorescence and by conducting chemical modification studies with *N*-bromosuccinimide (NBS) and the tryptophan-selective reagent 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl). To directly study the role of a tryptophan found to be at the active site, it was replaced by site-directed mutagenesis, and the kinetic properties and stabilities of the mutants were examined.

MATERIALS AND METHODS

Materials. L-(+)-Tartaric acid, NBS and DNPS-Cl were purchased from Aldrich; *p*-nitrophenyl phosphate (pNPP) and α-naphthyl phosphate were from Sigma. Tosylpheny-

lalanyl chloromethyl ketone (TPCK) and trypsin were from Boeringer-Mannheim; HPLC solvents were from Scientific Products; all the buffer compounds were of analytical grade. The fluorescence spectrophotometer was a Hitachi F-2000.

Purification of hPAP. Native hPAP was purified from seminal fluid by aminohexyltartramic acid affinity chromatography as described earlier (Van Etten & Saini, 1978), except that the protein was chromatographed twice and the solution was subsequently desalted by passage through a 30 cm × 1 cm Bio-Gel P-6DG column eluted with pH 5.0, 100 mM sodium acetate, 1 mM EDTA, I = 0.15 M buffer. The purified protein showed one band on SDS/PAGE and one peak on HPLC. Under the present assay conditions, the specific activity was 800 units/mg of protein.

Site-directed mutagenesis. The full length hPAP gene was cloned previously (Van Etten *et al.,* 1991). Mutations in the hPAP gene were generated as described by Kunkel (1985) and Ostanin *et al.,* (1994). The mutagenic primers were No. 1, 5′-GCCAGGACCTTTTTGGAATTTTGAGTAAAGTCTACGACC-3′; No. 2, 5′-GCCAGGACCTTTTTGGAATTTTCAGTAAAGTCTACGACC-3′. This gave rise to the following codon changes: TGG (Trp174) to TTG (Leu) or to TTC (Phe), respectively. The mutant proteins were purified as described earlier (Ostanin *et al.,* 1994), using a single step of tartramic acid affinity chromatography.

pH-dependence of phosphatase activity and of protein fluorescence. The phosphatase activity of hPAP was assayed from pH 3.0 to 9.0 using pNPP as substrate. The buffers used were as follows: from pH 3.0 to pH 3.8, 100 mM sodium formate; from pH 4.0 to pH 5.5, 100 mM sodium acetate; from pH 5.8 to pH 6.8, 100 mM Bis-Tris; from pH 6.9 to pH 7.9, 50 mM 2,2-diethylmalonate; from pH 8.0 to pH 9.0, 100 mM glycinamide. All the buffers contained 1 mM EDTA and were adjusted to an ionic strength of 0.15 M by addition of sodium chloride. When maintained in this way at constant ionic strength, changes in the concentration of the buffers used in this study do not appear to cause changes in the kinetic parameters of hPAP.

Activity assay procedures were as follows. Buffer containing the desired concentration of substrate was incubated at 37°C in a water bath, the reaction was initiated by adding solutions of hPAP such that the final volume of the reaction mixture was 400 µl. After incubating 4 min, the reaction was quenched by the addition of 1 ml of 1 M NaOH. The absorbance of the solution at 405 nm was measured and *p*-nitrophenolate ion was quantitated using an extinction coefficient of 18000 $M^{-1}cm^{-1}$. One unit of activity is defined as the amount of enzyme hydrolyzing one µmole of pNPP in 1 min. The rate of dephosphorylation of naphthyl phosphate was monitored by measurement of inorganic phosphate release. In this case 0.200 ml of 10% trichloroacetic acid was used to stop the reaction, followed by the assay of inorganic phosphate (Bradford, 1976; Black & Jones, 1983).

The pH dependence of the protein fluorescence studies were conducted with a 3 ml quartz cell in a Hitachi F-2000 fluorescence spectrophotometer. Enzyme (12 µg) stock was diluted to the final volume of 2 ml. The temperature was controlled by circulation of 25°C water around the cell for at least 10 min before measurement. The excitation wavelengths were either 295 nm (Trp) or 277 nm (excitation peak of hPAP). The emission was monitored at 342 nm and 330 nm.

Urea denaturation studies. Urea solutions of desired concentrations were made in volumetric flasks with fresh urea dissolved in pH 5.0 assay buffer. The solutions were filtered just before use. The denaturation was followed by measuring intrinsic tryptophan fluorescence. Fluorescence measurements of urea-induced protein denaturation were conducted with a 0.7 ml quartz cell in a Hitachi F-2000 fluorescence spectrophotometer. Protein samples (20 µl) were mixed with urea solutions (380 µl). The temperature was controlled by circulating 25°C water around the cell for 8 min before measurement. The excitation wavelength was 295 nm and the emission was monitored at 342 nm. Fluorescence was corrected for urea background.

Chemical modification of tryptophan residues by NBS. For NBS modification

experiments, protein solutions (1 mg/ml) were prepared in 100 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and the ionic strength adjusted to 0.15 M with sodium chloride. NBS was freshly recrystallized from water and aqueous solutions were prepared immediately before use and kept in an amber bottle. NBS was added from a 1.2 mM stock solution in water. The progress of the reaction was monitored at 280 nm with an IBM 9400 UV-visible spectrophotometer. After each addition of NBS 3 μl portions of the reaction mixture were withdrawn and diluted to 1 ml; then 10 μl portions were taken and assayed for phosphatase activity in pH 5.0 buffer using 10 mM pNPP as substrate. The modification by NBS was performed both in the absence and in the presence of 3 mM sodium L-tartrate.

DNPS-Cl modification of hPAP. The tryptophan residues of hPAP were modified with DNPS-Cl (Scoffone *et al.*, 1968). hPAP (1 mg) was modified by DNPS-Cl both in the absence and presence of 3 mM L-tartrate. After labeling, hPAP was precipitated with acetone and subsequently washed twice with acetone and ethyl ether. The labeled hPAP samples were then dissolved in 500 μl 100 mM ammonium bicarbonate, pH 7.8 buffer with 2 M urea in it. Dithiothreitol was added to both samples to a final concentration of 5 mM and the samples were incubated at 37°C for 1 h. Iodoacetate was added to both samples to a final concentration of 11 mM and the mixtures were incubated at 37°C in the dark for 20 min. The protein was precipitated again by addition of acetone to a final concentration of 65%. The precipitated hPAP samples were redissolved in 500 μl 100 mM ammonium bicarbonate, pH 7.8 buffer containing 2 M urea and the absorbancies at 350 nm of the samples were measured. The samples were used in subsequent proteolysis experiments.

Tryptic digestion and hPLC analysis. Trypsin was dissolved to a concentration of 1 mg/ml in 1 mM HCl solution and stored at −20°C. The hPAP samples obtained from DNPS-Cl modification were incubated at 37°C and trypsin solution (5 μl) was added to each labeled sample every 2 h for the first 12 h. Then the samples were further allowed to digest for an additional 24 h. Analysis of the digested samples was performed on an IBM LC/9533 ternary gradient liquid chromatography system equipped with two IBM LV/9523 variable-wavelength UV detectors set to monitor absorbance at 210 nm (peptide) and 350 nm (DNPS group). The samples were loaded on a Synchropak RP-P C18 column (4.6 mm × 250 mm) and eluted with a linear gradient generated from 0.1% TFA/H_2O and 0.1% TFA/CH_3CN with an increase of the latter solvent of 1% per min.

Amino acid sequencing and mass spectroscopy of the modified peptides. Gas phase sequencing of the purified labeled peptides was performed at the Laboratory of Macromolecular Structure, Purdue University, with an Applied Biosystems 470A gas phase sequenator. Plasma desorption mass spectroscopy was done on a Bio-Ion 20R mass spectrometer. Sample (1 μl) was applied to a nitrocellulose disk coated with Mylar. The disk was then washed with 20 μl 0.1% TFA/water solution. The spectra were collected for 30 min at an acceleration voltage of 17000 V.

RESULTS

pH Dependence of activity and fluorescence

Extensive data for the pH dependence of hPAP shows that, in contrast to the appearance of a simple "pH optimum" curve, V_{max} values are effectively constant over the pH range 3.5 to 7 (Van Etten & McTigue, 1977, Van Etten, 1982). In contrast, K_m is constant between pH 3.5 and pH 5.2 and increases above pH 5.2. This increase is probably due to the dissociation of the essential carboxylic acid that acts as a general acid to protonate the leaving alcohol or phenol (Saini & Van Etten, 1979, Ostanin, *et al.*, 1994). The pH dependence of the intrinsic fluorescence (Fig. 1) also suggests pH regions where ionizable groups such as histidines or carboxylic acids may directly alter the fluorescence of a nearby tryptophan, or where hPAP may undergo pH-dependent structural changes. When an excitation wavelength of 277 nm

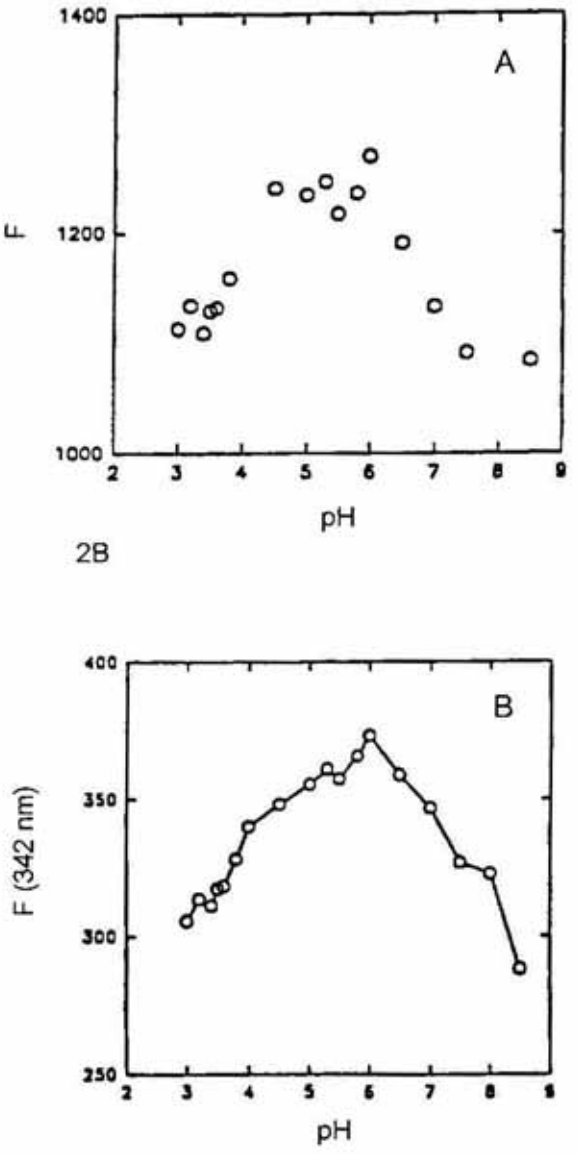

Figure 1. pH Dependence of hPAP fluorescence.

(A) The excitation wavelength is 277 nm and the emission wavelength is 342 nm; (B) the excitation wavelength is 295 nm and the emission wavelength is 342 nm. The buffers used are described in text.

was used, two pH regions were observed in which considerable fluorescence changes occur. As shown in Fig. 1, the fluorescence decreases above pH 6.5 and below pH 4.0. Those two pH points roughly coincide with pK_a regions indicated in the activity-pH dependence studies. When hPAP was excited at 295 nm, the pH dependence was more complex, perhaps due to the large number of tryptophans present in the molecule (7 per subunit). The pH 6.5 change was still evident. Towards both ends of the pH profile, there was an approximately 15–20% decrease in fluorescence, that is, somewhat more than 1/7th; this might be accounted for by the same tryptophan.

Determination of the exposed tryptophans of hPAP

The total number of accessible or reactive tryptophans was determined by spectrophotometric titration of the native enzyme with NBS. Tryptophan oxidization was monitored at 280 nm and the number was calculated from the equation $\Delta n = (1.31 \times \Delta A_{280})/(5500 \times [E])$, where Δn is the number of tryptophans oxidized, ΔA_{280} is the decrease in absorbance at 280 nm, and [E] is the enzyme molarity. The hPAP subunit molecular mass used was 45 kDa (Van Etten *et al.*, 1991). For NBS concentrations of up to 4 times the enzyme concentration, the number of tryptophans modified was proportional to the NBS concentration. Further increases of the NBS concentration did not result in further decreases of the 280 nm absorbance. This indicates that 2 out of 7 tryptophans (per subunit) are accessible to NBS modification.

Effect of tryptophan modification on phosphatase activity

Modification of tryptophans was carried out in order to determine whether tryptophan residues are essential for the phosphatase activity. Incubation of hPAP with increasing concentrations of NBS resulted in progressive inactivation of the phosphatase (Fig. 2). However, the inactivation curve did not coincide with the tryptophan loss curve, which indicates that the two tryptophans that are being modified are not equally important for retention of activity. The loss of phosphatase activity was more rapid than the tryptophan loss, indicating that one tryptophan is more reactive than the other and is responsible for the activity loss. The oxidation of tryptophan was also accompanied by a decrease in the intrinsic fluorescence, but the spectra remained very much similar to that of the native enzyme without any shift in the emission peak (not shown).

Protection by inhibitor of NBS-inactivation of hPAP

In order to study the possible positions of these two tryptophan residues, the competitive inhibitor L-tartrate (K_i = 15 μM at pH 5.0) was included in the incubation buffer during modification by NBS. When 3 mM tartrate was included, the phosphatase activity loss curve was noticeably shifted, from above to below the tryptophan loss curve, indicating considerable protection against phosphatase activity loss. However, the tryptophan loss curves, in the presence or absence of tartrate, were relatively similar to one another (Fig. 2). This result strongly suggests that one tryptophan is at the active site and can directly affect phosphatase activity while another tryptophan is accessible to NBS modification but is not essential for phosphatase activity.

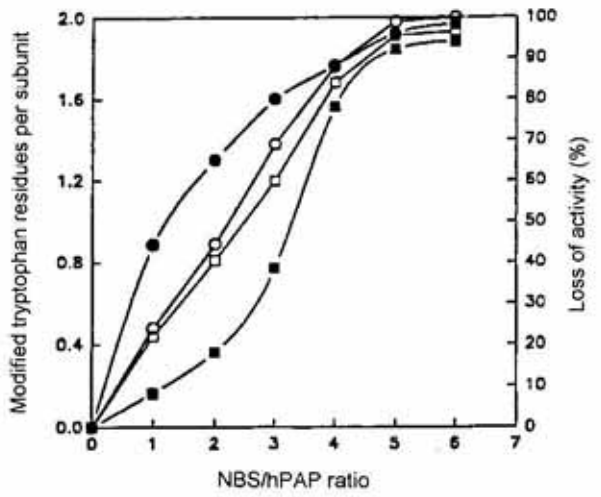

Figure 2. NBS modification of hPAP in 100 mM sodium acetate buffer, pH 5.0, with and without the presence of 3 mM tartrate.

The circles are in the absence of L-tartrate; the squares are in the presence of 3 mM tartrate. Symbols: ● and ■, phosphatase activity loss without and with L-tartrate present, respectively (right axis); ○ and □, number of tryptophans modified in the absence and presence of L-tartrate, respectively (left axis).

Identification of the tryptophans at the active site and at the surface

In order to identify the specific tryptophans that were subject to modification, we carried out specific labeling of tryptophan residues of hPAP by 2,4-dinitrophenylsulfenyl chloride. The labeled hPAP was digested with trypsin, and the DNPS-labeled tryptic peptides were fractionated by reverse phase HPLC. Because the DNPS functional group absorbs at 350 nm, peptide elution was monitored at 350 nm and 210 nm. A typical elution profile of the peptides is presented in Fig. 3. The incorporation of DNPS appeared to be relatively specific: only two major DNPS-labeled peptides were seen. In a similar manner, modification of the tryptophan residues of hPAP by DNPS-Cl was also carried out in the presence of the competitive inhibitor L-tartrate and the extent of DNPS incorporation was also examined by HPLC analysis of the tryptic peptides. As shown in Fig. 4, one peptide peak (peak D) absorbing at 350 nm was absent compared to the profile shown in Fig. 3, suggesting that in the presence of the competitive inhibitor L-tartrate, a specific tryptophan was rendered inaccessible towards labeling with DNPS-Cl. This evidence, combined with NBS modification data, suggests that a tryptophan is at or close to the hPAP active site and is essential for enzymatic activity.

Amino acid sequencing and mass spectroscopic analysis

Tryptic peptide peaks C and D were further purified by reverse phase C-18 column chromatography using a slower gradient (0.4 ml/min increase of acetonitrile) as shown in Figs. 5 and 6. The isolated peptide samples (E and F) were sequenced on an Applied Biosystem 470A gas phase sequenator. The amounts of both peptides were similar from sequencing analysis and corresponded to a 10% yield based on the amount of hPAP used in the initial modification reaction. The first 25 residues of peptide E were Phe-Ala-Glu-Leu-Val-Gly-Pro-Val-Ile-Pro-Gln-Asp-X(Trp-DNPS)-Ser-Thr-Glu-X(Cys)-Met-Thr-Thr-Asn-Ser-His-Glu-Gly. This sequence corresponds to the C-terminal region of hPAP. Peak F contained two peptides in similar amounts. The sequencing results showed the presence of the peptides Leu-Ser-Gly-Leu-His-Gly-Gln-Asp-Leu-Phe and Gly-Ile-X-Ser-Lys. These sequences correspond to hPAP residues 162 to 171 and 172 to 176, respectively. To further confirm these obser-

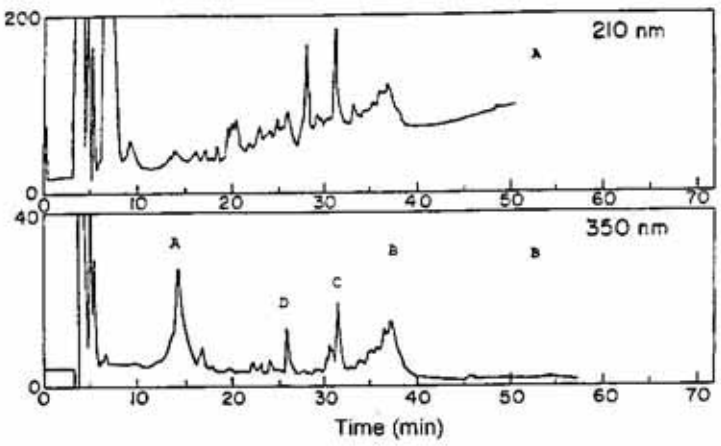

Figure 3. High-pressure liquid chromatography profiles of the tryptic peptides obtained after modification of hPAP by DNPS-Cl.

A total digest was applied to a Synchropak C-18 reverse phase column and eluted with a linear gradient of 2%–67% acetonitrile containing 0.1% TFA in 65 min. (A) Elution monitored at 210 nm; (B) elution monitored at 350 nm. Peaks: A, hydrolysed DNPS-Cl; B, uncleaved and partially cleaved PAP; C and D modified peptide segments.

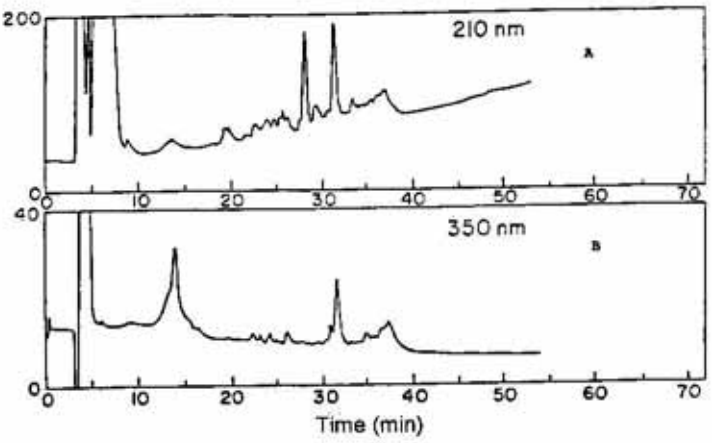

Figure 4. HPLC profiles of the tryptic peptides of hPAP modified by DNPS-Cl in the presence of 3 mM tartrate.

The column and elution conditions were the same as in Fig. 3. (A) Monitored at 210 nm; (B) monitored at 350 nm. Compared to Fig. 3, peak D is missing.

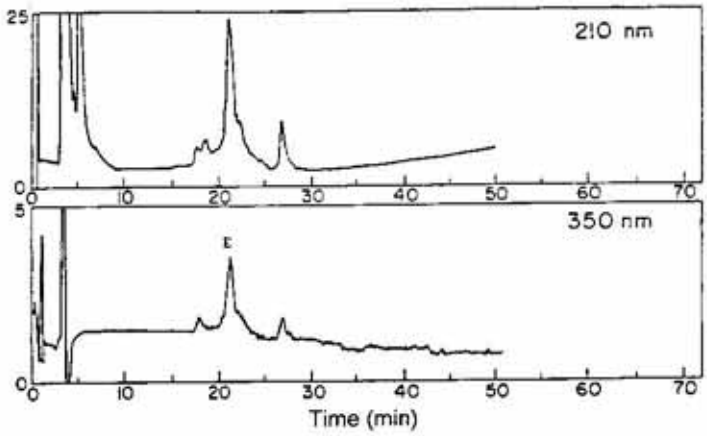

Figure 5. Further purification profile of peak C.

The column used was C-18 and the elution gradient was 0.4% increase of acetonitrile. Peptide E was isolated for sequencing.

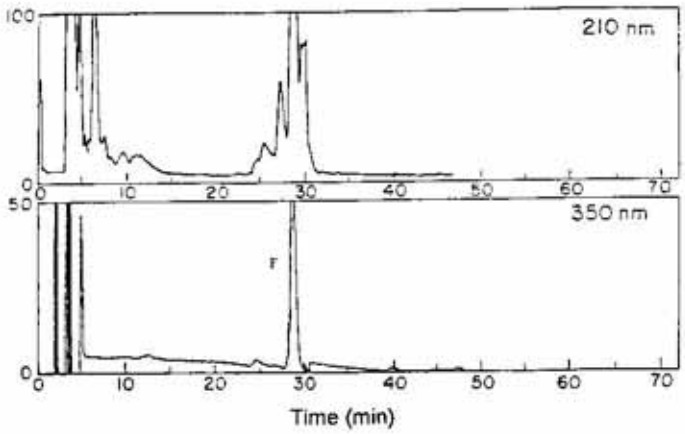

Figure 6. Further purification profile of peak D.

The column and elution conditions were the same as in Fig. 5. Peptide peak F was sequenced.

vations, the sample was submitted for plasma desorption mass spectroscopic analysis (Fig. 7). Such spectra generally give an M+1 peak with an occasional 2M+1 peak for peptides, consistent with the present results. Two major peaks of comparable abundance were found and corresponded to the masses predicted for the two peptides.

Gly-Ile-(DNPS)Trp-	calculated mass = 788.5
Ser-Lys	observed M+1 = 789.1
Leu-Ser-Gly-Leu-His-	calculated mass = 1086.2
Gly-Gln-Asp-Leu-Phe	observed M+1 = 1087.4

Kinetic parameters and stabilities of hPAP mutants

Because the preceding experiment indicated that Trp174 was at the active site, site-directed mutagenesis of hPAP was utilized in conjunction with a yeast expression system (Ostanin *et al.*, 1994) in order to examine the effects of Trp174Phe and Trp174Leu mutations on the properties of the mutant enzymes. Both mutants showed altered kinetic parameters toward pNPP and α-naphthyl phosphate. The mutants exhibited somewhat reduced V_{max} values as compared with the wild-type enzyme (Table 1). Interestingly, the two mutant proteins also exhibited smaller K_m values (Table 1) and also bound L-tartrate more strongly. Thus, Trp174Phe and Trp174Leu mutant proteins bound tartrate with dissociation constants K_i = 1.5 mM and 7.5 mM, respectively, compared to 15 μM for wild-type enzyme at pH 5.0.

The urea denaturation curves were analyzed as described by Pace *et al.* (1989). The mutant proteins showed marked differences in stability as compared to the wild type enzyme (Table 2). The mutants were much more susceptible to urea denaturation and exhibited decreased stability.

DISCUSSION

From the pH-dependence of activity and of fluorescence, a decrease in the catalytic activity of hPAP roughly coincides with a decrease of fluorescence, especially at higher pH. One possible interpretation is that around pH 6.5, an ionization occurs that alters the fluorescence of a nearby tryptophan. Alternatively, pH-dependent structural changes of hPAP may result in the

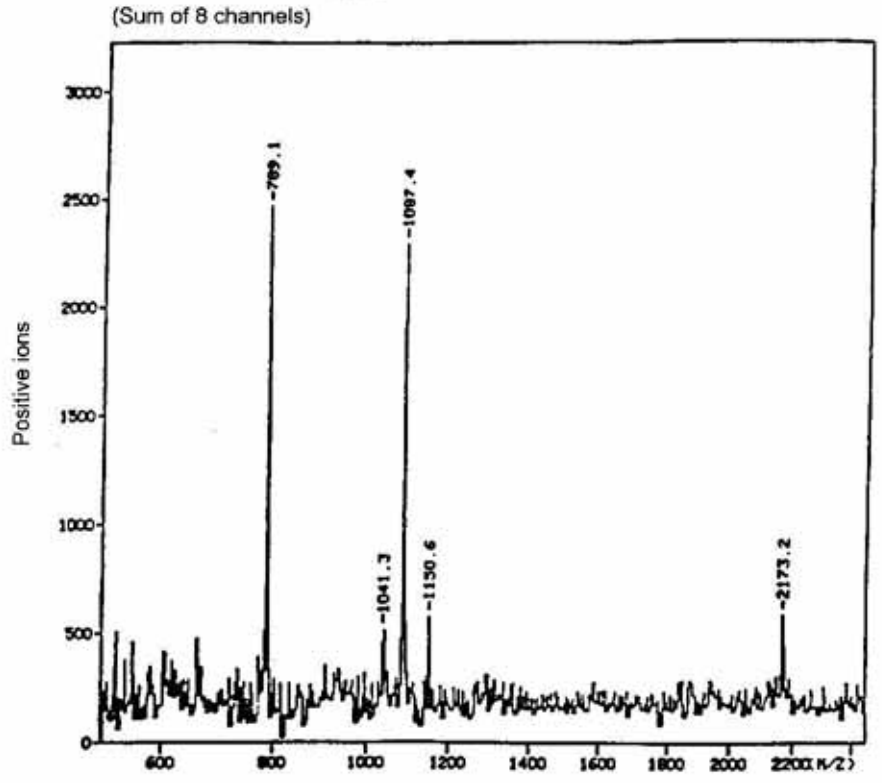

Figure 7. Mass spectrum of peptide peak F.

Table 1. Comparison of kinetic parameters of wild type and mutant hPAP[a]

	Enzyme		
	Wild type	Trp174Phe	Trp174Leu
pNPP as substrate			
V_{max}	800	430	540
K_m (mM)	0.28 ± 0.02	0.086 ± 0.009	0.17 ± 0.025
α-Naphthyl phosphate as substrate			
V_{max}	780	420	550
K_m (mM)	0.24 ± 0.05	0.019 ± 0.003	0.14 ± 0.04

[a]Activity was measured in 0.1 M sodium acetate pH 5.0 buffer.

movement of certain residues closer to tryptophan, thereby causing an increase in the quenching of tryptophan fluorescence. At the same time, such structural change results in the loss of enzymatic activity. It has been suggested that the hPAP structure undergoes a change between pH 5.5 and 7.0 and that this is in turn related to the fact that vanadate dimer inhibits hPAP at pH 5.5 while the monomer inhibits hPAP at pH 7.0 (Crans *et al.*, 1989).

Oxidation of the tryptophan indole moiety into an oxindole by NBS is particularly interesting since additional steric hindrance resulting from the substituted group is very limited, in contrast to other chemical modifiers (Lundblad & Noyes, 1984). This advantage sometimes presents a unique opportunity to study enzymatic properties with minimal perturbation of the site (Lundblad, 1991). At pH 5.0, where the modification of hPAP by NBS was carried out, the enzyme has an optimal activity towards pNPP and is also very stable. At pH 5.0, modification by NBS is generally considered to be relatively specific for tryptophan. The protein has 7 tryptophan residues per subunit (Van Etten *et al.*, 1991), a number that is similar to the 9 measured in earlier studies (Rybarska & Ostrowski, 1974). The NBS-dependent inactivation reaction is extremely rapid, reaching a plateau after only tens of seconds. The facile oxidation of two tryptophans indicates they are relatively exposed to the environment. Provided substantial excesses of NBS are avoided, the reaction is not accompanied by protein precipitation. Moreover, a specific modification of tryptophan residues is occurring, as indicated by the characteristic decrease in the 280 nm absorbance of the protein. The absorbance decrease indicates that only two out of seven tryptophans were modified. Obviously, this is a rapid and selective reaction.

The fact that one of the two rapidly modified tryptophans of hPAP is at the active site and

Table 2. Differences in stability of wild type and mutant hPAP.

Results from an analysis of urea denaturation for wild type hPAP and two mutants differing by one residue in amino-acid sequence are presented below.

Protein	$\Delta G(H_2O)$[a]	M[b]	$[urea]_{1/2}$[c]	$\Delta[urea]_{1/2}$[d]	$\Delta[\Delta G]$[e]
Wild-type	14.48	2220	6.48		
Trp174Leu	10.58	2130	5.05	1.43	3.9
Trp174Phe	9.05	2010	4.56	1.92	5.43

[a,b]From the equation $\Delta G = \Delta G(H_2O) - m$ [denaturant]; units of ΔG are kcal/mol, and of m are in cal/mol. [c]Midpoint of the urea denaturation curve in M. [d]Difference between the $[urea]_{1/2}$ values of the wild type protein and the mutant protein. [e]Difference between the $\Delta G(H_2O)$ values in kcal/mol.

is essential for activity is demonstrated by experiments involving the inclusion of L-tartrate, in which the loss of enzymatic activity is significantly decreased compared to the modification of total tryptophans. Apparently, in the absence of L-tartrate, the tryptophan near the active site is more accessible than the other one. Although L-tartrate had previously been observed to reduce the extent of oxidation by NBS, the large excess of NBS needed in those earlier experiments resulted in substantially reduced selectivity (Rybarska & Ostrowski, 1974). The present results with NBS are similar to those observed by Chen *et al.* (1993) on a purple acid phosphatase, which is a metalloenzyme that is structurally unrelated to hPAP. It was found that one tryptophan was susceptible to NBS modification and was essential for phosphatase activity. That tryptophan was suggested to be at the active site of the phosphatase.

DNPS-Cl is considered to react selectively with tryptophan in acidic solutions (Scoffone *et al.*, 1968). In the present case, the incorporation of DNPS groups in the protein was highly specific, with about 2.3 moles of DNPS incorporated per subunit when tartrate was not included in the modification buffer. This specificity for the modification of hPAP permitted the identification of two specific tryptophan-containing peptides, one presumably from the active site region because its formation was sensitive to tartrate binding, while the other one was evidently on the protein surface but was not part of the active site. The DNPS-labeled tryptic peptides were readily separated by HPLC and recovered in moderate yield. The specific location of the respective peptides was established with reference to the amino-acid sequence of hPAP, which has been determined from the nucleotide sequence of a full length cDNA clone and confirmed by mass spectroscopic studies of the protein itself (Van Etten *et al.*, 1991). Amino-acid sequencing and plasma desorption mass spectrometry identified one peptide as residues 172 to 176, and the residue modified by DNPS-Cl was identified as Trp174. The other peptide (E) corresponded to the C-terminal of hPAP, and the modified residue was identified as Trp336. In view of the sequence around Trp336, namely Gln-Asp-Trp-Ser-Thr-Glu, it would be reasonable to assume that it is at or near the surface since it resides in the middle of a hydrophilic segment. The use of mass spectroscopy in addition to amino acid sequencing provided positive confirmation of the presence of the modifying moiety. This is a substantial advantage compared to amino acid sequencing alone, which gives a negative result in the case of most amino acid modification reactions.

Site-directed mutagenesis of the active site Trp174 revealed effects on both reactivity and stability. The mutant Trp174Phe showed a markedly increased affinity for the inhibitor L-tartrate as well as for substrates, particularly the large, hydrophobic substrate α-naphthyl phosphate. The mutant Trp174-Leu showed smaller changes from the wild-type. The bulkiness of the tryptophan residue at the active site thus serves as part of the mechanism of substrate selection, maintaining the preference of the native enzyme for less hindered substrates. Among the more interesting findings of the present study is the more than 10-fold lower K_m value for α-naphthyl phosphate that is observed with the Trp174Phe mutant. The fact that L-tartrate binds with a more than 10-fold greater affinity indicates that the effect on K_m is due ultimately to an effect on the true E•S dissociation constant rather than on the k_3/k_2 ratio. This and related mutants may thus offer important experimental advantages in certain types of kinetic analyses such as stopped-flow studies.

Both mutants showed a marked decrease in structural stability, in comparison with wild-type hPAP. This is evident from the large difference of free energy of 5.4 kcal/mol between Trp174Phe mutant and wild-type hPAP in denaturation by urea. This must be primarily due to the creation of a hydrophobic hole upon replacement of tryptophan by the smaller or differently shaped amino acids, but the loss of a hydrogen bond may be an additional factor. Replacements of hydrophobic residues by smaller residues typically have significant effects on the stability of proteins. For example, the average change in the free energy of unfolding following the

deletion a single methylene group is 1.3 ± 0.5 kcal/mol (cf. Jackson *et al.*, 1993).

The present results are nicely consistent with the recently described 3 Å crystal structure of the rat prostate enzyme (Schneider *et al.*, 1993). Considering the fact that the mature human and rat enzymes exhibit 85% identical residues, it is likely that they possess very similar structures. Both Trp174 and Trp336 are conserved between the two enzymes, with the first being located at the active site while the second is located on the surface, approximately 20 Å from the active site (Fig. 8). It is of interest to consider the role of the active site tryptophan in more detail.

A surprising number of reports indicate that tryptophan is often found in phosphotransferase active sites, although its general role is not yet clear. The presence of one tryptophan at the ATP binding site of myosin has well been established (Johnson *et al.*, 1991) and the perturbation of its absorbance and emission upon ligand binding has been used to analyze the kinetics of the enzyme. An earlier suggestion that the tryptophan ring forms a charge transfer complex with the purine ring of ATP has been recently discounted, with the authors suggesting instead field effects, based on the results of calculations and molecular modeling (Bivin *et al.*, 1993). Tryptophan residues are also known to stabilize protonated side chains of other amino acids. Research done on barnase (Loewenthal *et al.*, 1992) indicates that tryptophan stabilizes a protonated histidine by one pH unit. The stabilization was suggested to require a specific orientation of the two residue side chains. In lysozyme, Trp108 stabilizes the free acid form of Glu48 by one pH unit (Inoue *et al.*, 1992). The notion that tryptophan stabilizes protonated forms of weak acids and perhaps holds them in specific orientations could certainly be true for hPAP, since that enzyme uses an aspartic acid to donate a proton to the leaving group (Schneider *et al.*, 1993, Ostanin *et al.*, 1994). However, the observed presence of trypto-

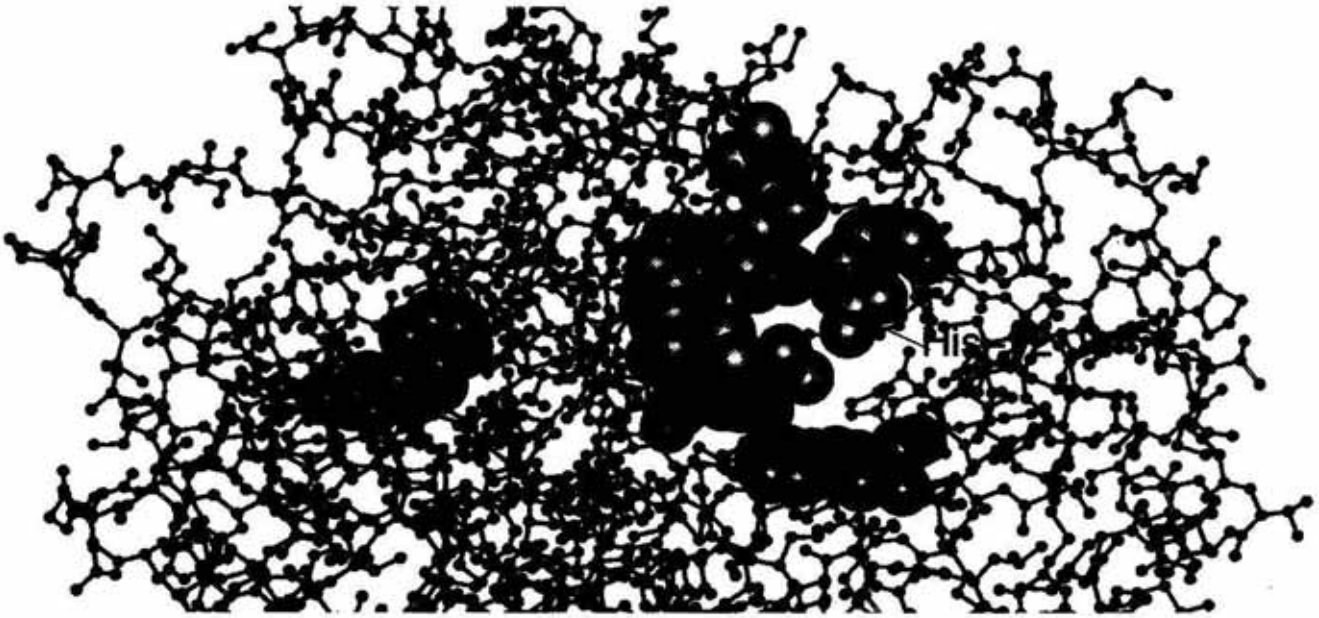

Figure 8. A portion of a structural model of hPAP, showing the locations of the two tryptophans studied here.

His12, which is the nucleophile that attacks the substrate in an $S_N2(P)$ reaction, is surrounded by a complex of three Arg residues (11,15 and 79). Nearby is His257 as well as Asp258, which functions as a general acid to donate a proton to the leaving alcohol or phenol. Trp174 is shown lining the bottom of the active site pocket, while Trp336 (to the left in this diagram) is located on the surface, approximately 20 Å away from the active site. The model was constructed using Insight/Discover software (BioSym/MSI, Inc.) based on the structure of the rat prostate enzyme (Schneider *et al.*, 1993) and on results obtained for site-directed mutagenesis of the human enzyme (Ostanin *et al.*, 1994).

phan in so many other phosphate binding sites may mean that tryptophan is used to modulate the pK_a values of bound phosphate (pK_{a2}) or of a covalent phosphoenzyme intermediate in the case of hPAP. Both of the mutants studied here exhibit only small decreases in V_{max}, which may indicate minor structural changes or perhaps that Trp174 may affect the electrostatic environment important in the dephosphorylation step. Considering the fact that tryptophan can stabilize protonated carboxylate and imidazole side chains, it may be speculated that tryptophan could facilitate the dephosphorylation in two ways. One way is to stabilize the protonated covalent phospho-intermediate and thus make it more susceptible to attack by water. Another way is to keep the imidazole ring of the covalent intermediate protonated and thus to make it a better leaving group. Finally, a second imidazole side chain is known to be present directly at the active site of the human enzyme (Moller & Van Etten, 1997), and its protonation state would be expected to affect the energetics of both reaction steps.

In summary, it is clear from the substantially reduced stabilities of both mutants that Trp174 serves an important structural role leading to the stabilization of the enzyme. However, additional effects, including the creation of an apolar environment as noted earlier by Rybarska & Ostrowski (1974), are also present. Finally, the detailed kinetic properties of these and related mutants merit further study.

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