A single domain of human prostatic acid phosphatase shows antibody-mediated restoration of catalytic activity

(limited proteolysis/active site/antibody-mediated restoration)

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ABSTRACT By limited proteolysis with mouse submaxillaris protease, human prostatic acid phosphatase (EC 3.1.3.2) was cleaved into three fragments, Sp1, Sp2, and Sp3, which individually had no enzymatic activity. One of the fragments, Sp3, regained enzymatic activity after interaction with rabbit antibody to prostatic acid phosphatase. The Sp3 fragment was purified and characterized as to its molecular weight, amino acid composition, and carbohydrate content. The Sp3 fragment behaved like the parent molecule in L(+)-tartrate affinity and in trapping of a phosphoryl intermediate. The same Sp3 fragment also bears the most prominent antigenic determinants. This evidence suggests that Sp3 is the enzymatically active domain of prostatic acid phosphatase.

Human prostatic acid phosphatase (EC 3.1.3.2) is a glycoprotein of M_r 102,000 (1) that consists of two identical subunits of M_r 45,000 (2). During the last several years the kinetic properties (3, 4) and antigenic structure (5–7) of this enzyme have been elucidated. However, studies concerning the structural organization of the enzyme have been scarce. Therefore an attempt was made to delineate the functional and antigenic domains of the enzyme molecule by carefully controlled cleavage of prostatic acid phosphatase (PAPase) by cyanogen bromide. We showed that one of the CNBr-derived polypeptides regains catalytic activity after interaction with specific antibodies to this enzyme (6). The active site fragment produced by CNBr cleavage was unstable and the yield in general was very low.

The constraints imposed by the folded conformation of several proteins have made possible their selective cleavage with proteinases. This same conformational constraint appears to direct the reassembly of the resulting fragments by noncovalent interactions in some instances (8, 9). Therefore, we have examined the possibility of limited digestion of PAPase to generate large fragments preserving their native conformations. Submaxillaris protease digestion has produced a consistent profile of PAPase fragments (Sp1, Sp2, and Sp3 fragments, PAPase fragments 1, 2, and 3, respectively, generated by submaxillaris protease digestion) in reasonable yield, and thus appears to be a generally useful procedure. One of the fragments was found to regain a portion of its catalytic activity after interaction with antibodies to PAPase. This paper describes the structural and functional characteristics of this fragment. Evidence presented here indicates that the fragment represents the enzymatically active domain of PAPase.

MATERIALS AND METHODS

Human PAPase. Methods of purification and physicochemical and antigenic characterization of this enzyme have been described (10).

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Limited Proteolysis by Submaxillaris Protease (11). PAPase (10 mg) was incubated with 200 units of submaxillaris protease (Pierce) in 2 M urea/1% NaHCO_3 , pH 8.0, for 16–48 hr at 4°C. A small amount of phenol red was added to monitor the pH. The digest was then applied on a Sephadex G-75 superfine column and eluted with 1% NaHCO_3 at pH 8.0. The column had been calibrated with the use of reference proteins of known molecular weights (12).

NaDodSO₄/Polyacrylamide Gel Electrophoresis. Slab polyacrylamide gel (10% or 20%) electrophoresis was performed according to Laemmli (13) in the presence of 0.1% NaDodSO₄.

Peptide Maps. For the two-dimensional peptide mapping, various radioactively labeled or unlabeled polypeptides (subunits, CNBr cleavage polypeptides, or submaxillaris protease digest polypeptides) were used. In general, 1–2 mg of polypeptides was digested with 1 ml of 50 mM ammonium bicarbonate containing 20 μg of *N*-tosylphenylalanine chloromethyl ketone (TPCK)-trypsin for 24 hr at 37°C and the supernatant was lyophilized. The lyophilized samples were dissolved in 20 μl of deionized water. One microliter of sample (or, in the case of radioactively labeled polypeptides, aliquots containing 100 cpm/mol of original polypeptides) was spotted on 20 $\times$ 20 cm cellulose-coated chromatograms (Eastman, 13255). Electrophoresis was carried out in pyridine/acetic acid/ H_2O , 40:14:1,946 (vol/vol), for 60 min at 500 V in a Shandon TLE electrophoresis apparatus with cooling. After drying, the chromatograms were developed in the second dimension in butanol/pyridine/acetic acid/ H_2O , 150:100:30:120 (vol/vol). Chromatograms were visualized by the fluorescamine method (14). Radioactive tryptic peptides were autoradiographed (Kodak X-Omat R film with fluorescent intensifying screens) for 1–18 hr.

Amino Acid Analysis. Proteins and polypeptides were hydrolyzed with 6 M HCl for 22 hr at 105°C and analyzed for amino acids by using a Beckman 221 M amino acid analyzer.

Concanavalin A-Sepharose 4B Affinity Chromatography. The procedure for concanavalin A affinity chromatography has been described (10, 15).

L(+)-Tartrate Affinity Chromatography. L(+)-Tartrate-Sepharose 4B affinity column matrix was prepared according to the procedure described by van Etten and Saini (16). Polypeptides applied to the affinity chromatography column were radioiodinated according to the procedure described by Hunter and Greenwood (17).

Trapping of a Covalent Phosphoryl-Enzyme Intermediate (18). One to 4 mg of protein (PAPase or Sp3 fragment) was in-

Abbreviations: PAPase, prostatic acid phosphatase; Sp1, Sp2, and Sp3 fragments, PAPase fragments 1, 2, and 3, respectively, generated by submaxillaris protease digestion.

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cubated with 1 mCi ($1 \text{ Ci} = 3.7 \times 10^{10}$ becquerels; 3.35 Ci/mmol) of carrier-free inorganic [^{32}P]pyrophosphate (New England Nuclear) in 200 μl of 0.2 M NaHCO_3 at pH 8.6 for 60 sec at room temperature. At the end of the incubation period, 0.5 ml of 0.2 M sodium phosphate at pH 7.5 was added. The proteins were precipitated with 20 vol of ice-chilled acetone. Acetone precipitation was repeated four times. The proteins were denatured by boiling, subjected to tryptic digestion, and lyophilized. Two-dimensional peptide mapping and autoradiography were performed as described above.

RESULTS

Limited Digestion of PAPase by Submaxillaris Protease.

For the separation of antigenic and functional fragments of PAPase several methods of protein cleavage were tried, and submaxillaris protease gave the best results. Submaxillaris protease digestion consistently yielded three large fragments of PAPase upon gel filtration (Fig. 1). Incubation of samples for varying time periods and varying enzymes/substrate ratio did not significantly alter the fragments obtained. These fragments were relatively resistant to further digestion by submaxillaris protease at 0–4°C as is characteristic of a structural domain. However denatured PAPase yielded over 12 small peptides after the digestion by submaxillaris protease at 37°C.

When the three PAPase fragments generated by submaxillaris protease digestion were separated by gel filtration, the first fraction (Sp1) contained a mixture of fragments of M_r 33,000, 31,000, and 18,000. The second (Sp2; M_r 18,000) and the third (Sp3; M_r 11,000) fractions were homogeneous in NaDodSO₄/polyacrylamide gel electrophoresis. Details of structural studies of the enzyme will be presented elsewhere and only experiments involving the Sp3 fragment will be described here.

Characterization of the Sp3 Fragment. The Sp3 fragment was a homogeneous polypeptide with an apparent M_r of 11,000–13,000, judging from rechromatography on Sephadex 75 and NaDodSO₄/polyacrylamide gel electrophoresis. About 73 amino acid residues were found to be present (Table 1), giving a calculated M_r of 9,910. The difference between this value and the molecular weight estimated by gel electrophoresis

FIG. 1. Separation of fragments of PAPase by gel filtration on superfine Sephadex G-75 after the limited digestion with submaxillaris protease. (Inset) Calibration of Sephadex G-75 column with marker proteins; ovalbumin (o), carbonic anhydrase (a), myoglobin (m), and cytochrome c (c). Arrows indicate the molecular weight (Inset) and elution position of fraction 3 (Sp3 fragment). This Sp3 fragment was used in the subsequent investigations.

Table 1. Amino acid composition of PAPase and its Sp3 fragment

Residues	Whole PAPase	Sp3
Aspartic acid	51.0	5
Threonine	46.0	3
Serine	51.7	3
Glutamic acid	100.0	12
Proline	52.0	4
Glycine	42.7	4
Alanine	45.2	1
Valine	35.5	6
Methionine	20.0	2
Isoleucine	38.1	3
Leucine	95.0	9
Tyrosine	37.0	3
Phenylalanine	30.5	5
Histidine	25.1	3
Lysine	44.0	7
Arginine	29.0	3
Tryptophan	ND	ND
Cysteic acid	ND	ND
Total	742.8	73.0

Values represent average of two determinations for each polypeptide. The summation of amino acid residues gave calculated M_r of 97,344 for PAPase and 9,910 for the Sp3 fragment. ND, not determined.

seems to be accounted for by the weight of the carbohydrate. The Sp3 fragment yielded 3 methionyl peptides upon CNBr cleavage and 8–13 tryptic peptides upon tryptic digestion which were compatible with the compositional analyses (Table 1). Concanavalin A-Sepharose 4B affinity chromatography of ^{125}I -labeled PAPase and ^{125}I -labeled Sp3 fragment showed that both polypeptides bind to this affinity matrix. Therefore, the glycosylated amino acid residues seem to be clustered on the Sp3 segment of the enzyme (1, 15).

Sp3 Fragment as the Active Site Domain. Dissociation of PAPase by 8 M urea into subunits or prolonged incubation (over 48 hr) of PAPase with submaxillaris protease leads to the total loss of catalytic activity. However, the catalytically inactive PAPase subunit and Sp3 fragments regain a portion of their enzymatic activity after the interaction with anti-PAPase antibodies (Table 2). The maximal level of restoration, in excess antibody, of enzyme activity by Sp3 fragments was ≈ 2 –3% of native enzyme, suggesting that Sp3 had decreased enzymatic potency even upon activation. Therefore, substrate concentrations and catalytic activity of Sp3–antibody complexes were investigated by using native PAPase–antibody complexes as a control (Fig. 2). Both polypeptide antibody complexes seemed to

Table 2. Antibody-mediated restoration of enzyme activity of PAPase and fragments

Polypeptides	Polypeptide concentration, ng/100 μl	Enzyme activity of immune complexes, $\mu\text{mol/min}^*$
PAPase	10	1.228 ± 0.022
Subunit	50	0.486 ± 0.024
Sp1	500	0.007 ± 0.004
Sp2	500	0.005 ± 0.006
Sp3	500	0.086 ± 0.004

* Four sets of reaction mixtures for each polypeptide were incubated with 1 μg of rabbit antibodies to PAPase at 4°C for 16 hr. Enzyme activities of immune complexes were determined by double-antibody immunoassay (10) and are expressed as μmol of *p*-nitrophenol produced per min.

have similar affinities for substrates (K_m); however, the catalytic activity (V_{max}) of Sp3-antibody complexes was only 1/10 of that of native PAPase-antibody complexes. These data suggest that substrates bind to Sp3 fragment as efficiently as to native enzyme but that the hydrolytic function is less in the Sp3 fragment-catalyzed reaction. It is possible that the binding of activating antibody to the determinant(s) on Sp3 affects the conformation of the polypeptide in such a way that Sp3 partially regains its hydrolytic activity.

L(+)-Tartrate Affinity Column Chromatography. L(+)-Tartrate binds to the active site of human PAPase as a transition state analog (19). As a probe of the active site conformation, interaction of Sp3 fragment with L(+)-tartrate was investigated (Fig. 3). Approximately 70% of the 125 I-labeled Sp3 fragment showed specific binding to this affinity column, indicating that the Sp3 fragment maintained a conformation to interact with L(+)-tartrate.

Occurrence of a Covalent Phosphoryl-Enzyme Intermediate. The results shown in Fig. 2 suggest that both the Sp3 fragment and native enzyme have similar affinities for substrate. Therefore, we investigated the phosphoryl group transfer catalyzed by Sp3, related to the possibility of participation of a phosphorylated polypeptide as an intermediate.

The PAPase and the Sp3 fragment were incubated with 32 P-labeled inorganic pyrophosphate at pH 8.5 and phosphoproteins were precipitated with acetone. No significant incorporation occurred with bovine serum albumin or with PAPase in the presence of 10 mM inorganic phosphate. The minute levels of radioactivity above background in those controls were of the order to be expected for residual adhering phosphate, in view of the enormous numbers of cpm in the initial incubation mixtures. As shown in Table 3, at least 84% of the 32 P was incorporated into PAPase or into Sp3. The quantity of radioactive polypeptides thus obtained was limited by the amount of phosphate rather than by the amount of protein. The 32 P-labeled proteins were further subjected to proteolytic digestion with trypsin. Both PAPase and Sp3 showed only two radioactive tryptic peptide spots in the autoradiograms (Fig. 4).

DISCUSSION

The present study provides evidence that limited digestion of PAPase with submaxillary protease consistently yields large

FIG. 2. Lineweaver-Burk plot for an antibody-mediated restoration of catalytic activity of Sp3 fragment. PAPase (○): K_m , 0.20 mM; V_{max} , 150 μ mol/min $\cdot$ mg $^{-1}$. Sp3 (●): K_m , 0.22 mM; V_{max} , 3 μ mol/min $\cdot$ mg $^{-1}$.

FIG. 3. Affinity chromatography (pH 7.0) of 125 I-labeled PAPase (□) and 125 I-labeled Sp3 fragment (■) on a L(+)-tartrate-Sepharose 4B column. Arrow indicates the start of sodium phosphate (0–20 μ M) gradient elution.

antigenic polypeptide fragments and that they may represent domain structures of PAPase. Furthermore, evidence was presented that one of the fragments, Sp3, represents an active site domain on the basis of the following experimental approaches: (i) antibody-mediated restoration of catalytic activity; (ii) L(+)-tartrate affinity chromatography; and (iii) 32 P-labeling of Sp3 fragment.

The mechanism of antibody-mediated restoration of catalytic activity of Sp3 is not yet clear. The best studied example of this phenomenon is the *Escherichia coli* β -galactosidase system, in which defective enzyme molecules that originated from various structural gene mutations were shown to regain catalytic activity through the interaction with specific antibodies to wild-type enzymes (20–22). In this case, the enzyme activation reaction is a single-hit event in which binding of one antibody molecule to an antigenic determinant seems to favor the correct conformation of one active center of the enzyme (20).

The antigenic mapping study of PAPase (7) showed that at least one major antigenically active determinant is localized on the Sp3 fragment. Therefore, it is possible that the binding of one antibody to this determinant on Sp3 favors, for thermodynamic reasons, a relatively slow rearrangement of the polypeptide conformation resulting in one active center.

Table 3. Covalent labeling of PAPase and Sp3 fragment

Protein				
Kind	Amount, mg	Incubation conditions*	32 P incorporation, %	$[^{32}\text{P}]\text{PP}_i$ incorporation, nmol
Albumin	3	0.3	0	—
PAPase	1	0.1†	0	—
PAPase	1	0.3	85	0.25
PAPase	2	0.3	96	0.29
Sp3	2	0.3	94	0.28
Sp3	4	0.3	94	0.28

Albumin, bovine serum albumin; PP_i , inorganic pyrophosphate.

* Values are expressed as nmol of $[^{32}\text{P}]\text{PP}_i$.

† With 10 mM P_i .

FIG. 4. Two-dimensional tryptic peptide maps of PAPase and Sp3 fragments. (Upper) Tracing of a peptide map of PAPase; arrow indicates the spots equivalent to ^{32}P -labeled tryptic peptides shown in Lower. Shaded spots in Upper show the tryptic peptides corresponding to tryptic peptides of Sp3 fragments. $\times$ indicates point of application. (Lower) Autoradiogram of tryptic peptide map of ^{32}P -labeled PAPase or ^{32}P -labeled Sp3 fragment; PAPase and Sp3 fragment both yielded identical ^{32}P -labeled tryptic peptides.

L(+)-Tartrate is a strong competitive inhibitor of PAPase. It has been suggested that the structure of L(+)-tartrate resembles that of the transition state for the rate-limiting step in the catalytic process of the enzyme, and thus that the inhibitory interaction of L(+)-tartrate is attributable to its action as a transition state analog (19). Following this reasoning, van Etten and

Saini (16) developed tartrate affinity chromatography for single-step purification of PAPase. Therefore, binding of ^{125}I -labeled Sp3 to the L(+)-tartrate affinity column demonstrates the structural similarities of active centers on Sp3 and the native enzyme.

van Etten and co-workers (18, 23) and Ostrowski (3) have shown that catalytic activity of PAPase involves phosphorylation of the active-site histidine residue. Specific labeling of the Sp3 fragment with ^{32}P and subsequent tryptic digestion resulted in peptides with covalently linked [^{32}P]orthophosphate. This further supports the view that Sp3 bears the catalytically active site.

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