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Bovine Liver Phosphoamidase as a Protein Histidine/Lysine Phosphatase

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A 13-kDa phosphoamidase was isolated as a single band on SDS-PAGE from bovine liver. Its Stokes' radius, sedimentation coefficient, molecular mass, and optimal pH were estimated to be 1.6 nm, 1.8 *s*, 13 kDa, and 6.5, respectively. The enzyme released P_i from 3-phosphohistidine, 6-phospholysine, and amidophosphate at rates of 0.9, 0.6, and 2.6 μmol/min/mg protein, respectively. However, it did not dephosphorylate phosphocreatine, N^{ω}-phosphoarginine, imidodiphosphate, or *O*-phosphorylated compounds including inorganic pyrophosphate. It also dephosphorylated succinic thiokinase and nucleoside diphosphate kinase autophosphorylated at His residues, indicating that it works as a protein histidine phosphatase. A thiol reagent, 30 μM *N*-ethylmaleimide, depressed the activity by half, while a thiol compound, 2-mercaptoethanol, protected the enzyme from heat-inactivation. Five millimolar divalent cations, such as Mg^{2+} and Mn^{2+}, and 5 mM EDTA, had no effect on the activity.

Key words: characterization, nucleoside diphosphate kinase, protein histidine/lysine phosphatase, purification, succinic thiokinase.

Phosphoamidases are enzymes that hydrolyze the nitrogen-phosphorus bond (N-P bond) of given *N*-phosphorylated compounds. The phosphoamidase classified as [EC 3.9.1.1] dephosphorylates phosphocreatine and N^{ω}-phosphoarginine (P-Arg), while the 56-kDa inorganic pyrophosphatase (PPase) from bovine liver also dephosphorylates amidophosphate (NP), imidodiphosphate (PNP), 3-phosphohistidine (P-His), and 6-phospholysine (P-Lys) (*1, 4*). However, the phosphoamidases also hydrolyze *O*-phosphorylated substrates such as glucose 6-phosphate (*2*) and phosvitin (*3*), while PPase hydrolyzes inorganic pyrophosphate (PP_i) (*1, 4*). In light of these findings, the possibility that phosphoamidases are identical to glucose 6-phosphatase [EC 3.1.3.9], phosphoprotein phosphatase [EC 3.1.3.16], and inorganic pyrophosphatase [EC 3.6.1.1] cannot be ignored. Moreover, alkaline phosphatase [EC 3.1.3.1] from rat renal microsomes also hydrolyzes phosphocreatine, P-Arg, glucose 6-phosphate, PP_i, and phosphotyrosine, in addition to other substrates (*5*). These findings support the idea that phosphoamidases do not form a group of enzymes distinct from phosphatases (*6*).

However, the above idea was challenged by the first report of a real phosphoamidase, N^{ω}-phosphoarginine phosphatase (PAPase). The enzyme is a 17-kDa phosphoamidase that is specific only for P-Arg and is free of the activity that hydrolyzes *O*-phosphorylated compounds and *N*-phosphorylated compounds including P-His, P-Lys, NP, and phosphocreatine (*7, 8*). It also dephosphorylates P-Arg residues in artificial peptides, suggesting that it works as a protein arginine phosphatase (*9*). Moreover, further studies of PAPase revealed that it is an *N*-acetylated product from the genes for the ubiquitin-conjugating enzymes, UBC 4A/10A and UBC2E (*10*).

Here, we report that a 13-kDa phosphoamidase specific for P-His, P-Lys, and NP occurs in bovine liver. It does not hydrolyze P-Arg, phosphocreatine, PNP, PP_i, or *O*-phosphorylated compounds. It also dephosphorylates both succinic thiokinase [EC 6.2.1.4] (STK) and nucleoside diphosphate kinase [EC 2.7.4.6] (NDPK) autophosphorylated at His residues. These results indicate that the 13-kDa phosphoamidase is also a real phosphoamidase and works as a protein histidine phosphatase. The possible biochemical significance of the 13-kDa phosphoamidase is discussed.

MATERIALS AND METHODS

Materials—Q-Sepharose Fast Flow, Sephadex G-75, and mono Q were products of Pharmacia LKB Biotechnology. Fluoromonophosphate was the product of Aldrich. 5′-Adenylylimidodiphosphate, PNP, P-Arg, porcine heart STK, and bovine liver NDPK were purchased from Sigma Chemical. [γ-^{32}P]ATP (222 TBq/mmol) and [γ-^{32}P]GTP (1.11 TBq/mmol) were purchased from Amersham Life Science and NEN Life Science Products, respectively. Other chemicals were of analytical grade.

Preparation of Phosphorylated Compounds and PPase—P-His, P-Lys, and NP were prepared according to the

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Abbreviations: P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; NDPK, nucleoside diphosphate kinase; NP, amidophosphate; PAGE, polyacrylamide gel electrophoresis; PAPase, N^{ω}-phosphoarginine phosphatase; P-Arg, N^{ω}-phosphoarginine; P-His, 3-phosphohistidine; P-Lys, 6-phospholysine; PNP, imidodiphosphate; PPase, 56-kDa inorganic pyrophosphatase; SDS, sodium dodecyl sulfate; STK, succinic thiokinase; N-P bond, nitrogen-phosphorus bond; FPLC, fast protein liquid chromatography.

methods of Hultquist *et al.* (*11*), Zetterqvist and Engström (*12*), and Sheridan *et al.* (*13*), respectively. STK (170 μg) or NDPK (680 μg) was incubated for 1 h at 25°C in buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and 1 μM [γ-^{32}P]GTP (1.11 TBq/mmol) or 1 μM [γ-^{32}P]ATP (1.11 TBq/mmol), respectively. Protein fractions were concentrated by a Microcon 10 concentrator (Amicon) in 50 mM Tris-HCl (pH 8.0) to serve as ^{32}P-labeled substrates for hydrolases. Protein concentration was determined by the method of Bradford (*14*). PPase was purified from bovine liver according to the method described previously (*4*). PPase hydrolyzes P-His at a rate of 0.4 μmol/min/mg protein.

Phosphoamidase Assay—A standard reaction mixture (25 μl) containing 50 mM Tris-HCl (pH 7.0), 1 mM substrate, and purified hydrolase was incubated for 30 min at 30°C. The released P_i was measured by the malachite green method as described previously (*7*). When ^{32}P-STK and ^{32}P-NDPK were employed as substrates, ^{32}P-STK (1.3 μg, 500 Bq) or ^{32}P-NDPK (5.9 μg, 1.8 kBq) was incubated at 25°C for 0, 0.5, 1, or 2 h with PPase (3.1 μg) or 13-kDa phosphoamidase (step 5, 2.3 μg), respectively, in a 15-μl reaction mixture containing 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. Upon incubation, the hydrolases showed the same P-His hydrolyzing activities at pH 8.0. After the addition of 15 μl of sample buffer (pH 8.8) to the reaction mixtures, 25 μl aliquots were subjected to basic 15% SDS-PAGE (*15*). The proteins on the gel were transferred to an Immobilon P membrane (Millipore) which was exposed to X-ray film for autoradiography.

High Voltage Paper Electrophoresis of Reaction Mixtures—Reaction mixtures (25 μl) containing 50 mM Tris buffer (pH 7.0) and 10 mM *N*-phosphorylated amino acids were incubated for 180 min at 30°C with 9 μg of 13-kDa phosphoamidase. The reaction was terminated by the addition of 3 μl of 40 mM *N*-ethylmaleimide. A control experiment was performed without 13-kDa phosphoamidase. An aliquot (5 μl) of each reaction mixture was subjected to high voltage paper electrophoresis according to the method of Hultquist *et al.* (*11*). Amino acids and P_i on the electrophoretograms were visualized by spraying with ninhydrin reagent and Rosenberg's reagent (*16*), respectively.

Treatment of ^{32}P-Labeled Proteins on Immobilon P Membranes with Acid and Base—After ^{32}P-STK or ^{32}P-NDPK on the Immobilon P membrane was located by short exposure to X-ray film, the membrane was cut into several strips and each strip was subjected to incubation for 1 h at 25°C in 0.1 M Tris-HCl (pH 7.5), 1 N KOH (pH 14), 1 N HCl (pH 1), and 0.8 M hydroxylamine (pH 5.4). After incubation, the strips were washed with water and 20 mM Tris-HCl (pH 8.0), and exposed again to X-ray film (*17*).

Phosphoamino Acid Analysis—^{32}P-STK or ^{32}P-NDPK on the Immobilon P membrane was hydrolyzed in 3 M KOH for 4 h at 110°C. A portion of the hydrolysate (10%) was neutralized with 10% perchloric acid, mixed with standard phosphoamino acids (P-Lys, P-His, and P-Ser), and analyzed directly on a silica gel sheet (Kodak) with two successive solvent (ethanol : 25% ammonia, 3.5:1.6) cycles (*18*). After the sheet was exposed to X-ray film, the positions of the standard amino acids were located with ninhydrin reagent.

Determination of Stokes' Radius—The Stokes' radius of the purified phosphoamidase (step 5, 0.5 mg) was determined using ubiquitin (1.3 nm), cytochrome *c* (1.6 nm),

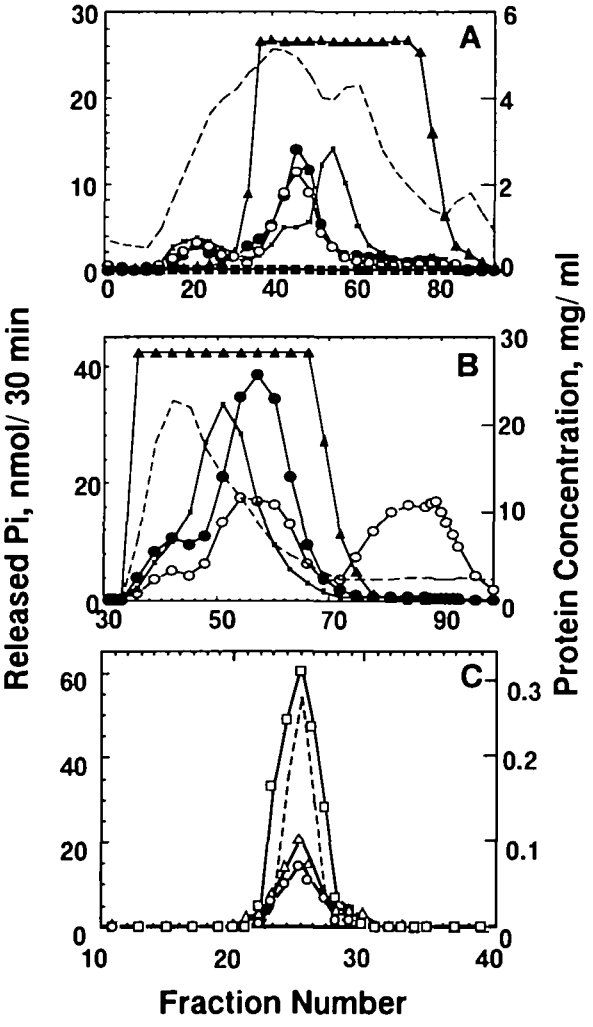

Fig. 1. **Isolation of 13-kDa phosphoamidase from bovine liver.** Substrates employed were P-Lys (open circles), P-His (open triangles), P-Arg (closed squares), PNP (closed circles), NP (open squares), PP_i (closed triangles), and *p*-nitrophenyl phosphate (×). Total volume of the reaction mixture was 25 μl for all assays of hydrolysis. The hydrolyses of PNP, PP_i, and *p*-nitrophenyl phosphate were assayed in the presence of 1 mM $MgCl_2$. Protein concentration is shown by the dashed line. (A) Q-Sepharose Fast Flow column chromatography: The dialysate from Step 2 was applied to a Q-Sepharose Fast Flow column (2.7×18 cm). The column was washed with buffer A, and an 800-ml linear gradient of NaCl from 0 to 400 mM was applied. The hydrolyzing activities were assayed using 10 μl of eluate. (B) Sephadex G-75 column chromatography: The dialysate from Step 3 was applied to a Sephadex G-75 column (1.6×95 cm). The hydrolyzing activities were assayed using 20 μl of eluate. The column was calibrated with standard markers: ovalbumin (molecular mass, 43 kDa; Stokes' radius, 2.7 nm), cytochrome *c* (13 kDa; 1.6 nm), and ubiquitin (8 kDa; 1.3 nm) eluted at fractions 54, 76, and 114, respectively, while blue dextran 2000 and phenol red eluted at fractions 36 and 130, respectively. The 13-kDa phosphoamidase eluted after cytochrome *c* and its molecular mass was estimated to be 12 kDa by the method of Porath (*19*). (C) monoQ FPLC: MonoQ FPLC (1×5 cm) was carried out with a 50-ml linear gradient of NaCl from 0 to 200 mM in buffer A. Assays were performed using 3 μl of eluate. The protein profile is also shown in Fig. 2A.

and ovalbumin (2.7 nm) as internal standards, according to the method of Porath (*19*). The elution peaks of phosphoamidase and each standard protein were determined by phosphoamidase assay and 15% SDS-PAGE (*20*), respectively.

Determination of the Sedimentation Coefficient—The sedimentation coefficient was estimated according to the method of Martin and Ames (*21*). Briefly, a 0.2-ml mixture containing purified phosphoamidase (step 5, 0.5 mg) and standard proteins such as ubiquitin (1.3 *s*), cytochrome *c* (1.8 *s*), and myoglobin (2.1 *s*), were layered on top of a 5 to 20% linear gradient of sucrose (11 ml) containing 50 mM Tris-HCl (pH 7.5) and 10 mM 2-mercaptoethanol. After centrifugation for 70 h at 120,000 × *g* in a Beckman SW 40 rotor, 10 drop fractions were collected from the bottom of the tubes. The elution peaks of phosphoamidase and standard proteins were determined by phosphoamidase assay and 15% SDS-PAGE (*20*), respectively.

RESULTS

Purification of 13-kDa Phosphoamidase—All procedures were performed in a cold room.

Step 1 (Extraction): Bovine liver (100 g) was homogenized in 400 ml of buffer A (50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine hydrochloride, and 5 mM EDTA in a Waring blender. The homogenate was centrifuged for 40 min at 10,000 × *g*.

Step 2 (Ammonium sulfate fractionation): The supernatant was fractionated with ammonium sulfate from 40 to 60%. The precipitate was dissolved in a minimal volume of buffer A and dialyzed overnight against the same buffer.

Step 3 (Q-Sepharose fast flow column chromatography): The dialysate was applied to a Q-Sepharose column (2.7 × 18 cm) equilibrated with buffer A. After washing the column with buffer A, the column was eluted with an 800-ml linear gradient of NaCl from 0 to 400 mM in buffer A. Fractions of 8 ml were collected. The P-Lys hydrolyzing activity was eluted as two peaks at fractions 22 and 46 (Fig. 1A). The second peak at fraction 46 also contained PNP, *p*-nitrophenyl phosphate, and PP_i hydrolyzing activities. It also showed P-His and NP hydrolyzing activities (data not shown). Fractions 38 to 58 were combined and precipitated with 80% ammonium sulfate. The precipitate was dissolved in a minimal volume of buffer A and dialyzed against 150 mM NaCl in buffer A.

Step 4 (Sephadex G-75 column chromatography): The dialysate was applied to a Sephadex G-75 column (1.6 × 95 cm) equilibrated with 150 mM NaCl in buffer A. Fractions of 3 ml were collected. The P-Lys hydrolyzing activity was eluted as two peaks at fractions 57 and 84, while the other activities and most proteins were eluted before the second peak (Fig. 1B). The enzyme in the first peak that hydrolyzes PP_i, PNP, and P-Lys was purified, and identified as the PPase reported previously (*4*). In this manuscript, the second peak including fractions 72 to 98 was combined and applied to a hydroxylapatite column (0.7 × 9 cm) equilibrated with buffer A. After application, the column was washed with 25 ml of buffer A. Most proteins were retained in the column, while most of the activity was eluted in the flow through volume and wash fraction. Therefore, the fractions not retained on the hydroxylapatite column were combined and dialyzed against buffer A.

Step 5 [monoQ Fast Protein Liquid Chromatography (FPLC)]: The dialysate was applied to a monoQ column (1 × 5 cm) equilibrated with buffer A. After washing the column with buffer A, a 50-ml linear gradient of NaCl from 0 to 200 mM in buffer A was applied. Fractions of 1 ml were collected (Fig. 1C). The P-Lys hydrolyzing activity was

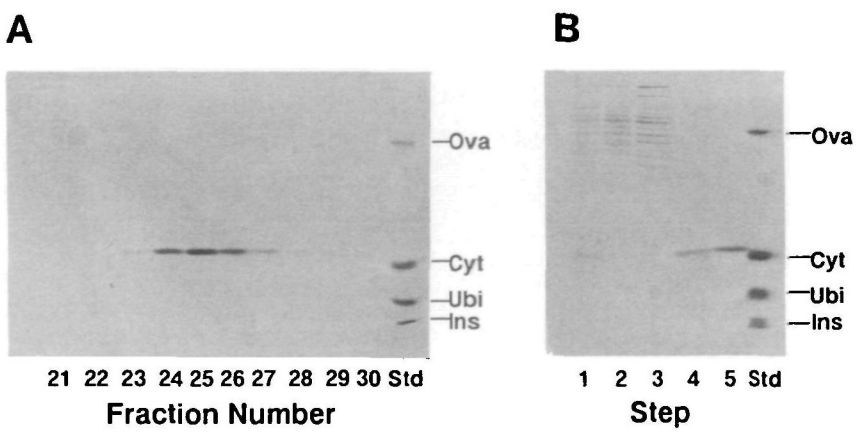

Fig. 2. **Identification of the 13-kDa phosphoamidase.** (A) Purity of the 13-kDa phosphoamidase at Step 5. The eluate (20 μl) from monoQ FPLC was subjected to 15% SDS-PAGE (Fig. 1C). Numbers under the lanes indicate fraction numbers in Fig. 1C. (B) Protein profile during each purification step. The preparation (7 μg) at each step was subjected to 15% SDS-PAGE. The 13-kDa protein band was visible at the Sephadex G-75 step (Step 4), and free of other protein bands at Step 5. The migration positions of standard proteins are denoted in the right margin; Ova (ovalbumin), Cyt (cytochrome *c*), Ubi (ubiquitin), and Ins (insulin). The migration position of the purified protein was slower than that of cytochrome *c*, and its molecular mass was estimated to be 13 kDa.

TABLE I. **Summary of the purification of the 13-kDa phosphoamidase from bovine liver.**

Step	Total protein (mg)	Total activity[a] (P_i, nmol/min)	Yield (%)	Specific activity (P_i, nmol/min/mg)	Purity (fold)
1. Extraction	19,305	49,421	100	2.56	1.0
2. Ammonium sulf.	6,521	24,975	51	3.83	1.5
3. Q-Sepharose	375	4,792	10	12.8	5.0
4. Seph. G-75	5.8	2,291	5	395	154
5. mono Q FPLC	0.67	382	0.8	570	223

[a]Activity was assayed with P-Lys as a substrate.

eluted as a single peak at 75 mM NaCl from fraction 22 to 29. P-His and NP hydrolyzing activities, in addition to the P-Lys hydrolyzing activity, were also detected, and the changes in these three activities agreed with the protein changes (Fig. 1C).

Identification of 13-kDa Phosphoamidase—15% SDS-PAGE revealed that the phosphoamidase activity coincided with the amount of the 13-kDa protein and, thus, this hydrolase was tentatively designated as a 13-kDa phosphoamidase (Fig. 2). During purification, the 13-kDa protein band was visible at step 4 and was free of other protein bands at step 5 (Fig. 2B). The results of the above purification steps are summarized in Table I. The yield was 0.8% with a 223-fold purification. The most effective step was Sephadex G-75 column chromatography (Step 4). In this step, about half the total activity was eluted in fractions where most proteins and other activities were absent, resulting in a 31-fold increase in the specific activity.

Physicochemical Properties of the 13-kDa Phosphoamidase—The Stokes' radius and sedimentation coefficient were estimated to be 1.6 nm and 1.8 *s*, respectively (data not shown). The optimal pH was 6.5, as shown in Fig. 3.

Substrate Specificity of the 13-kDa Phosphoamidase—The purified phosphoamidase produced P_i and histidine from P-His (Fig. 4, panel A and B, lanes 3 and 4) at a rate of 0.9 μmol/min/mg protein, and P_i and lysine from P-Lys (Fig. 4, panel A and B, lanes 5 and 6) at a rate of 0.6 μmol/min/mg protein. Moreover, this enzyme hydrolyzed NP to P_i and ammonia at a rate of 2.6 μmol/min/mg protein, whereas N^{ω}-phosphoarginine was not hydrolyzed (Fig. 4, panel A and B, lanes 1 and 2). The substrate concentration curves are given in Fig. 5. K_m values were estimated to be 3.3, 5.0, and 1.7 mM for P-His, P-Lys, and NP, respectively. Other *N*-phosphorylated compounds including phosphocreatine, PNP, and 5′-adenylylimidodiphosphate were not hydrolyzed. *O*-Phosphorylated compounds, including phosphotyrosine (Fig. 4, panel A and B, lanes 7 and 8), phosphothreonine, phosphoserine, nucleoside monophosphates, nucleoside diphosphates, nucleoside triphosphates, *p*-nitrophenyl phosphate, glucose 6-phosphate, and PP_i, were not hydrolyzed. Moreover, acetylphosphate and fluoromonophosphate were not hydrolyzed.

Effect of Thiol Reagents and Thiol Compounds on the 13-kDa Phosphoamidase—*N*-Ethylmaleimide (30 μM) inhibited the P-His and NP hydrolyzing activities by half and the P-Lys hydrolyzing activity by about 90% (Fig. 6). However, thiol reagents including *p*-chloromercuriphenyl

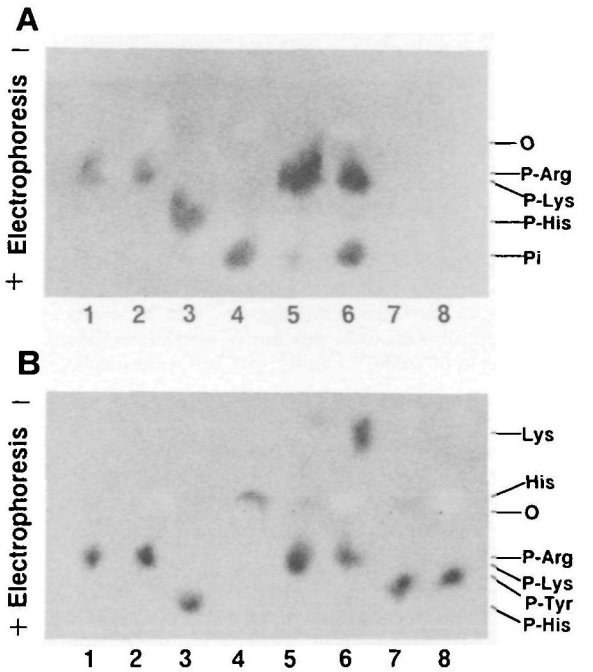

Fig. 4. **Product analysis after the incubation of *N*-phosphorylated amino acids with the 13-kDa phosphoamidase.** A 25-μl reaction mixture containing 50 mM Tris-HCl (pH 7.0), 10 mM *N*-phosphorylated amino acid, and 9 μg of 13-kDa phosphoamidase was incubated for 180 min at 30°C. The reaction was terminated by adding 3 μl of 40 mM NEM. A control reaction mixture was incubated for 180 min at 30°C without 13-kDa phosphoamidase. An aliquot (5 μl) of the reaction mixture was applied to a filter paper (20×20 cm) and high-voltage paper electrophoresis was performed for 30 min at 15°C according to the method of Hultquist *et al.* (*11*). (A) Rosenberg's staining; (B) Ninhydrin staining. Substrates employed: lane 1, P-Arg/control; lane 2, P-Arg; lane 3, P-His/control; lane 4, P-His; lane 5, P-Lys/control; lane 6, P-Lys; lane 7, P-Tyr/control; lane 8, P-Tyr. The position of the origin (O) and the migration positions of standard compounds are denoted in the right margin.

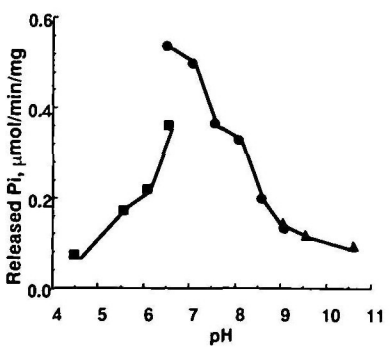

Fig. 3. **pH curve of the 13-kDa phosphoamidase.** Reaction mixtures (25 μl) containing 1 mM P-Lys and purified phosphoamidase (0.56 μg) in 50 mM Mes buffer (closed squares), Tris buffer (closed circles), or Gly buffer (closed triangles) were incubated for 30 min at 30°C.

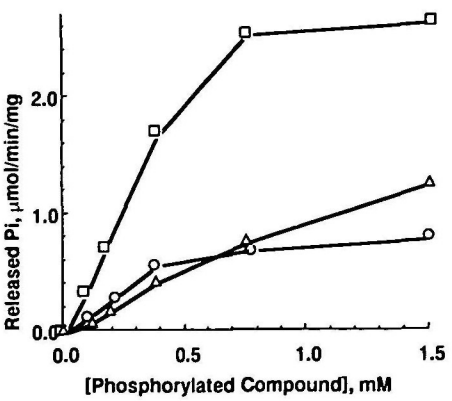

Fig. 5. **Substrate concentration curve of the 13-kDa phosphoamidase.** Reaction mixtures (25 μl) containing P-Lys (open circles), P-His (open triangles), or NP (open squares) at the indicated concentrations, and purified phosphoamidase (0.56 μg) in 50 mM Tris buffer (pH 7.0), were incubated for 30 min at 30°C.

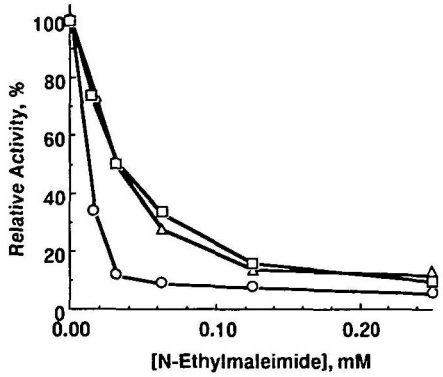

Fig. 6. **Effect of *N*-Ethylmaleimide on the 13-kDa Phosphoamidase.** Reaction mixtures (25 μl) containing 1 mM P-Lys (open circles), 1 mM P-His (open triangles), or 1 mM NP (open squares), purified phosphoamidase (0.56 μg), and *N*-ethylmaleimide at various concentrations in 50 mM Tris buffer (pH 7.0) were incubated for 30 min at 30°C.

sulfonate and idoacetamide at 1 mM had no effect on the hydrolyzing activity.

The addition of thiol compounds such as dithiothreitol and 2-mercaptoethanol to the reaction mixture had no effect on the phosphoamidase activity. However, the presence of 16 mM 2-mercaptoethanol protected the enzyme from heat-inactivation (data not shown). The stabilizing effect of 2-mercaptoethanol was dependent on the concentration of the thiol compound (data not shown).

Effect of Other Reagents on the 13-kDa Phosphoamidase—Sodium fluoride at 5 mM had no inhibitory effect. The addition of 5 mM EDTA had no effect on the phosphoamidase activity. Divalent cations such as Mg^{2+} and Mn^{2+} also had no effect on the hydrolysis of P-His, P-Lys, or NP by the phosphoamidase. No substrate tested was hydrolyzed after the addition of Mg^{2+} or Mn^{2+}.

Sodium vanadate and sodium molybdate at 1 mM inhibited the P-Lys, P-His, and NP hydrolyzing activities by 20–50% (data not shown). Fifty percent inhibition of the P-Lys, P-His, and NP hydrolyzing activities were observed in the presence of 0.6 M guanidine hydrochloride and 1.3 mM sodium dodecyl sulfate (data not shown). These results suggest that these three activities reside in the 13-kDa phosphoamidase molecule.

Dephosphorylation of ^{32}P-STK and ^{32}P-NDPK by the 13-kDa Phosphoamidase and PPase—The phosphorus incorporated into autophosphorylated STK and NDPK on the Immobilon P membrane (Fig. 7A) was stable to 0.1 M Tris-HCl (pH 7.5) (lane 1) and 1 N KOH (pH 14) (lane 2), and labile to treatments with 1 N HCl (pH 1) (lane 3) and 0.8 M hydroxylamine (pH 5.4) (lane 4), suggesting that the phosphorylated amino acid residues are His and Lys. P-His was identified in the alkaline hydrolysate of both substrates (Fig. 7B). The alkaline hydrolysate of ^{32}P-NDPK also yielded P-Lys (Fig. 7B). Both ^{32}P-labeled proteins were dephosphorylated by a 56-kDa inorganic pyrophosphatase (56-kDa PPase) (Fig. 7C, lanes 1-4) and the 13-kDa phosphoamidase (Fig. 7C, lane 1 and lanes 5-7). Since the employed hydrolases showed the same P-His hydrolyzing activity, both were better substrates for the 56-kDa PPase than the 13-kDa phosphoamidase. These findings indicate that both hydrolases function as protein histidine (lysine) phosphatases.

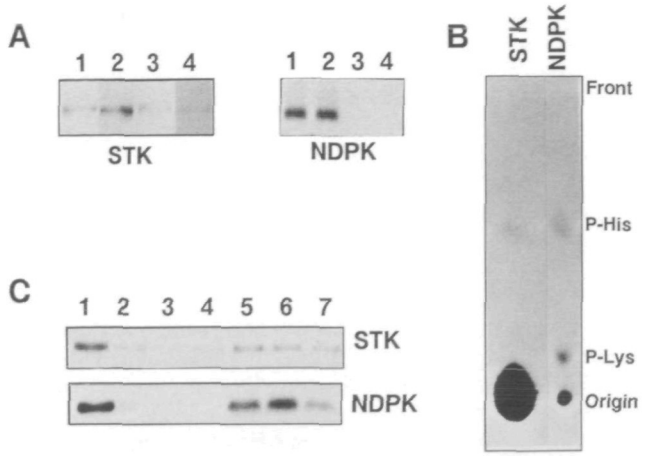

Fig. 7. **^{32}P-STK and ^{32}P-NDPK as substrates for the 13-kDa phosphoamidase and 56-kDa PPase.** A: Stability of ^{32}P-labeled proteins to alkaline and acid treatment. The protein bands of ^{32}P-STK and ^{32}P-NDPK on basic SDS-PAGE were transferred to an Immobilon P membrane and exposed for 1 h at 25°C to 0.1 M Tris (pH 7.5) (lane 1), 1 N KOH (pH 14) (lane 2), 1 N HCl (pH 1) (lane 3), or 0.8 M hydroxylamine (pH 5.4) (lane 4). Bands were visualized by autoradiography. B: Identification of ^{32}P-labeled amino acids. ^{32}P-labeled protein substrates on an Immobilon P membrane were hydrolyzed for 4 h at 110°C, neutralized with 10% perchloric acid, and centrifuged. The supernatant was subjected to thin layer chromatography on silica gel with two successive solvent cycles, as described in "MATERIALS AND METHODS." This chromatogram was subjected to autoradiography. C: Time courses for the incubation of ^{32}P-proteins with two kinds of hydrolases. ^{32}P-STK (1.3 μg, 500 Bq) or ^{32}P-NDPK (5.9 μg, 1.8 kBq) in 15-μl reaction mixtures containing 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol was incubated at 25°C for 0 h (lane 1), 0.5 h (lanes 2 and 5), 1 h (lanes 3 and 6), or 2 h (lanes 4 and 7) with no hydrolase (lane 1), PPase (3.1 μg, lanes 2-4), or the 13-kDa phosphoamidase (Step 5, 2.3 μg, lanes 5-7). During incubation, the hydrolases showed the same P-His hydrolyzing activities. After the addition of 15 μl sample buffer (pH 8.8) to the reaction mixtures, 25 μl aliquots were subjected to basic 15% SDS-PAGE. Proteins on the gel were transferred to an Immobilon P membrane which was then exposed to X-ray film for autoradiography.

DISCUSSION

The 13-kDa phosphoamidase in this report hydrolyzes not only amidophosphate (NP) but also *N*-phosphorylated amino acids such as P-His and P-Lys instead of P-Arg in the case of PAPase, and does not hydrolyze other *N*-phosphorylated substrates including phosphocreatine or *O*-phosphorylated substrates including phosphotyrosine and glucose 6-phosphate. Accordingly, this 13-kDa phosphoamidase is a second real phosphoamidase. This phosphoamidase has an estimated molecular mass of 13 kDa, sedimentation coefficient of 1.8 *s*, Stokes' radius of 1.6 nm, and pH optimum of 6.5. Moreover, the hydrolase is not inhibited by pCMPS or iodoacetoamide, but only by 30 μM *N*-ethylmaleimide. This suggests that this hydrolase is distinct from the phosphoamidases reported thus far. Although there is no report of the natural occurrence of free P-His or P-Lys, P-His (*15, 22-32*) and P-Lys (*12, 25, 27*) residues in proteins have been reported. In this study, P-His residues were also detected in ^{32}P-STK and ^{32}P-NDPK, while P-Lys was identified in ^{32}P-NDPK (Fig. 7B), as reported previously (*25*). Freije *et al.* have shown that

one human NDPK, Nm23-H1, autophosphorylates a His residue and this autophosphorylated NDPK transfers its phosphoryl group to the His residue of porcine heart STK (*33*). In this paper, the autophosphorylated forms of these proteins are shown to be substrates for PPase and the 13-kDa phosphoamidase, indicating that both hydrolases work as protein histidine (or lysine) phosphatases, as in the case of the 10.1-kDa phosphoprotein phosphatase of *Pseudomonas aeruginosa* (*34*). The P-His hydrolyzing activity is equivalent to the P-Lys hydrolyzing activity of PPase. In contrast, the P-His hydrolyzing activity was 1.5-fold higher than the P-Lys hydrolyzing activity of the 13-kDa phosphoamidase. This seems to be related to the affinities of both enzymes for P-NDPK and P-STK (PPase hydrolyzes P-NDPK and P-STK more actively than the 13-kDa phosphoamidase). As described above, the 13-kDa phosphoamidase appears to have different affinities than PPase for recognition sequences that include phosphorylation sites when they hydrolyze the same P-His or P-Lys residues in identical substrate proteins.

P-His residues are especially interesting for two reasons. First, the P-His residue is an intermediate in the phosphotransfer reactions of several enzymes including NDPK (*25*), STK (*22*), histidine-containing protein (HPr) (*35*), ATP-citrate lyase (*36*), fructose 2,6-bisphosphatase (*37*), and isocitrate lyase (*38*). Second, the phosphate moiety of the P-His residue in the sensor protein is transferred to acidic amino acid residues, Asp or Glu, in the response regulator in the two component systems of bacteria (*39*), yeast (*40*), and plants (*41*). Recently, in this system, Ogino *et al.* have reported that one protein histidine phosphatase from *Escherichia coli*, SixA protein, is involved in the His-Asp phosphorelay by dephosphorylating an HPt domain phosphorylated at His-717 (*42*). However, it does not dephosphorylate EnvZ phosphorylated at His-273, and has a specific sequence motif of Arg-His-Gly, which presumably functions as a nucleophilic phosphoacceptor, in the N-terminal region (*42*). On the other hand, the mammalian p36 protein, which is induced by peroxisome proliferators, is phosphorylated on a His residue *in vitro* and dephosphorylated by PP2C (*43*). It has also been shown that p36 histidyl phosphorylation occurs in rat hepatoma cells *in vivo* (*32*). In another case, P-His in proteins is hydrolyzed by *O*-phosphorylated protein phosphatases such as PP1, PP2A, and PP2C (*43-45*). In addition, it has been shown that nm23H-1 can transfer a phosphate moiety from its catalytic His to Asp or Glu residues on a 43-kDa membrane protein from bovine brain (*46*). Although the physiological significance of the 13-kDa phosphoamidase remains unknown at present, there is the possibility that it also plays a role like that of SixA in *Escherichia coli*, in regulating the phosphorylation state of proteins phosphorylated at His residues (for example, in the phosphorelay from nm23H-1 to the 43-kDa membrane protein described above).

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