A protamine kinase has been purified to apparent homogeneity from extracts of the cytosol of bovine kidney cortex. This protamine kinase exhibited an apparent Mr = 43,000 as estimated by gel permeation chromatography on Sephacryl S-200 and an apparent Mr = 45,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified protamine kinase exhibited about 5% activity with casein, 8% with histone H2B, and <0.1% with histone H1, histone H4, glycogen synthase a from rabbit skeletal muscle, ovalbumin, bovine serum albumin, and phosvitin. The activity of the highly purified protamine kinase was unaffected by cyclic AMP (up to 0.1 mM), cyclic GMP (up to 0.1 mM), the heat-stable protein inhibitor of cyclic AMP-dependent protein kinase (up to 100 μg/ml), heparin (up to 100 μg/ml), EGTA (up to 1 mM), Ca2+ (up to 1 mM), calmodulin (up to 0.5 μM) in the absence or presence of Ca2+ (0.05 mM), and phosphatidylserine (up to 40 μg/ml) and/or diolein (up to 1 μg/ml) in the absence or presence of Ca2+ (up to 0.5 mM). Experiments in which extracts of kidney cytosol were incubated with [γ-32P]ATP and MgCl₂ revealed that the phosphorylation of numerous polypeptides was markedly increased in the presence of the purified protamine kinase. The results indicate that this protamine kinase of kidney cytosol is a novel protein kinase.

The regulation of enzyme activity by phosphorylation/dephosphorylation is an important mechanism for the coordinated control of cellular activity in response to extracellular stimuli (1, 2). Over the last few years, it has become evident that cells contain numerous protein kinases. The substrate specificity and subunit composition of many of these enzymes have been determined (reviewed in Ref. 3). The availability of this information has provided a firm molecular basis for further characterization of the structure, physiological role, and regulation of these kinases.

Recently, two forms of a protamine kinase have been identified from the soluble fraction of bovine kidney mitochondria (4). Both forms of this protamine kinase were present in an inactive form in mitochondrial extracts and were only detected following an initial chromatography on poly(L-lysine)-agarose. The two forms of the mitochondrial protamine kinase were separated by chromatography on protamine-agarose, and each form was purified about 80,000-fold to apparent homogeneity. Both forms of the protamine kinase exhibited an apparent Mr = 45,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography on Sephacryl S-200. Both forms of the protamine kinase underwent autophosphorylation and exhibited identical substrate specificities. The properties of the two forms of the protamine kinase of bovine kidney mitochondria distinguished these enzymes from previously described protein kinases.

In studies designed to determine the subcellular distribution of the mitochondrial protamine kinase in bovine kidney, we detected 40-100-fold higher protamine kinase activity in extracts of cytosol. In order to determine the relationship, if any, of cytosolic protamine kinase to the two forms of the mitochondrial protamine kinase, the protamine kinase of kidney cytosol was examined. In this paper, we report the purification of the cytosolic protamine kinase to apparent homogeneity and describe studies on the substrate specificity of this protamine kinase. The results indicate that the cytosolic protamine kinase is distinct from the two forms of the mitochondrial protamine kinase and also from previously described protein kinases. The results also indicate that the cytosolic protamine kinase exhibits activity toward a large number of physiological polypeptides.

**Experimental Procedures**

**Materials—**Sephacryl S-200, poly(L-lysine)-agarose, protamine-agarose, dephosphorylated casein, protamine sulfate, the catalytic subunit, and the heat-stable protein inhibitor of cyclic AMP-dependent protein kinase, phospholysine b, phosvitin, glycogen synthase a, ovalbumin, bovine serum albumin, poly(γ-ethylglycol) (4.1), and histones were from Sigma. Q-Sepharose was from Pharmacia LKB Biotechnology Inc. Poly(ethylene glycol) 8000 was obtained from J. T. Baker Chemical Co. Bovine kidney mitochondrial protamine kinase and type II casein kinase were purified to apparent homogeneity as described (4). Other materials are given in Refs. 4 and 5.

**Cytosolic Protamine Kinase Assay—**The protamine sulfate used in the following assay was near homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7). When purified protamine kinase preparations were employed in the incubations, this protamine was the only phosphate acceptor as shown by autoradiography of sodium dodecyl sulfate-polyacrylamide gels. The assay mixture (0.1 ml) contained 50 mM imidazole chloride, pH 7.0, 10% glycerol, 1 mM benzamidine, 0.25 mg of bovine serum albumin, 14 mM β-mercaptoethanol, 1.0 mg of protamine sulfate, 10 mM MgCl₂, 0.2 mM [γ-32P]ATP (150-300 cpm/pmol), and kinase sample. The ATP and MgCl₂ were added after equilibration of the mixture for 5 min at 30°C in a plastic microcentrifuge tube. After a 10-min reaction period, 1 ml of 10% trichloroacetic acid was added. The mixture was centrifuged at 10,000 rpm for 2 min in an Eppendorf microcentrifuge. The supernatant fluid was discarded, and the pellet was washed four times with 1-ml portions of trichloroacetic acid. Aqueous counting scintillant (1 ml) (Ready-solv™, Beckman) was added to the tube, and radioactivity was determined. Kinase sample was omitted from the control. One unit of cytosolic protamine kinase activity is defined as the amount of enzyme that incorporated 1 μmol of 32P into protamine/min. To ensure linearity, the extent of incorporation of
phosphoryl groups was limited to <3 nmol. This is equivalent to <20% conversion of the limiting substrate.

Other Protein Kinase Assays—Determination of mitochondrial protamine kinase activity was performed as described for the cytosolic protamine kinase except that 0.25 mg of ovalbumin and 1.5 mM MgCl₂ were used instead of bovine serum albumin and 10 mM MgCl₂. One unit of mitochondrial protamine kinase activity is defined as the amount of enzyme that incorporated 1 μmol of ³²P into protamine/min. The assay for cyclic AMP-dependent protein kinase activity was as described for the protamine kinase except that 1.0 mg of histone H₂B was used instead of protamine, and 0.5 mM CaCl₂ and 40 μg/ml phosphatidylserine were included in the incubations. One unit of protein kinase C activity is defined as the amount of enzyme that incorporated 1 μmol of ³²P into histone H₁/min.

Protein was determined as described (6). Polyacrylamide gel electrophoresis was performed in slab gels (12% acrylamide) with 0.1% sodium dodecyl sulfate and Tris/glycine buffer, pH 8.3 (7). Protein bands were detected by staining with Coomassie Blue. Radioactive bands were located with Kodak X-Omat AR-5 film.

RESULTS

Purification of the Cytosolic Protamine Kinase—All operations were carried out at 2-5 °C. Fresh bovine kidney was obtained from a local abattoir and transferred to the laboratory on ice. The kidney cortex was removed and homogenized with 2 liters of buffer A and then developed with a 1.0-liter linear gradient from 0.0 to 1.0 M NaCl. This step was effective in removing traces of cyclic AMP-dependent protein kinase and protein kinase C-like activities (not shown). The active fractions from Sephacryl S-200 were collected. The active fractions eluting from the column at about 0.35 M NaCl were pooled (step 5), diluted with 1 volume of buffer B, and chromatographed on a column (2.0 × 8 cm) of protamine-agarose equilibrated in buffer B. The column was washed with 2 liters of buffer B containing 0.4 M NaCl and then developed with a 300-mL linear gradient from 0.4 to 1.2 M NaCl. The flow rate was 80 ml/h, and 2.8-ml fractions were collected. The active fractions eluting from the column at about 0.7 M NaCl were pooled (step 6), diluted with 4 volumes of buffer B, and concentrated on a column (0.5 × 1 cm) of Q-Sepharose equilibrated in buffer B. The column was washed with 30 ml of buffer B, and protamine kinase was eluted with buffer B containing 0.8 M NaCl. The active fractions were combined (~3 ml) and subjected to gel permeation chromatography on a column (2.5 × 95 cm) of Sephacryl S-200 equilibrated and developed with buffer B containing 0.5 M NaCl. The active fractions (step 7) were combined and concentrated using a small column of Q-Sepharose as described above and then rechromatographed on Sephacryl S-200 (step 8, Fig. 1). A summary of the purification is presented in Table I.

Homogeneity and Composition of the Cytosolic Protamine Kinase—The apparent M₉ of the purified protamine kinase as estimated by gel permeation chromatography on a calibrated Sephacryl S-200 column was about 43,600 (Fig. 1). The highly purified enzyme showed a single band on sodium dodecyl sulfate-slab polyacrylamide gel electrophoresis with apparent M₉ = 45,000 (Fig. 1, inset). The protocol for the purification of the cytosolic protamine kinase has been successfully used 12 times. In three of these preparations, the kinase was probably partially proteolysed and exhibited an apparent M₉ = 42,500 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown). None of the purified preparations underwent autophosphorylation in the presence of 10 μg of ³²P-labeled ATP.

![Fig. 1. Gel permeation chromatography of protamine kinase on Sephacryl S-200](image-url)
Protamine was preferentially phosphorylated by the purified cytosolic and mitochondrial protamine kinases. The two forms of the mitochondrial protamine kinase exhibited activity toward histone H1 (≈90%), bovine serum albumin (≈48%), and glycogen synthase from rabbit skeletal muscle (≈15%). With these substrates the cytosolic protamine kinase was essentially inactive. The cytosolic protamine kinase exhibited some activity with histone H2B (≈8%) and casein (≈5%). With these substrates the mitochondrial protamine kinases were inactive. The cytosolic and mitochondrial protamine kinases exhibited little activity (<0.1%) with histone H4, ovalbumin, phospho, and the synthetic polypeptide poly (Glu,Tyr) (4:1). With protamine as substrate, each of the purified enzymes exhibited a broad pH optimum of 5.7–9.0. Differences in the substrate specificities of the purified cytosolic and mitochondrial protamine kinases are summarized in Table II.

The activity of the cytosolic protamine kinase, like the activities of the mitochondrial protamine kinases (4), was unaffected by cyclic AMP (up to 0.1 mM) or cyclic GMP (up to 0.1 mM). Similarly, the heat-stable inhibitor of cyclic AMP-dependent protein kinase at concentrations (up to 100 µg/ml) that completely inhibited the catalytic subunit of cyclic AMP-dependent protein kinase with either histone H2B or protamine as substrate was without effect on the activity of the cytosolic protamine kinase with protamine, histone H2B, or casein. Heparin up to 100 µg/ml had no effect on the cytosolic enzyme with protamine or casein as a substrate. The activity of the cytosolic protamine kinase, like the activities of the two forms of the mitochondrial protamine kinase (this study and Ref. 4), was also unaffected by calmodulin (up to 0.5 µM) with or without Ca²⁺ (0.05 mM). In addition, phosphatidylycerine (up to 40 µg/ml) and/or diolein (up to 1 µg/ml), in the presence or presence of Ca²⁺ (up to 0.5 mM), were without effect on the activities of the cytosolic protamine kinase and the two forms of the mitochondrial kinase. At 0.2 mM, GTP did not replace ATP in the cytosolic or mitochondrial protamine kinase reactions.

**Physiological Role of the Cytosolic Protamine Kinase**—As a first step in our studies on the physiological role of the cytosolic protamine kinase, extracts of kidney cytosol were incubated with [γ⁻³²P]ATP and MgCl₂ in the absence and

| Substrate | Mitochondrial protamine kinase I | Mitochondrial protamine kinase II | Cytosolic protamine kinase |
|-----------|---------------------------------|---------------------------------|---------------------------|
| Protamine | 100                             | 100                             | 100                       |
| Histone H1| 86                              | 93                              | <0.1                      |
| Bovine serum albumin | 45                              | 51                              | <0.1                      |
| Glycogen synthase a | 12                              | 9                               | <0.1                      |
| Histone H2B | <0.1                           | <0.1                            | 8                         |
| Casein   | <0.1                            | <0.1                            | 5                         |

* The final concentration of all the proteins tested was 10 mg/ml except for glycogen synthase a (1 mg/ml) and casein (6 mg/ml). The activity of the kinases with bovine serum albumin was determined as described (4). All other reactions were performed as described under "Experimental Procedures.

* Activity is expressed as percent of the activity observed with protamine.

* Mitochondrial protamine kinase I and mitochondrial protamine kinase II refer to the forms of this enzyme which elute at 0.6 and 0.8 M NaCl from protamine-agarose, respectively (4).

The relative activity of the cytosolic protamine kinase with the proteins shown was not altered when the activity of this enzyme was determined at 1.5 mM Mg²⁺ (not shown).
presence of highly purified preparations of the protamine kinase (Fig. 3). A marked increase in the phosphorylation of numerous polypeptides was observed in the incubations which contained the purified protamine kinase (Fig. 3B).

DISCUSSION

The protamine kinase described in this paper was detected in studies designed to determine the subcellular distribution of two forms of a protamine kinase that were identified and purified to apparent homogeneity from extracts of bovine kidney mitochondria (4). We estimate that extracts of bovine kidney cytosol contained between 40- and 100-fold higher protamine kinase activity than was present in mitochondrial extracts. Based on the total recoveries following fractionation of extracts on DEAE-cellulose, we estimate that about 30% of the total cytosolic protamine kinase activity was due to cyclic AMP-dependent protein kinase, protein kinase C, and an unidentified protamine kinase which did not bind to DEAE-cellulose (not shown). The remaining protamine kinase activity (~70%) was due to the protamine kinase described in this paper.

In order to provide a firm molecular framework to examine the properties of cytosolic protamine kinase, a procedure was developed to purify this enzyme about 30,000-fold to apparent homogeneity from extracts of bovine kidney cytosol (Table I). The purified cytosolic protamine kinase exhibited an apparent $M_r$ of 43,000 as determined by gel permeation chromatography on Sephacryl S-200 (Fig. 1) and an apparent $M_r$ of 45,000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1, inset). Although the apparent $M_r$ of the cytosolic protamine kinase is similar to the apparent $M_r$ of the mitochondrial protamine kinases (4), in contrast to these enzymes (4), the cytosolic enzyme did not undergo autophosphorylation. In addition, although the steps employed to purify the major protamine kinase of kidney cytosol (Table I) were similar to the steps used in the purification of the mitochondrial protamine kinases (4), the cytosolic enzyme exhibited several distinct chromatographic properties. Thus, by contrast to the two forms of the mitochondrial protamine kinase which coeluted from Q-Sepharose at about 0.2 M NaCl (4), the cytosolic protamine kinase was eluted from this column at about 0.35 M NaCl. In addition, by contrast to the two forms of the mitochondrial protamine kinase recovered at about 0.6 and 0.8 M NaCl from protamine-agarose (4), only one form of the cytosolic kinase was recovered from protamine-agarose at about 0.7 M NaCl.

The substrate specificity of the highly purified preparations of the cytosolic protamine kinase indicates that this enzyme is distinct from the two forms of the mitochondrial protamine kinase (Table II). Thus, by contrast to the two forms of the mitochondrial protamine kinase, the cytosolic enzyme was inactive with histone H1, bovine serum albumin, and glycogen synthase a from rabbit skeletal muscle. In addition, by contrast to the two forms of the mitochondrial protamine kinase, the cytosolic enzyme exhibited some activity with histone H2B (~8%) and casein (~5%). Moreover, the optimal concentration of Mg$^{2+}$ for the cytosolic protamine kinase was 10 mM as compared to 1.5 mM for the mitochondrial protamine kinases (Fig. 2). At 10 mM Mg$^{2+}$, the activity of the mitochondrial protamine kinase was inhibited by 40% (Fig. 2). Furthermore, the activities of the two forms of the mitochondrial protamine kinase were inhibited 80% by 1 mM Ca$^{2+}$. In contrast, the activity of the cytosolic protamine kinase was unaffected by this divalent cation.

The catalytic properties of the highly purified preparations of the cytosolic protamine kinase also differentiate this enzyme from previously described cytosolic protein kinases. Thus, by contrast to cyclic AMP-dependent protein kinase, phosphorylase kinase, Ca$^{2+}$/calmodulin-dependent multiprotein kinase, glycogen synthase kinase ~3 and ~4, type I and type II casein kinases, protein kinase C, and a new glycogen synthase kinase (Refs. 2, 4, 8-11, and references therein), the cytosolic protamine kinase was essentially inactive with glycogen synthase a from rabbit skeletal muscle. In addition, the purified protamine kinase was unaffected by 0.1 mM cyclic AMP, 0.1 mM cyclic GMP, or by the heat-stable protein inhibitor of the cyclic AMP-dependent protein kinase (up to 100 μg/ml), and the enzyme exhibited only about 8% activity with histone H2B, a good substrate for cyclic AMP-dependent protein kinase (this study and Ref. 12). These properties also indicate that this protamine kinase is distinct from cyclic AMP-dependent protein kinase (13) and cyclic GMP-dependent protein kinase (14). Similarly, the activity of the protamine kinase was unaffected by EGTA (up to 1 mM), Ca$^{2+}$ (up to 1 mM), or calmodulin (up to 0.5 μM) with or without Ca$^{2+}$ (0.05 mM), and only 0.15 mol of phosphoryl groups was incorporated per mol of phosphorylase b monomer following 1 h of incubation with the kinase. These properties differentiate the protamine kinase from phosphorylase kinase (15) and other Ca$^{2+}$/calmodulin-dependent protein kinases (e.g. Ref. 16). The cytosolic protamine kinase also exhibited <0.1% activity with histone H1 in the absence or presence of phosphatidylserine (up to 40 μg/ml) and/or diolein (up to 1 μg/ml), with or without Ca$^{2+}$ (up to 0.5 mM). These properties distinguish the protamine kinase from the catalytic domain and native forms of protein kinase C (17), as well as other histone H1 kinases (18, 19). The purified cytosolic protamine kinase was also inactive with histone H4, a substrate of S6 kinase (20), and protease-activated kinase I (21) and II (22, 23). Similarly, by contrast to the type I and type II casein kinases (8), the cytosolic protamine kinase exhibited only about 8% activity with casein and was essentially inactive with phosvitin. Heparin (up to 100 μg/ml), a potent inhibitor of type II casein kinases (8), also had no effect on protamine kinase 

![Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns (A1, B1, C1) and autoradiograms (A2, B2, C2) of 50 μg of extract (A1, A2), 50 μg of extract in the presence of 0.01 unit of highly purified protamine kinase (B1, B2), and 0.01 unit of protamine kinase (C1, C2). Each sample (final volume = 0.05 ml) was incubated in the presence of 0.2 mM [γ-32P]ATP (180 cpm/pmol) and 10 mM MgCl2. After 5 min of incubation at 30 °C, reactions were terminated by the addition of an equal volume of denaturing sample buffer, heated for 5 min at 100 °C, and then subjected to polyacrylamide gel electrophoresis (7). The gel was stained with Coomassie Blue, washed extensively, dried, and exposed to x-ray film. The position of marker proteins, $M_r$ values from top to bottom are phosphorylase, bovine serum albumin, ovomuculin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin. The protamine kinase used in these experiments was from step 7, Table I.]

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kinase activity with either protamine or casein as substrates. The physiological role and regulation of the protamine kinase of kidney cytosol remain to be elucidated. The experiments presented in Fig. 3, however, indicate that this protamine kinase exhibits activity toward a large number of physiological proteins. These results raise the possibility that this protamine kinase may occupy a central position in the coordinated regulation of a number of metabolic processes and that by analogy to other protein kinases (1–3), this protamine kinase may be a key target for regulation by hormones and other extracellular stimuli.

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