Purification and Properties of a Heat-stable Protein Inhibitor of Phosphoprotein Phosphatase from Rabbit Liver*

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A heat-stable protein inhibitor of phosphoprotein phosphatase has been purified to homogeneity from rabbit liver extract by heating to 95°C followed by ion exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-200. The purified inhibitor showed a single band when examined by gel electrophoresis. $a_{280}$ and Stokes radius values were 1.45 and 25.5, respectively. Using these two values, the molecular weight and frictional ratio was calculated to be 15,500 and 3.40, respectively. The molecular weight determined by sodium dodecyl sulfate-gel electrophoresis was found to be 14,200.

The inhibition of phosphoprotein phosphatase was linear up to 40% inhibition with respect to inhibitor concentration. The percentage of inhibition was constant with time of incubation for at least 30 min. The optimum pH for the inhibition was between 6.8 and 7.6. A kinetic analysis of the effect of the inhibitor on the dephosphorylation of $[^{32}P]$phosphorylase $a$ by rabbit liver phosphoprotein phosphatase indicated a noncompetitive inhibition with respect to phosphorylase $a$. Purified liver inhibitor inhibited the phosphoprotein phosphatase activity in all rat tissues examined. Utilizing purified rabbit liver phosphoprotein phosphatase, the presence of inhibitor activity was also demonstrated in all rat tissues tested.

In recent years, it has been suggested that a heat-stable protein inhibitor of phosphoprotein phosphatase plays an important role in the regulation of this enzyme (1-5). The presence of this inhibitor was first observed by Brandt et al. (1, 2) during the purification of phosphorylase phosphatase from rabbit liver. In addition to liver, the presence of protein inhibitor was also demonstrated by these authors in skeletal muscle and heart. Huang and Glinsmann (3, 4, 6) reported the presence of two heat-stable inhibitors of phosphorylase phosphatase in skeletal muscle. The inhibitor $1$ was only inhibitory when it was phosphorylated by cyclic AMP-dependent protein kinase, whereas the inhibitory activity of inhibitor $2$ was not dependent on such phosphorylation. Recently, Cohen et al. (5) reported a partial purification of a heat-stable inhibitor presumably like inhibitor $2$ from rabbit skeletal muscle. The inhibitor was most effective against a low molecular weight phosphatase III and was at least 200 times less effective against higher molecular weight phosphatase I and II (5, 7).

We also observed the presence of some interfering proteins in crude extracts during the purification of phosphoprotein phosphatase from rabbit liver (6). The present communication reports the purification to homogeneity of a heat-stable protein inhibitor of phosphoprotein phosphatase from this tissue. The study has been extended to examine some of the properties of this inhibitor as well as its presence in a number of rat tissues.

EXPERIMENTAL PROCEDURES

Materials
Crystalline rabbit skeletal muscle phosphorylase $b$ was prepared as described by Fischer and Krebs (9). Rabbit skeletal muscle phosphorylase kinase was prepared according to Hayakawa et al. (10) and was a kind gift from Dr. J. H. Wang and Mr. T. Singh, Department of Biochemistry, Faculty of Medicine. $[^{32}P]ATP$ was obtained from ICN, and phosphorylase kinase as described by Krebs et al. (11). The preparation of rabbit liver phosphoprotein phosphatase has been described previously (8). Frozen rabbit livers (type II or IC) were obtained from Pel-Freez Biologicals, Inc. $[^{32}P]ATP$ was obtained from ICN, and PCS (phase-combining system) solvent for liquid scintillation was a product of Amersham/Searle. Wool fast blue dye was a kind gift from Dr. B. L. Lambert (Naval Dental Research Institute, Great Lakes, III.) to our colleague Dr. C. Dawes. All other products were obtained from commercial sources.

Methods
Assay of Phosphoprotein Phosphatase Inhibitor—Inhibitor activity was determined by its ability to inhibit the dephosphorylation of $[^{32}P]phosphorylase a$ catalyzed by rabbit liver phosphoprotein phosphatase. The reaction mixture contained 50 mM Tris (pH 7.5) buffer, 0.5 mM dithiothreitol, 0.4 mM of bovine serum albumin, 1 mM caffeine, 0.5 mg/ml of $[^{32}P]phosphorylase a$, phosphoprotein phosphatase, and inhibitor preparation in a total volume of 50 ml. The

1 For terminology of these phosphatases, see Ref. 7.
2 During the purification of inhibitor, partially purified phosphoprotein phosphatase after the DEAE-Sephadex step (see Ref. 8) was used in the assays. However, with purified inhibitor, all studies were done using homogeneous phosphoprotein phosphatase II from rabbit liver.
amount of phosphatase used per assay was sufficient to give an activity of 1 pmol of $[^{32}P]$P released from $[^{32}P]$P phosphatase $\alpha$ per min without inhibitor. The incubation was done at 30° for 30 min and the released $[^{32}P]$P was separated and counted as described previously (8). One unit of inhibitor activity was defined as that amount which would inhibit 20% dephosphorylation of $[^{32}P]$P phosphatase $\alpha$ under the defined conditions.

**Determination of Molecular Weight** — Molecular weight of protein was determined by the method of Siegel and Monty (12) from $s_{20,w}$ and Stokes radius. The $s_{20,w}$ value was determined by sucrose density gradient according to the method of Martin and Ames (13) using a IEC SB-283 rotor run at 39,000 rpm for 45 h at 4°. Approximately 100 μg of purified inhibitor together with marker proteins, muscle phosphorylase $b$ (0.9 mg), bovine serum albumin (1.0 mg), peroxidase (0.125 mg), and myoglobin (0.25 mg) were applied to each gradient in a total volume of 200 μl. Phosphorylase $b$ and bovine serum albumin were identified by optical density of 280 nm. Peroxidase activity was measured by the guaiacol method as described by George (14). Myoglobin was identified by optical density of 412 nm. Stokes radius was determined by gel filtration using a column of Sephadex G-100 (2.5 × 87.5 cm). One milligram of purified inhibitor together with 5 mg each of marker proteins, bovine serum albumin, ovalbumin, and myoglobin was applied to the column in a total volume of 1.0 ml. Marker proteins were identified by optical density of 280 nm for bovine serum albumin and ovalbumin and of 412 nm for myoglobin.

Molecular weight of the protein inhibitor was also determined by the method of Weber and Osborn (15) employing 10% sodium dodecyl sulfate-polyacrylamide gels with phosphorylase $b$ ($M_w = 92,500$), bovine serum albumin ($M_w = 68,000$), ovalbumin ($M_w = 43,000$), chymotrypsinogen ($M_w = 27,000$), and myoglobin ($M_w = 17,200$) as the marker proteins. After the electrophoresis, gels were stained with 0.25% Coomassie brilliant blue for 4 h and were then destained.

**Analytical Methods** — Disc gel electrophoresis was carried out by the method of Davis (16). Acrylamide gels (7.5%) were used and stained, just after the electrophoresis, with 0.5% wool fast blue stain, and the standard.

**Preparation of Heat-stable Protein Inhibitor**

**Preparation of Crude Liver Extract** — Frozen rabbit livers were homogenized in an osterizer blender at high speed for 1 min in 4 volumes of 20 mM Tris (pH 7.5) containing 0.25 M sucrose and 0.5 mM dithiothreitol (Buffer A). The homogenate was centrifuged at 10,000 × g for 30 min and the supernatant solution was collected after filtering through glass wool and four layers of cheesecloth. This step and all subsequent steps for the purification were carried out at 4°.

**Preparation of Boiled Extract** — Crude extracts as described above were heated at 95° in a stainless steel beaker with stirring. The resultant suspension was cooled in an ice bath and then filtered through glass wool and four layers of cheesecloth.

**First Ion-Exchange Chromatography on DEAE-Cellulose (DE52)** — The boiled extract was adjusted to 15% (w/v) with respect to the acid, by the addition of a cold solution of 100% (w/v) trichloroacetic acid. After 30 min, the precipitate, which contained the inhibitor protein, was collected by centrifugation at 20,000 × g for 30 min. The precipitate was dissolved in minimal volume of 20 mM Tris (pH 7.5) containing 2 mM EDTA (Buffer B) by keeping a constant pH 7.5 with the addition of 1 N NaOH. This step was primarily used for concentrating the large volume of boiled extract and resulted in neither any decrease in total protein nor in the inhibitor activity. The suspension was applied to a column of DEAE-cellulose equilibrated with Buffer B. The inhibitor protein was eluted from the column using a linear gradient from 0 to 0.5 M NaCl in Buffer B (Fig. 1A). The fractions containing

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![Fig. 1. Purification of heat-stable protein inhibitor of phosphoprotein phosphatase from rabbit liver. A, 1st ion-exchange chromatography on DEAE-cellulose (DE52) at pH 7.5. The trichloroacetic acid precipitate fraction was chromatographed as described in the text. Fractions of 80 drops were collected. $A_{190}$ (O-O) and phosphatase activity in the presence of different fractions (10 μl) (○-○). B, gel filtration on Sephadex G-200. Pooled fractions from DE52 column were precipitated with 40 g of solid (NH₄)₂SO₄/100 ml of solution. The pellet obtained after centrifugation, dissolved in 5 ml of Buffer C, and incubated at 30° for 30 min was applied to a column (2.5 × 65 cm) of Sephadex G-200 and eluted with Buffer C. The flow rate was 15 ml/h. Fractions of 80 drops were collected. Optical density (O-O) and phosphatase activity in the presence of different fractions (10 μl) (○-○). C, 2nd ion-exchange chromatography on DEAE-cellulose (DE52) at pH 7.5. The inhibitor of phosphatase activity after Fraction 55 was due to the high concentration of NaCl present in the fractions. For further purification, Fractions 22 to 42, as shown by solid bar, were pooled. B, gel filtration on Sephadex G-200. Pooled fractions from DE52 column were precipitated with 40 g of solid (NH₄)₂SO₄/100 ml of solution. The pellet obtained after centrifugation, dissolved in 5 ml of Buffer C, and incubated at 30° for 30 min was applied to a column (2.5 × 65 cm) of Sephadex G-200 and eluted with Buffer C. The flow rate was 15 ml/h. Fractions of 80 drops were collected. Optical density (O-O) and phosphatase activity in the presence of different fractions (10 μl) (○-○). The inhibition of phosphatase activity after Fraction 38 was due to the high concentration of NaCl present in the fractions. Fractions 24 to 33, as shown by solid bar, were pooled and concentrated by ultrafiltration using Amicon UM-2 membrane.](http://www.jbc.org/content/6/18/561.full)
The amount of liver used was 130 g.

was not incubated at 30°, most of the inhibitor protein was

Boiled superna-

191

1st DE52

2nd DE52

1C). The fractions containing inhibitor activity were pooled

inhibitor protein, eluted as a broad peak, were pooled. The
pooled fraction was then mixed with solid ammonium sulfate
(40 g/100 ml) with continuous stirring. After 30 min, the
mixture was centrifuged at 40,000 × g for 30 min and the
pellet was dissolved in 20 mM Tris/glycine (pH 9.0) buffer
containing 2 mM EDTA (Buffer C). The incubation mixture
with trypsin was terminated with the addition of 300-fold excess of soy bean
trypsin inhibitor. Inhibitor activity was determined as described
under "Methods."

Purified inhibitor (3 µg) was incubated at 30° for 30 min. The incubated
sample was then applied to a Sephadex G-200 column (2.5 × 65 cm) previously equilibrated with Buffer C. Inhibitor protein
was eluted as a single peak in the retarded fractions of this
column (Fig. 1B). It should be noted, however, if the sus-
pended ammonium sulfate pellet was not raised to pH 9.0 and
was not incubated at 30°, most of the inhibitor protein was
eluted with the major protein peak. The fractions containing
inhibitor protein were pooled.

Sephadex G-200 Gel Filtration—The suspended pellet as
described above was incubated at 30° for 30 min. The incubated
sample was then applied to a Sephadex G-200 column (2.5 × 65 cm) previously equilibrated with Buffer C. Inhibitor protein
was eluted as a single peak in the retarded fractions of this
column (Fig. 1B). It should be noted, however, if the sus-
pended ammonium sulfate pellet was not raised to pH 9.0 and
was not incubated at 30°, most of the inhibitor protein was
eluted with the major protein peak. The fractions containing
inhibitor protein were pooled.

Second Ion-Exchange Chromatography on DEAE-Cellu-
lose—The pooled Sephadex G-200 fraction was applied to a
column of DEAE-cellulose that had been equilibrated with
Buffer C. The inhibitor protein from this column was eluted
with a linear gradient from 0 to 0.5 M NaCl in Buffer C (Fig.
1C). The fractions containing inhibitor activity were pooled

TABLE I

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<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
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<td>10.2</td>
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<td>Boiled supernanta</td>
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<td>80</td>
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<td>43</td>
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<td>8,600</td>
<td>845</td>
<td>27</td>
</tr>
</tbody>
</table>

*Activity expressed as a percentage of that at zero time incubation.

Characterization of Inhibitor

The activity of inhibitor was completely destroyed by incu-
bation with different concentrations of trypsin but was not
affected by DNase or RNase (Table II). Following disc gel
electrophoresis, the inhibitor stained with the protein dyes,
wool fast blue, and Coomassie brilliant blue. These data,
along with the precipitation of the inhibitor with trichloro-
acetic acid, would suggest that inhibitor is a protein.

The inhibitor was essentially homogeneous as it migrated
as a single band on a regular disc gel electrophoresis (Fig. 2).
Inhibitor of Phosphoprotein Phosphatase 563

FIG. 3. A, inhibition of phosphoprotein phosphatase activity by varying amounts of the inhibitor using [32P]phosphorylase a as the substrate. The assay conditions were as described under "Methods" except that varying amounts of inhibitor were used. Percentage of inhibition was calculated from the phosphatase activity determined in the absence of the inhibitor. B, effect of inhibitor on the rate of 32P released from [32P]phosphorylase a by phosphoprotein phosphatase. The rate of dephosphorylation of phosphorylase a was examined in the absence (○) or in the presence of 0.32 μg (Δ) and 0.64 μg (□) of inhibitor. Other assay conditions were as described under "Methods."

6.0 6.8 7.6 8.4 9.2

FIG. 4. The effect of the inhibitor on the pH optima for phosphoprotein phosphatase. A, phosphatase activity in the absence (○) or in the presence (●) of 1.62 μg of inhibitor was carried out as described under "Methods" except that a 50 mM Tris-2-(N-morpholino)ethanesulfonic acid buffer at the different pH was used. B, percentage of inhibition of phosphatase activity by the inhibitor as calculated from A.

Employing different concentrations of gel (6, 7.5, and 10%), the inhibitor always moved as a single band. In all conditions, the inhibitor activity migrated with the protein band. The sedimentation constant \( s_{20,w} \) as determined by sucrose density gradient was 1.45. The Stokes radius as determined by gel filtration on Sephadex G-100 was 25.5. Molecular weight and frictional ratio calculated by using these two values were 15,500 and 3.40, respectively (12). The molecular weight by the sodium dodecyl sulfate-gel electrophoresis method was found to be 14,200.

Optimal Assay Conditions

Inhibitor activity for the inhibition of dephosphorylation of [32P]phosphorylase a by phosphoprotein phosphatase was linear up to approximately 40% inhibition (Fig. 3A). Within this range, the inhibited reaction remained linear for at least a 30-min incubation period (Fig. 3B). The percentage of inhibition calculated from these curves was constant at all times of incubation. The optimum pH for the phosphatase was 7.2 to 8.0 and 8.0 to 8.4 in the absence or in the presence of the inhibitor, respectively (Fig. 4A). The maximum percentage of inhibition by the inhibitor for the dephosphorylation of [32P]phosphorylase a by phosphoprotein phosphatase, however, was between pH 6.8 and 7.6 (Fig. 4B). In all assays, therefore, reactions were carried out at pH 7.4 with less than 40% inhibition for an incubation period of 30 min.

Kinetics of Inhibition

Employing different concentrations of [32P]phosphorylase a with a fixed amount of phosphoprotein phosphatase, the inhibition by the inhibitor was shown to be noncompetitive (Fig. 5). As can be seen, the inhibitor increased the \( K_m \) for phosphorylase a and decreased the \( V_{max} \) of the reaction. When varying amounts of phosphatase were used in the reaction, similar kinetic data were observed (not shown).

Effect of Liver Inhibitor on Phosphoprotein Phosphatase from Different Tissues

The effect of purified liver inhibitor on the dephosphorylation of [32P]phosphorylase a by phosphoprotein phosphatase from a number of different tissues was examined. Tissues from the rat were cut into small pieces and homogenized in 4 volumes of 20 mM Tris (pH 7.4) containing 0.25 M sucrose and 1 mM dithiothreitol. The homogenate was centrifuged at 10,000 × g for 30 min and the supernatant, after filtering through glass wool and four layers of cheesecloth, was used as the source of phosphoprotein phosphatase. The effect of varying amounts of purified liver protein inhibitor using these phosphatase preparations for the dephosphorylation of phosphorylase a is shown in Fig. 6. The purified inhibitor was essentially equally effective for the inhibition of phosphatase from all of the tissues tested. A comparison of Fig. 3 and Fig. 6 would indicate that the inhibition of crude rat liver phosphatase was greater than of the purified rabbit liver phosphatase by the same amount of the inhibitor. The reason

FIG. 6. A, inhibition of phosphoprotein phosphatase activity by varying amounts of inhibitor using [32P]phosphorylase a as the substrate. The assay conditions were as described under "Methods" except that varying amounts of inhibitor were used. Percentage of inhibition was calculated from the phosphatase activity determined in the absence of the inhibitor. B, effect of inhibitor on the rate of 32P released from [32P]phosphorylase a by phosphoprotein phosphatase. The rate of dephosphorylation of phosphorylase a was examined in the absence (○) or in the presence of 0.32 μg (Δ) and 0.64 μg (□) of inhibitor. Other assay conditions were as described under "Methods."

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Inhibitor of Phosphoprotein Phosphatase

Double reciprocal plots of $\frac{1}{[\text{32P}]\text{Phosphorylase a}}$ versus initial velocity of the dephosphorylation of $[\text{32P}]\text{Phosphorylase a}$ by phosphoprotein phosphatase in the absence (O-O) or in the presence of 0.6 $\mu$g (△-△) and 1.60 $\mu$g (□-□) of purified inhibitor. Other assay conditions were as described under "Methods."

Liver Inhibitor $[\mu$g$]$  

Presence of Inhibitor Activity in Different Tissues

By employing the purified liver phosphoprotein phosphatase, the presence of inhibitor activity was also tested in a number of rat tissues. The preparation of inhibitor from these tissues was carried out as described for the purification of inhibitor in the text. The assay conditions were as described under "Methods" except that the varying amounts of inhibitor prepared from different tissues were used. Source of the inhibitor was brain (△-△), kidney (△-△), liver (O-O), heart (O-O), skeletal muscle (O-O), and adipose tissue (W-B).

As can be seen in Fig. 7, the inhibitor was present in all of the tissues examined. The inhibitor activities (units per g of tissue) were also calculated in different rat tissues. The values were as follows: brain, 7260; kidney, 6740; heart, 3760; liver, 2740; adipose tissue, 2700; and skeletal muscle, 2240. As can be seen, the activity was higher in brain and kidney followed by heart, liver, adipose tissue, and skeletal muscle.

DISCUSSION

Brandt et al. (9), while studying the properties of a crude inhibitor preparation from rabbit liver, observed that gel filtration of inhibitor on a column of Bio-Gel A-0.5m resulted in elution of the activity with seemingly multiple molecular forms. We also encountered similar problems during our initial attempts to purify the inhibitor from this tissue. In the course of these attempts, it was discovered that the incubation of partially purified inhibitor preparation at alkaline pH (>8.0) resulted in the conversion of these multiple molecular forms into a single lower molecular form of the inhibitor. This property of the inhibitor was successfully utilized in the purification procedure outlined in this paper (Fig. 1B).

The anomaly between the sedimentation value ($s_{20,w} = 1.45$) and Stokes radius ($A = 25.5$) determined for this protein might be explained if the protein is very asymmetrical. This assumption is supported by a high frictional ratio ($f/f_0 = 3.40$) calculated for this protein. The possibility that this divergence might also be due to a bound carbohydrate or lipid moiety also exists. Similar properties of another heat-stable protein, the inhibitor of cyclic AMP-dependent protein kinase, were also observed by Walsh et al. (18). The molecular weight
determined in the present study for the liver inhibitor was lower than the previously reported values for a similar inhibitor in skeletal muscle (4, 5). Huang and Glinsmann (4) reported a value of 33,000, whereas Cohen et al. (5) observed an apparent molecular weight of 65,000. The reason for this difference is not clear, but it might be due to the difference in the inhibitor isolated from the different tissues, i.e. the skeletal muscle and the liver.

The biochemical mechanisms for the action of phosphatase inhibitor for the dephosphorylation of phosphoproteins remain unknown. In 1975, Brandt et al. (2) observed that crude phosphatase inhibitor from rabbit liver inhibited the phosphatase activity with phosphorylase a or glycogen synthase D as substrates. Recently, Cohen et al. (5) partially purified a heat-stable inhibitor from rabbit skeletal muscle and reported that it inhibited the dephosphorylation of phosphorylase a, glycogen synthase D, and β subunit of phosphorylase kinase catalyzed by a lower molecular weight phosphatase. The inhibitor was at least 200 times less effective in inhibiting the activity of higher molecular weight phosphatases. These observations along with the noncompetitive inhibition with respect to phosphorylase a as observed in the present study (Fig. 5) and as reported by Brandt et al. (2) would indicate that the inhibitor probably interacts with the enzyme and not the phosphoprotein substrates. Furthermore, it has been suggested by Brandt et al. (2) that the inhibitor interacts with low molecular weight active phosphoprotein phosphatase and converts it into multiple inactive (or less active) higher molecular forms of the enzyme. If the inhibitor is a regulatory subunit of a holoenzyme, the interaction between regulatory and catalytic subunit has to be studied. In addition to this, the factors which regulate the interaction between these two subunits have to be found. With the availability of homogeneous inhibitor protein as described in this paper and of phosphoprotein phosphatase (8, 19, 20), these studies are now possible.

The physiological significance of phosphatase inhibitor has to be elucidated. There are, however, certain indications that the inhibitor plays a regulatory role in the control of phosphoprotein phosphatase activity. First, recently we have observed that streptozotocin-induced diabetic animals have approximately 30% higher inhibitor levels than control or insulin-treated diabetic animals (21). Secondly, the inhibitor is widely distributed and is present in all of the various tissues examined (Fig. 7). Future work is, however, needed to determine the exact role of this inhibitor in the regulation of phosphoprotein phosphatase activity in vivo.

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