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A potent, heat-stable protein inhibitor of [branched-chain α-keto acid dehydrogenase]-phosphatase from bovine kidney mitochondria

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ABSTRACT **A heat- and acid-stable protein inhibitor of the [branched-chain α-keto acid dehydrogenase]-phosphatase was purified over 100,000-fold from extracts of bovine kidney mitochondria. The nearly homogeneous protein was recovered with a yield of 4–8%. The apparent molecular weight of the inhibitor is about 36,000. This protein is a noncompetitive inhibitor of the phosphatase, and the inhibitor constant (K_I) is about 0.13 nM. The inhibition was reversed 50% by about 1.3 mM Mg^{2+} and about 0.1 mM spermine. This protein inhibitor is different from the cytosolic protein phosphatase inhibitors 1 and 2.**

The mammalian branched-chain α-keto acid dehydrogenase [BCKDH; 3-methyl-2-oxobutanoate:lipoamide oxidoreductase (decarboxylating and acceptor-2-methylpropanoylating), EC 1.2.4.4] complex is located within the mitochondrial inner membrane–matrix compartment and is subject to regulation by a phosphorylation–dephosphorylation cycle. Phosphorylation and concomitant inactivation of the BCKDH component of the multienzyme complex is catalyzed by a kinase that copurifies with the complex (1–4). Dephosphorylation and concomitant reactivation is catalyzed by a specific phosphatase that has been isolated in a highly purified state from bovine kidney mitochondria (5). During development of procedures for isolation of BCKDH phosphatase, a marked increase in its activity was observed upon dilution of gel-filtered mitochondrial extracts. This observation suggested the presence of an inhibitor. Further study revealed that the inhibitor is a heat-stable protein. This paper reports the purification to near homogeneity and some properties of this inhibitor.

MATERIALS AND METHODS

Polyethylene glycol 8000 was obtained from Baker; [γ-^{32}P]ATP was from New England Nuclear; hydroxylapatite (high-resolution grade) was from Calbiochem; Sephacryl S-200, pepsin, and imidazole (grade III) were from Sigma; *Staphylococcus aureus* V8 protease was from Miles; and cytosolic protein phosphatase inhibitors 1 and 2 were gifts from Philip Cohen. All other reagents and materials were also of the purest grades available commercially.

Purification, Labeling, and Assay of Enzymes. Highly purified pyruvate dehydrogenase complex, [pyruvate dehydrogenase (lipoamide)]-phosphatase (EC 3.1.3.43), and BCKDH phosphatase were prepared from bovine kidney and heart mitochondria essentially as described (5–7). Purified BCKDH complex containing active BCKDH kinase was prepared from polyethylene glycol precipitate A (see below). This precipitate was suspended, by means of a glass/Teflon homogenizer, in 250 ml of buffer A′ [0.1 M potassium phosphate buffer, pH 7.3/0.5 mM phenylmethanesulfonyl fluoride ($PhMeSO_2F$)/1 mM benzamidine/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol] containing 0.01 mM benzyloxycarbonylphenylalanylalanine diazomethyl ketone (Z-Phe-AlaCHN_2), a thiol-protease inhibitor (8). The suspension was clarified by centrifugation at 18,000 rpm for 20 min in a Beckman J-20 rotor.

The supernatant fluid was diluted with buffer A′ to 4 mg of protein per ml, and 0.09 vol of a 25% (wt/vol) aqueous solution of polyethylene glycol was added, with stirring. After 20 min, this mixture was centrifuged at 14,000 rpm for 15 min, and the precipitate was discarded. To the supernatant fluid was added 0.5 mM $PhMeSO_2F$ and 0.01 mM Z-Phe-AlaCHN_2 (final concentrations). The solution was warmed to 20°C, and 0.06 vol of 25% polyethylene glycol was added, with stirring. After 20 min, the mixture was centrifuged at 14,000 rpm and 20°C for 15 min. The precipitate was discarded. To the supernatant fluid was added again 0.5 mM $PhMeSO_2F$ and 0.01 mM Z-Phe-AlaCHN_2 (final concentrations), and the solution was cooled to 5°C and stirred for 20 min. The mixture was centrifuged at 14,000 rpm for 20 min. The precipitate (B) contained 80–90% of the BCKDH complex activity, and the supernatant fluid contained 80–90% of the pyruvate dehydrogenase complex activity.

The pyruvate dehydrogenase complex was precipitated with 0.09 vol of 25% polyethylene glycol, and the precipitate was extracted with buffer A′ containing 0.01 mM Z-Phe-AlaCHN_2. After clarification, the solution was centrifuged at 40,000 rpm for 4 hr in a Beckman type 40 rotor to sediment the pyruvate dehydrogenase complex. The pellets were dissolved in buffer A′, and the solution was subjected to acid fractionation (9). The pyruvate dehydrogenase complex precipitated at pH 5.4. These preparations were nearly homogeneous, as shown by $NaDodSO_4$/polyacrylamide gel electrophoresis and were essentially free of [pyruvate dehydrogenase]-phosphatase activity. Precipitate B was extracted with buffer A′ containing 0.01 mM Z-Phe-AlaCHN_2; after clarification, the solution was chromatographed on a column (2.5 × 5 cm) of hydroxylapatite essentially as described (5). After an extensive washing of the column with buffer A′ containing 0.03 M and then 0.15 M potassium phosphate buffer (pH 7.3), BCKDH complex was eluted with buffer A′ containing 0.35 M potassium phosphate.

The active fractions were combined and centrifuged at 40,000 rpm for 4 hr to sediment the BCKDH complex. The pellets were dissolved in buffer C (50 mM imidazole chloride, pH 7.3/10% glycerol/0.1 mM EDTA/0.1 mM EGTA/0.1 mM $PhMeSO_2F$/1 mM benzamidine/1 mM dithiothreitol). The purified BCKDH complex had a specific activity of 8–13 units/mg of protein, and the overall recovery was 20–50%. These preparations were apparently homogeneous, as shown by $NaDodSO_4$/polyacrylamide gel electrophoresis. ^{32}P-labeled, inactive BCKDH complex and ^{32}P-labeled, inactive pyruvate dehydrogenase complex were prepared as de-

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Abbreviations: BCKDH, branched-chain α-keto acid dehydrogenase; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; Z-Phe-AlaCHN_2, benzyloxycarbonylphenylalanylalanine diazomethyl ketone.

scribed (5), except that the [γ-^{32}P]ATP had a specific radioactivity of 2–4 × 10^6 instead of 2 × 10^5 cpm/nmol.

BCKDH phosphatase activity was assayed essentially as described (5), except that the specific radioactivity of the ^{32}P-labeled BCKDH complex was increased 10- to 20-fold to increase the sensitivity of the assay and to permit diluting out any inhibitor protein that might be present. The standard assay for inhibitor protein contained 0.001–0.003 unit of BCKDH phosphatase and additions as noted in the text in 40 μl of buffer C containing bovine serum albumin (1 mg/ml). The solution was incubated for 5 min at 30°C in a plastic microcentrifuge tube. The reaction was initiated with 10 μl of ^{32}P-labeled BCKDH complex (2.5 mg/ml; 1.2–2.4 × 10^7 cpm/mg). After 5 min at 30°C, 0.1 ml of 10% trichloroacetic acid was added. The mixture was centrifuged at 10,000 rpm for 2 min in an Eppendorf microcentrifuge. A 0.12-ml aliquot of the supernatant fluid was transferred to a plastic microcentrifuge tube containing 1 ml of scintillant (DuPont Formula 963). The tube was placed in a plastic vial and radioactivity was assayed. One unit of BCKDH phosphatase activity is the amount of enzyme that releases 1 nmol of [^{32}P]P_i per min at 30°C. One unit of protein inhibitor is defined as the amount of protein that inhibits 1 unit of BCKDH phosphatase by 50% in the standard assay. Protein concentrations were determined by using the Coomassie blue G-250 dye-binding procedure (10) and by the fluorescamine technique (11). Desalting of protein solutions was accomplished by centrifugation of the sample through a minicolumn of Bio-Gel P-4 (12). Polyacrylamide gel electrophoresis was performed in slab gels (12.5% acrylamide) with 0.1% $NaDodSO_4$ and Tris glycine buffer (pH 8.3) (13). Protein bands were detected by the silver-staining technique (14) except that the potassium dichromate/nitric acid wash was omitted.

RESULTS

Purification of Inhibitor Protein. The BCKDH phosphatase activity of bovine kidney mitochondrial extracts (5) was increased up to 4-fold by dilution (Fig. 1), suggesting the presence of an inhibitor of the phosphatase. The inhibitor was nondialyzable, and its activity was not affected by heating the extract to 90°C for 5 min or by keeping the heated extract at pH 2.0 for 1 hr at 30°C. A sample of the heated extract was passed through a column of Sephadex G-50 (fine). Aliquots of the fraction containing inhibitor activity were incubated for 1 hr at 30°C with pepsin at pH 2.0 and with *S. aureus* V8 protease at pH 7.3. The protein-to-protease ratio was 20:1 (wt/wt). The controls lacked protease or contained inactivated pepsin (by adjusting the pH to 7.3) or heat-inactivated *S. aureus* protease. In the presence of either pepsin or *S. aureus* protease, 50–80% of the inhibitor activity was destroyed. These observations indicated that the inhibitor is a heat- and acid-stable protein.

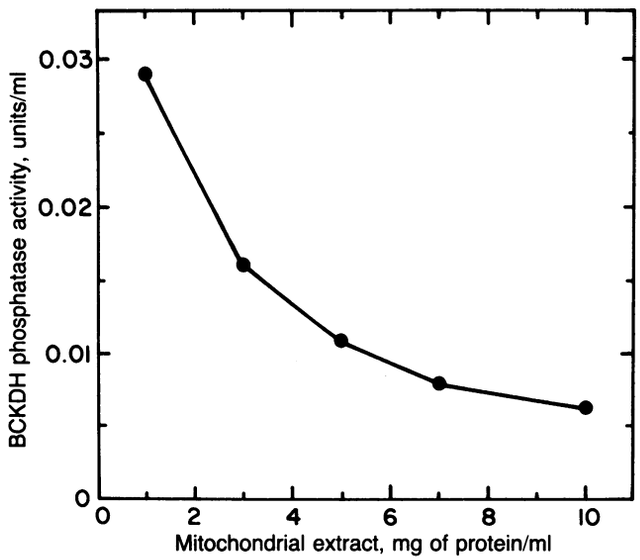

FIG. 1. Stimulation of BCKDH phosphatase activity in mitochondrial extract by dilution. The extract was gel-filtered on Sephadex G-50 to remove inorganic orthophosphate and other low molecular weight inhibitors of BCKDH phosphatase. Serial dilutions of the gel-filtered extract were made with assay buffer, and 40-μl aliquots were assayed for BCKDH phosphatase activity as described (5).

An assay for the inhibitor protein was developed, as described under *Materials and Methods*. To increase the yield of inhibitor protein extracted from bovine kidney mitochondria and to facilitate separation of inhibitor protein, BCKDH phosphatase, and BCKDH complex at an early step in the purification procedure, some modifications were made in the procedure developed for isolation of BCKDH phosphatase (5). Unless specified otherwise, all operations were performed at 2–5°C. The washed mitochondrial paste (5, 6) was suspended in 50 mM potassium phosphate buffer (pH 7.1) containing 0.1 mM $PhMeSO_2F$, 0.01 mM Z-Phe-AlaCHN_2, and 1 mM benzamidine. The mixture was shell-frozen and thawed once. The thawed suspension was diluted with 0.6 vol of 50 mM phosphate, pH 7.3/0.1 mM $PhMeSO_2F$/0.01 mM Z-Phe-AlaCHN_2/1 mM benzamidine/0.1 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol. NaCl was added to a final concentration of 100 mM, the pH was adjusted to 7.3, and the mixture was stirred for 20 min. The mixture was centrifuged at 14,000 rpm for 30 min in a Beckman JA-14 rotor, and the pellets were discarded.

To the mitochondrial extract (step 1) was added, with stirring, 0.08 vol of 50% (wt/vol) aqueous polyethylene glycol 8000. After 20 min, the precipitate was collected by centrifugation at 14,000 rpm for 15 min. This precipitate (A) contained essentially all of the BCKDH complex activity and the pyruvate dehydrogenase complex activity. The supernatant fluid was diluted with 1 vol of solution A (0.1 mM EDTA/0.1 mM EGTA/0.1 mM $PhMeSO_2F$/1 mM benzamidine/1 mM dithiothreitol). This solution was passed at a flow rate of 5–10 liters/hr through a column (7 × 14.5 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with buffer C in a Büchner funnel with a fritted disc. The effluent contained essentially all of the BCKDH phosphatase activity, which was not increased by dilution, indicating that the phosphatase and the inhibitor protein were separated by chromatography on DEAE-cellulose. BCKDH phosphatase in the effluent was precipitated by addition of 0.18 vol of polyethylene glycol and purified further by chromatography on ADP-Sepharose as described (5). After separation of BCKDH phosphatase, the DEAE-cellulose column was washed with about 20 liters of buffer C containing 0.1 M NaCl and then with 200 ml of buffer C containing 0.3 M NaCl. Inhibitor protein was eluted with an additional 400–500 ml of buffer C containing 0.3 M NaCl (step 2).

The active fractions were combined and diluted with 2 vol of solution B (1 mM EDTA/1 mM EGTA/0.1 mM $PhMeSO_2F$/1 mM benzamidine/0.01 mM Z-Phe-AlaCHN_2/1 mM dithiothreitol). The solution was applied to a column (2.5 × 12 cm) of DEAE-cellulose equilibrated with buffer C. The column was washed with 6 liters of buffer D (50 mM imidazole chloride, pH 5.0/solution B/30% ethylene glycol/0.03% Brij 35) containing 0.05 M NaCl and was developed with a 500-ml salt gradient from 0.05–0.5 M NaCl. The active fractions emerging from the column at ≈0.3 M NaCl were pooled (step 3) and diluted with 3 vol of buffer E (50 mM imidazole chloride, pH 7.3/solution A/30% ethylene

glycol/0.03% Brij 35). The solution was applied to a column (2.5 × 6 cm) of DEAE-cellulose equilibrated with buffer E. The column was washed with about 400 ml of buffer E containing 0.05 M NaCl and developed with a 280-ml salt gradient from 0.05–0.5 M NaCl. The active fractions were pooled (step 4) and diluted with 1 vol of buffer E.

The solution was applied to a column (2.5 × 3 cm) of hydroxylapatite equilibrated with 50 mM potassium phosphate, pH 7.3/solution B/30% ethylene glycol/0.03% Brij 35. The column was washed with the same buffer until the absorbance of the effluent at 280 nm and 260 nm approached zero (about 1 liter). Inhibitor protein was eluted stepwise with 0.15 M potassium phosphate and then 0.3 M potassium phosphate/solution B/30% ethylene glycol/0.03% Brij 35. The activity was divided equally between the 0.15 M and 0.3 M potassium phosphate eluates. The 0.3 M phosphate eluate (step 5) was diluted with 8 vol of buffer E and concentrated by using a small column (1 × 2.5 cm) of DEAE-cellulose equilibrated with buffer E. The column was washed with about 50 ml of buffer E, and inhibitor protein was eluted with buffer E containing 0.4 M NaCl. The concentrated inhibitor protein (1.5 ml) was applied to a column (2 × 90 cm) of Sephacryl S-200 equilibrated and developed with buffer E containing 0.2 M NaCl (Fig. 2) (step 6). After removing aliquots for $NaDodSO_4$/gel electrophoresis, the active fractions were combined and concentrated about 8-fold, prior to protein determination, using a small column of DEAE-cellulose as described above. Protein determinations by the Bradford procedure (10) and by the fluorescamine technique (11) gave similar values. A summary of the purification is presented in Table 1.

Properties of Inhibitor Protein. Samples (80 μl) of fractions across the peak of inhibitor activity from the Sephacryl S-200 column (Fig. 2) were subjected to $NaDodSO_4$/slab gel electrophoresis. The gel was stained for protein by the silver staining technique (Fig. 3). A major band of M_r ≈36,000 was seen, which appeared to be a closely spaced doublet. It is not yet known whether one of these bands might represent a contaminant or whether it might be due to a proteolytic product or an interconvertible form of the inhibitor protein. By gel-permeation chromatography on a calibrated column of Sephadex G-100, the apparent molecular weight of the inhibitor protein was estimated to be ≈33,000 (data not shown). Partially purified preparations of inhibitor protein were subjected to $NaDodSO_4$/slab gel electrophoresis, the gel was sliced at 5-mm intervals, the individual slices were macerated and extracted with buffer E, and the extracts were assayed for inhibitor protein activity. Of the activity applied to the gel, 30–60% was recovered in the position corresponding to a M_r of ≈36,000.

Table 1. Purification of inhibitor protein

Step	Vol, ml	Protein, mg	Specific activity*	Recovery, %
1. Mitochondrial extract	5550	84,095	0.19	100
2. DEAE-cellulose, pH 7.3	865	4,481	4.2	118
3. DEAE-cellulose, pH 5.0	110	59	119	44
4. DEAE-cellulose, pH 7.3	25	20.3	212	27
5. Hydroxylapatite	260	7.2	272	12
6. Sephacryl S-200	8	0.029†	22,860	4

Approximately 22 kg of kidney cortex (≈2.5 kg of mitochondria, wet weight) was used. Mitochondria obtained from ≈11 kg of cortex were processed through steps 1 and 2, and the active fractions from two runs were combined prior to step 3.

*Units per mg of protein. Inhibitor protein activity in mitochondrial extracts was determined after heat treatment (90°C for 5 min) and gel filtration on Sephadex G-50 or Bio-Gel P-4.

†Protein was determined after concentrating the solution about 8-fold as described in the text.

Highly purified inhibitor protein gave 50% inhibition of BCKDH phosphatase in the standard assay at about 6 ng/ml (0.17 nM) (Fig. 4). Because the protein substrate is present in the assay at a concentration of 2–3 μM, these data indicate that the inhibitor protein acts on the BCKDH phosphatase rather than on its protein substrate. In support of this conclusion is the observation that with BCKDH phosphatase acting on an alternate substrate, ^{32}P-labeled pyruvate dehydrogenase complex (5), inhibitor protein gave 50% inhibition of the phosphatase at about 4.5 ng/ml.

Preliminary experiments indicated that inhibition of BCKDH phosphatase by inhibitor protein was noncompetitive with respect to the protein substrate and that the apparent K_m was 0.4–0.8 μM. Because of tight binding of the inhibitor protein to the phosphatase, data were plotted as described by Henderson (15). The results (Fig. 5) show that

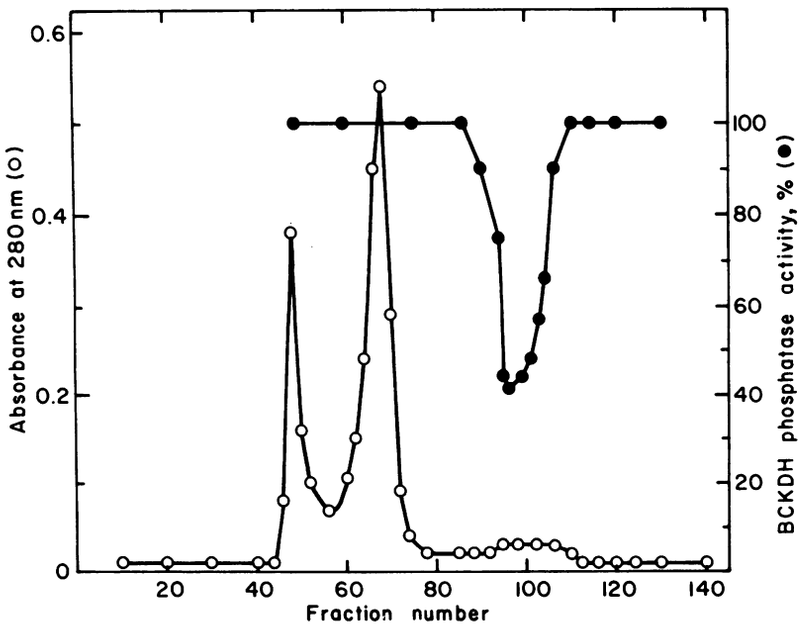

FIG. 2. Gel-permeation chromatography of inhibitor protein on Sephacryl S-200. The active fractions from the hydroxylapatite column (step 5) were concentrated to about 1.5 ml as described, and the concentrate was applied to the column (2 × 90 cm). The flow rate was 11 ml/hr, and fractions of 0.9 ml were collected.

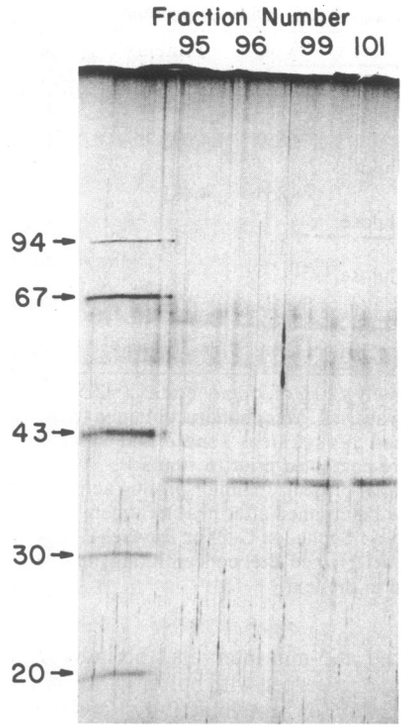

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis patterns of active fractions from Sephacryl S-200 column. Aliquots (80 μl) of fractions 95, 96, 99, and 101 (Fig. 2) were subjected to NaDodSO$_4$/slab gel electrophoresis with 12.5% acrylamide. The gel was silver-stained. The protein standards in $M_r \times 10^{-3}$ are, from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

the apparent K_i (given by the slope) is independent of substrate concentration, indicating that the inhibition of BCKDH phosphatase by inhibitor protein is indeed noncompetitive. The apparent K_i calculated from the slope was 0.13 nM.

BCKDH phosphatase was not inhibited by protein phosphatase inhibitors 1 or 2 at 0.1–1.0 μg/ml. The inhibitor protein had little effect, if any, on [pyruvate dehydrogenase]-phosphatase activity in amounts up to 60 ng/ml in the standard assay, with either ^{32}P-labeled pyruvate dehydrogenase complex or ^{32}P-labeled BCKDH complex as substrate. The inhibitor protein at 60 ng/ml had no effect on the activity of the BCKDH complex or its endogenous kinase (5).

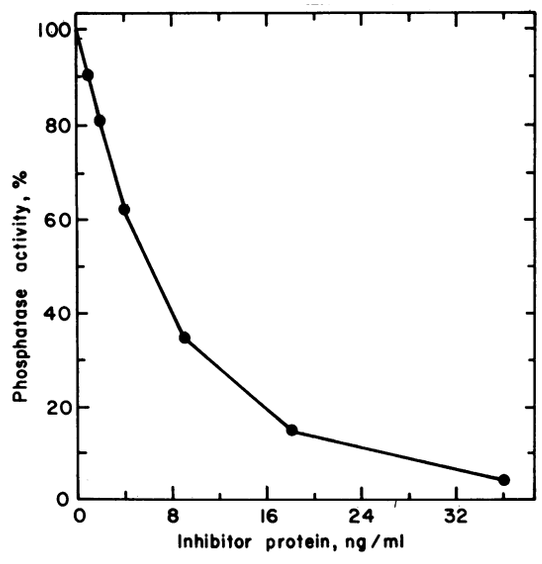

FIG. 4. Inhibition of BCKDH phosphatase by highly purified inhibitor protein.

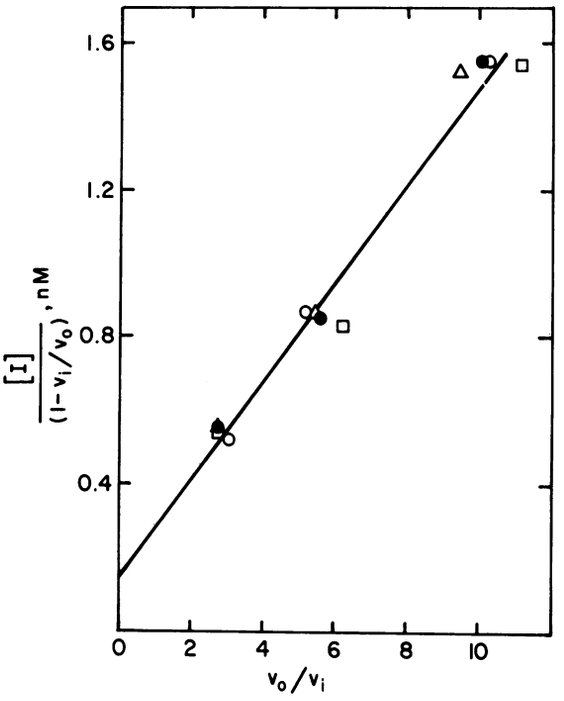

FIG. 5. Henderson plot showing noncompetitive inhibition of BCKDH phosphatase by inhibitor protein. The ^{32}P-labeled BCKDH concentrations were 0.057 (●), 0.17 (△), 0.52 (□), and 1.5 μM (○). [I] is the concentration of inhibitor protein and v_o and v_i are the BCKDH phosphatase activities in the absence and presence of inhibitor protein.

Inhibitor protein activity was reversed by Mg^{2+} (Fig. 6*A*). Half-maximal reversal was obtained at ≈1.3 mM, near physiological concentration. Reversal of inhibitor protein activity was also obtained with the polyamine spermine (Fig. 6*B*), with 50% reversal at ≈0.1 mM. Basic polypeptides such as protamine sulfate, poly(L-lysine), and poly(L-arginine) were also capable of reversing the inhibition of BCKDH phosphatase by the inhibitor protein. Half-maximal reversal was observed at ≈36 μg/ml.

DISCUSSION

The specific activity of BCKDH phosphatase in gel-filtered mitochondrial extracts was observed to increase as much as 4-fold upon dilution of the extracts. This observation suggested the presence of an inhibitor of the phosphatase. After further purification of the phosphatase by DEAE-cellulose chromatography, BCKDH phosphatase activity was no longer increased by dilution, indicating that the inhibitor had been separated from the phosphatase. Indeed, a fraction exhibiting inhibitor activity was eluted from DEAE-cellulose with 0.3 M NaCl. This inhibitor activity was heat- and acid-stable. The activity was destroyed by treatment with pepsin or *S. aureus* V8 protease, demonstrating that the inhibitor is a protein. A purification procedure was then developed that gave nearly homogeneous preparations of the protein inhibitor with an overall yield of 4–8%. This purification procedure also provides near homogeneous preparations of BCKDH complex and pyruvate dehydrogenase complex and highly purified preparations of BCKDH phosphatase. Inclusion of ethylene glycol and Brij 35 in the buffers was found to be necessary to stabilize the protein inhibitor in dilute solutions,

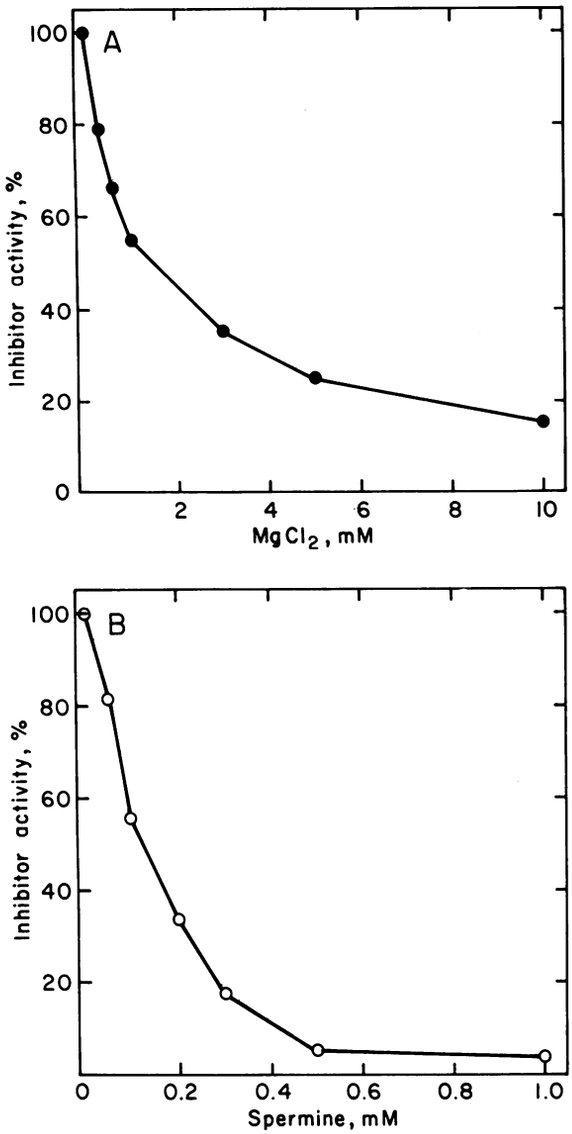

FIG. 6. Effect of Mg^{2+} (*A*) and spermine (*B*) on inhibition of BCKDH phosphatase activity by the inhibitor protein. The standard assay mixture contained 0.001 unit of BCKDH phosphatase, 0.005 unit of highly purified inhibitor protein, and the indicated concentrations of $MgCl_2$ or spermine. Other components and conditions were as described.

particularly in the late stages of the purification. Purification through the hydroxylapatite step resulted in a 1000- to 2000-fold purification and was effective in removing low molecular weight proteins and most of the nucleic acids. The Sephacryl S-200 gel filtration step, which has been repeated more than 10 times, provided a 75- to 300-fold purification.

Both Mg^{2+} and spermine reversed inhibitor protein activity. These substances do not exert a direct effect on BCKDH phosphatase, but they do affect [pyruvate dehydrogenase]-phosphatase directly. The latter phosphatase is Mg^{2+}-dependent, and its activity is stimulated markedly by spermine at physiological concentrations of Mg^{2+} (<1.0 mM) (16). Reversal of inhibitor protein activity by spermine suggests that the net effect of an increase in free spermine concentration in mitochondria would be to increase the activity of both BCKDH phosphatase and [pyruvate dehydrogenase]-phosphatase and hence to increase the activities of the pyruvate dehydrogenase and BCKDH complexes.

Reversal of inhibitor protein activity by Mg^{2+} was maximal at ≈10 mM Mg^{2+}. Measurement of BCKDH phosphatase activity in gel-filtered extracts in the presence of 10 mM Mg^{2+} was dose-dependent and gave values comparable to the activity of the phosphatase at the highest dilution of gel-filtered mitochondrial extracts.

The identification of the BCKDH phosphatase inhibitor protein and the demonstration of its potency (K_i ≈0.13 nM) may explain, at least in part, the difficulties encountered by other investigators in identifying BCKDH phosphatase activity in mitochondrial extracts and in its subsequent purification (2, 17). Contamination of either the phosphatase or its protein substrate by the inhibitor protein may have caused the difficulties.

An inhibitor of BCKDH phosphatase with an apparent M_r of ≈70,000 has also been detected in partially purified preparations of inhibitor protein by gel-permeation chromatography. The latter inhibitor is also heat-stable and pepsin-labile. Its relationship, if any, to the M_r 36,000 species is not yet known.

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1. Fatania, H. R., Lau, K. S. & Randle, P. J. (1981) *FEBS Lett.* **132,** 285–288.
2. Odessey, R. (1982) *Biochem. J.* **204,** 353–356.
3. Paxton, R. & Harris, R. A. (1982) *J. Biol. Chem.* **257,** 14433–14439.
4. Lawson, R., Cook, K. G. & Yeaman, S. J. (1983) *FEBS Lett.* **157,** 54–58.
5. Damuni, Z., Merryfield, M. L., Humphreys, J. S. & Reed, L. J. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4335–4338.
6. Pettit, F. H. & Reed, L. J. (1982) *Methods Enzymol.* **89,** 376–386.
7. Teague, W. M., Pettit, F. H., Wu, T.-L., Silberman, S. R. & Reed, L. J. (1982) *Biochemistry* **21,** 5585–5592.
8. Watanabe, H., Green, G. D. J. & Shaw, E. (1979) *Biochem. Biophys. Res. Commun.* **89,** 1354–1360.
9. Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148,** 327–342.
10. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
11. Böhlen, P., Stein, S., Dairwan, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155,** 213–220.
12. Mosbaugh, D. W. & Linn, S. (1983) *J. Biol. Chem.* **258,** 108–118.
13. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
14. Merrill, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211,** 1437–1438.
15. Henderson, P. J. F. (1972) *Biochem. J.* **127,** 321–333.
16. Damuni, Z.. Humphreys, J. S. & Reed, L. J. (1984) *Biochem. Biophys. Res. Commun.* **124,** 95–99.
17. Harris, R. A., Paxton, R. & Parker, R. A. (1982) *Biochem. Biophys. Res. Commun.* **107,** 1497–1503.