Guanine Deaminase Inhibitor from Rat Liver

ISOLATION AND CHARACTERIZATION

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1. An inhibitor of cytoplasmic guanine deaminase of rat liver was isolated from liver 'heavy mitochondrial' fraction after freezing and thawing and treatment with Triton X-100. 2. Submitochondrial fractionation revealed that the inhibitor was localized in the outer-membrane fraction. 3. The method of purification of inhibitor, involving precipitation with $(\text{NH}_4)_2\text{SO}_4$ and chromatography on DEAE-cellulose, its precipitability by trichloroacetic acid and the pattern of absorption in the u.v. indicated that the inhibitor was a protein. In confirmation, tryptic digestion of the isolated material resulted in destruction of the inhibitor activity. The inhibitor was stable to acid, but labile to heat. 4. The isolated inhibitor required phosphatidylcholine (lecithin) for activity. Phosphatidylcholine also partially protected the inhibitor against heat inactivation. 5. When detergent treatment was omitted, the inhibitor activity of frozen mitochondria was precipitated by $(\text{NH}_4)_2\text{SO}_4$ in a fully active form without supplementation with phosphatidylcholine, indicating that Triton X-100 ruptured the linkage between inhibitor and lipid. 6. A reconstituted sample of inhibitor-phosphatidylcholine complex was precipitated in a fully active form by dialysis against 2-mercaptoethanol, but treatment of the precipitate with NaCl yielded an extract which was inactive unless supplemented with fresh phosphatidylcholine. 7. We interpret the results as evidence that the inhibitor was present *in vivo* as a lipoprotein and that once the complex was dissociated by the action of detergent and the protein precipitated, there was an absolute need for exogenous phosphatidylcholine for its activity. The manner in which inhibitor associated with the outer membrane of rat liver mitochondria might regulate the activity of the enzyme in the supernatant has been suggested.

Inhibitors of proteinaceous nature, generally with low molecular weights, are known to occur in a variety of tissues and biological fluids. It is believed that these inhibitors play a role *in vivo* by regulating the activities of specific enzymes. Except in the case of the proteinase inhibitors, the reaction mechanism is not known with any certainty. Well known among the inhibitors are those of the serine proteinases (Vogel *et al.*, 1968; Laskowski & Sealock, 1971; Liener & Kakade, 1969); alkaline ribonuclease inhibitor (Roth, 1956; Gribnau *et al.*, 1969); ATPase* inhibitor isolated from mitochondria (Pullman & Monroy, 1963) and chloroplasts (Nelson *et al.*, 1972); globin inhibitor (Darnell *et al.*, 1971) from reticulocytes; inhibitor of cyclic AMP-dependent protein kinase isolated from muscle (Posner *et al.*, 1965); a component of troponin, an inhibitor from muscle (Ebashi & Kodama, 1965); phospholipase A inhibitor from venom (Braganca *et al.*, 1970; Vidal & Stoppani, 1971); protein methylase inhibitor from liver (Kim & Paik, 1971); amylase inhibitor from colocasia tuber (Rao *et al.*, 1970); chitin synthetase

inhibitor from yeast (Cabib & Keller, 1971); and inhibitors associated with blood coagulation (Yin *et al.*, 1971*a,b*). In addition to the above, another class of inhibitors is known, characterized by their being themselves enzymes and functioning in catalytic amounts. Examples of these are tryptophan synthetase inhibitor (Katsunuma *et al.*, 1972), glutamine synthetase inhibitor (Schutt & Holzer, 1972) and inhibitor of microsomal mixed-function oxidases (Krieger & Wilkinson, 1969).

The presence in rat liver mitochondria of an inhibitor of cytoplasmic guanine deaminase was reported from this laboratory (Kumar *et al.*, 1967). The present paper relates to the isolation and characterization of the inhibitor and the demonstration that the purified inhibitor is inactive unless supplemented with phospholipid.

Materials and Methods

Isolation of inhibitor

The 'heavy mitochondrial' fraction was isolated from 65g of liver from adult albino male rats by the method of Kumar *et al.* (1965), differing

* Abbreviation: ATPase, adenosine triphosphatase.

only in that the sedimented fraction was washed with 0.25M-sucrose five times instead of three and that the volume of solution used for washing was increased threefold. (Under these modified conditions, the final preparation was devoid of any guanine deaminase activity.) The particles were suspended in water and the volume made up to 163ml [equivalent to 40% (w/v) of the original tissue]. The suspension (fraction I) was frozen at -18°C overnight, thawed at $0-4^{\circ}\text{C}$ and ground in a VirTis homogenizer for two 1min periods, with an intervening gap of 1min, then 2-mercaptoethanol was added to a final concentration of 1mM and the mixture stored at $0-4^{\circ}\text{C}$ for 30min. The preparation was treated for 1h at $0-4^{\circ}\text{C}$ with Triton X-100 added to a final concentration of 1% (v/v) and the suspension was centrifuged at 15000g for 1h in rotor no. 856 of the International High-Speed refrigerated centrifuge model HR-1. To the supernatant (fraction II) solid $(\text{NH}_4)_2\text{SO}_4$ (enzyme-grade) was added to 40% saturation, the pH was adjusted to 7.0 with dilute aq. NH_3 , the suspension was then allowed to flocculate for 2h at $0-4^{\circ}\text{C}$ and centrifuged at 15000g for 30min. The sediment was suspended in about 20ml of 1mM-2-mercaptoethanol (fraction III). The supernatant was increased to 90% saturation with $(\text{NH}_4)_2\text{SO}_4$, then the pH was readjusted to 7.0 with dilute aq. NH_3 ; the suspension was left for 2h in the cold and centrifuged at 15000g for 30min. The sediment was dissolved in 10ml of 1mM-2-mercaptoethanol (fraction IIIa) and kept for testing for activity. Fraction III, the suspension of the 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate, was dialysed for 6h at $0-4^{\circ}\text{C}$ against 150vol. of 1mM-2-mercaptoethanol and the resulting suspension was centrifuged at 15000g for 40min. The inactive supernatant was discarded and the residue was suspended in 10ml of 1% (w/v) NaCl in 1mM-2-mercaptoethanol. After 1h in the cold with frequent stirring, the suspension was centrifuged at 15000g for 40min. The residue was washed in the centrifuge with 10ml of 1% NaCl in 1mM-2-mercaptoethanol. The wash fluid was pooled with the main extract (fraction IV) and the residue was discarded.

To test the solubility characteristics, a portion of fraction IV was centrifuged at 105000g for 1h in rotor no. 40 of Beckman model L preparative ultracentrifuge and the supernatant was analysed for inhibitor activity.

After an eightfold dilution with 20mM-potassium phosphate buffer, pH8.0 (prepared from KH_2PO_4 and K_2HPO_4), one-half of fraction IV was loaded on a column (1.5cm $\times$ 25cm) of DEAE-cellulose equilibrated with the 20mM-phosphate buffer, pH8.0. The column was flushed with 180ml of starting buffer and protein eluted stepwise from

the column with 80ml each of 50mM-, 100mM-, 150mM- and 200mM-potassium phosphate buffer, pH8.0, used consecutively. The original wash fluid and the eluates with 50mM-, 100mM- and 150mM-phosphate were collected in 10ml fractions each, and the eluates with 200mM-phosphate in 5ml fractions.

Submitochondrial localization of guanine deaminase inhibitor

The mitochondrial pellet prepared from 15.5g of liver (as above) was suspended in 0.25M-sucrose to give 2.6ml so as to contain 150mg of protein/ml. The method of Schnaitman & Greenawalt (1968) was used to separate the outer mitochondrial-membrane fraction. Digitonin (45mg; E. Merck A.G., Darmstadt, Germany), dissolved in 2.5ml of iso-osmotic sucrose, was added at 0°C to the suspension of mitochondria with constant stirring to give a final concn. of 1% (w/v) of digitonin and a ratio of 1.5mg of detergent/10mg of protein. The resulting suspension was gently stirred for 15min at $0-4^{\circ}\text{C}$ and diluted with 13.5ml of iso-osmotic sucrose solution. The suspension was homogenized in a hand-operated Teflon homogenizer and centrifuged at 12000g for 10min to sediment the inner-membrane-matrix fraction. The supernatant was drawn off and the pellet was resuspended in 13.5ml of iso-osmotic sucrose and the suspension centrifuged as above. The pellet was treated with Triton X-100 at a final concn. of 1% (v/v) for 1h. The two supernatants were combined and centrifuged at 105000g for 1h to sediment the outer-membrane fraction, which was washed once on the centrifuge with 12ml of iso-osmotic sucrose under the same conditions. The two supernatants (intermembrane-space material) were combined. The various fractions were tested for guanine deaminase inhibitor activity (without phosphatidylcholine supplementation). Monoamine oxidase, chosen as the marker enzyme for the outer-membrane fraction, was determined by the method of Tabor *et al.* (1954), with benzylamine sulphate as substrate. One unit of monoamine oxidase caused an increase of 0.001 in the extinction at 250nm/min at 30°C under the assay conditions.

Isolation of guanine deaminase

Guanine deaminase employed in monitoring the enrichment of inhibitor was a partially purified preparation. The 15000g $\times$ 1h supernatant from a 20% (w/v) iso-osmotic homogenate of rat liver in 0.25M-sucrose was fractionated with $(\text{NH}_4)_2\text{SO}_4$ at pH7.0 and 0°C and the 60-75%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate dialysed against 150vol. of water for 6h at $0-4^{\circ}\text{C}$ and the resulting suspension centrifuged.

The supernatant, which contained the enzyme activity, was stored at 0–4°C and was used for about 2 weeks. The preparation was freshly diluted 15–20-fold with water for use in each assay.

Guanine deaminase assay

The assay system for guanine deaminase activity consisted of 100 μ mol of Tris-HCl buffer, pH 8.0, water, enzyme preparation and 0.12 μ mol of guanine, in 2 ml. After incubation at 30°C for 15 min the system was inactivated with 1 ml of 10% (v/v) HClO₄. After centrifugal clarification, the E_{245} was measured in a Beckman model DU spectrophotometer. One unit of guanine deaminase converted 1 μ mol of guanine into xanthine under the assay conditions.

Guanine deaminase inhibitor assay

The inhibitor and the enzyme preparation were mixed for 3 min at 30°C and the mixture was assayed for residual guanine deaminase activity against a control enzyme sample assayed after 3 min of preincubation at 30°C with the same medium as was present in the inhibitor preparation. For determination of inhibitory activity in the presence of lipid, the inhibitor was kept mixed for 3 min with an ethanolic solution of the lipid at 30°C; the lipid-treated inhibitor was then preincubated with enzyme preparation for 3 min at 30°C and the residual enzyme activity determined. The control assay for uninhibited enzyme was with the enzyme preparation preincubated for 3 min at 30°C with lipid, followed by 3 min with the appropriate medium. The amount of inhibitor employed in the assay systems was generally adjusted to give 35–65% enzyme inhibition. One inhibitor unit affected 50% inhibition of one unit of enzyme activity under the assay conditions. The specific activity of inhibitor was expressed in terms of units of activity/mg of protein. In some experiments it was more convenient to express the activity of the inhibitor in terms of percentage inhibition of the enzyme activity in the assay.

Protein determination

The method of Lowry *et al.* (1951) was employed in determining the protein content in all the fractions except in the DEAE-cellulose column eluates, where the estimation was by the spectrophotometric method as employed as Kalckar (1947). Crystallized and freeze-dried bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used as standard in colorimetric determination of protein.

Results

Enrichment of inhibitor

A typical purification schedule is given in Table 1 and an elution profile from a DEAE-cellulose column shown in Fig. 1.

The inhibitor activity in Triton X-100-solubilized fraction (fraction II) was higher in all experiments than the activity in the untreated mitochondrial suspension (fraction I); 55–85% of the activity in fraction II was recoverable in the 40%-satd.-(NH₄)₂SO₄ precipitate (fraction III). The 40–90%-satd.-(NH₄)₂SO₄ precipitate (fraction IIIa) was inactive. The whole of the activity in fraction III passed into the NaCl extract (fraction IV) when the residue separating during dialysis of the protein fraction against water was treated with the NaCl solution. That the inhibitor was apparently in true solution at this stage followed from the finding that the entire activity was recovered from the supernatant when a portion of the extract was centrifuged at 105000g for 60 min. The inhibitor in fraction IV was retained on DEAE-cellulose at pH 8.0 and was eluted in a single peak with 200 mM-potassium phosphate buffer, pH 8.0, after the use of 50 mM-, 100 mM- and 150 mM-phosphate buffer, pH 8.0.

The pattern of enrichment of inhibitor was reproducible from experiment to experiment. The activity recovered from the pooled eluate fractions from the DEAE-cellulose column accounted for 55–70% of the inhibitory activity loaded on the column and 50–60% of the activity in fraction I. The overall enrichment in the pooled fractions was 595–623-fold, with the maximum enrichment in a single fraction eluted from the DEAE-cellulose column being 1126-fold.

Unless otherwise stated, the active fractions from the column eluates (in Table 1, fractions 32–35) were pooled and used as the source of inhibitor.

Stability of inhibitor

The mitochondrial fraction lost one-third of its activity at the end of 1 week's storage at 0–4°C or –18°C. The material frozen for 24 h at –18°C and thawed and stored for 1 week suffered 75% loss at 0–4°C and 40% at –18°C. After Triton X-100 treatment, there was complete loss of activity on storage as above. The (NH₄)₂SO₄-precipitated fraction was also not stable, but the extract in NaCl suffered only marginal loss at –18°C and less than 30% at 0–4°C at the end of 7 days. The addition of phosphatidylcholine afforded partial protection against the loss of activity on storage of the (NH₄)₂SO₄-precipitated fraction at either temperature and the NaCl extract at 0–4°C. An inhibitor preparation purified about 800-fold, containing 23 μ g of protein/ml, remained fully active up to 10

Table 1. *Purification of guanine deaminase inhibitor from rat liver mitochondria*

The starting material was the 5000g sediment isolated from the post-nuclear supernatant of 65g of liver homogenized in 0.25M-sucrose. The isolation schedule was as described in the text. All inhibitor assays were after treatment with 100 μ g of phosphatidylcholine. The guanine deaminase used in the assays contained 0.041 unit of activity/83.51 μ g of protein. The unit of inhibitor activity was as defined in the Materials and Methods section.

Fraction	Vol. (ml)	Total inhibitor activity (units)	Total protein (mg)	Sp. activity (units/mg of protein)	Recovery (%)	Purifi- cation
(I) Mitochondrial suspension, untreated	160	41.4	450	0.092	(100)	(1)
(II) Triton X-100-solubilized extract	192.4	49.5	301	0.165	120	1.8
(III) 40%-satd.-(NH ₄) ₂ SO ₄ precipitate	15.4	36.7	125	0.294	88.6	3.2
(IV) NaCl extract	21.9	37.6	5.5	6.836	90.8	74.3
(V) DEAE-cellulose column chromatography: eluate (5ml fractions) with 200mM-phosphate buffer, pH 8.0*						
No. 29		Nil	0.283	—	—	—
No. 30		1.9	0.342	5.556	4.6	60.4
No. 31		1.9	0.283	6.714	4.6	73
No. 32		2.9	0.084	34.52	7.0	375
No. 33		8.7	0.084	103.5	21	1126
No. 34		7.5	0.147	51.02	18.1	555
No. 35		2.5	0.084	29.75	6.0	324
No. 36		1.4	0.158	8.86	3.4	96.3
No. 37		1.4	0.232	5.98	3.4	65.0
No. 38		Nil	0.202	—	—	—

* Only half of fraction IV was chromatographed at a time, but the data have been calculated to correspond to the activity in the whole of the fraction.

days when stored at 0–4°C; at the end of 20 days about 50% loss in activity was observed. Another preparation which was about 1100-fold purified and which contained 16 μ g of protein/ml retained full activity when analysed at the end of 2 months of storage at –18°C.

Submitochondrial localization of inhibitor

The results of analysis of inhibitor activity in the submitochondrial fractions are recorded in Table 2.

On centrifugation of digitonin-treated mitochondria at 12000g, the original inhibitory activity was recovered in the supernatant fraction with about fourfold enrichment. Some activity was associated also with the residual pellet, but this represented only 6% of the original activity and the specific activity in this fraction was very low. The pellet resulting on centrifugation of the supernatant at 105000g contained 98% of the inhibitory activity of the digitonin-treated mitochondrial suspension and 106% of the activity present in the 12000g supernatant. As the protein recovered in the pellet was only

6.4% of that in the original mitochondria, the inhibitor was enriched 15.4-fold in this fraction. Monoamine oxidase activity was virtually completely localized in the 105000g pellet, proving the validity of the fractionation procedure for the isolation of the outer membrane.

Obligatory dependence on lipid, possibly phosphatidylcholine

In a number of preliminary experiments, there was almost complete failure to recover inhibitor activity after fractionation of Triton X-100 extracts with (NH₄)₂SO₄. In a trial experiment, an apparently inactive fraction was mixed with phosphatidylcholine and tested with the enzyme, whereupon considerable inhibitory activity was demonstrable.

To test for specificity of requirement, 100 μ g each of phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine and cholesteryl stearate, dissolved in 0.1ml of ethanol, were separately tested for their effect on the inhibitory activity of fraction III. Cholesteryl stearate was

Fig. 1. Elution pattern of rat liver mitochondrial inhibitor of guanine deaminase from a DEAE-cellulose column

Inhibitor preparation isolated from ‘heavy mitochondrial’ fraction after freezing and thawing, VirTis homogenization, Triton X-100 treatment and (NH₄)₂SO₄ fractionation was dialysed and the precipitated material from the bag was extracted with NaCl in 2-mercaptoethanol. The extract was diluted with 20mM-potassium phosphate buffer, pH 8.0, and loaded on a column (1.5cm×25cm) of DEAE-cellulose equilibrated with 20mM-potassium phosphate buffer, pH 8.0. Elution was carried out as described in the Materials and Methods section. ○, Protein; ▲, inhibitor activity.

Table 2. Submitochondrial localization of guanine deaminase inhibitor

‘Heavy’ mitochondria isolated from 15.5g of liver were fractionated for the separation of external membrane by the digitonin method and monitored by monoamine oxidase activity. The guanine deaminase preparation used in inhibitor assays contained 0.028 unit of activity in 78.2µg of protein.

Fraction	Protein (mg)	Inhibitor (units)	Monoamine oxidase (units)	Recovery (%)		
				Protein	Inhibitor	Monoamine oxidase
Suspension of ‘heavy mitochondria’ from rat liver	391.8	19.25	46.60	(100)	(100)	(100)
12000g pellet after digitonin treatment	271.4	1.10	7.50	68.0	5.7	16.1
12000g pellet treated with Triton X-100: whole suspension		1.25	8.0		6.5	17.1
12000g supernatant	100.8	20.55	49.95	25.7	106.8	107.2
105000g pellet	25.1	18.90	48.00	6.4	98.18	103.0
105000g supernatant	70.5	3.90	1.85	17.9	20.25	3.94

a product of BDH Chemicals Ltd., Poole, Dorset, U.K. The other lipids were products of V.P. Chest Institute, Delhi University, Delhi, India. Only phosphatidylcholine elicited inhibitory activity. Ethanol by itself used in 0.1ml portions/assay system did not influence the activity. In a later experiment, phosphatidic acid obtained from V.P.

Chest Institute was found to be inactive, tested with the inhibitor fractionated on DEAE-cellulose.

In all subsequent experiments, as in the experiment reported in Table 1, the assay of inhibitor activity was after preincubation with 100µg of phosphatidylcholine unless otherwise specified. Assays of inhibitor activity in preparations at various

stages of enrichment, in the presence and absence of added phosphatidylcholine (100 µg/assay system), revealed that fractions I and II did not need externally added phosphatidylcholine for the manifestation of the inhibitory activity, nor was there any increase in activity in its presence. But fraction III and fraction IV were totally inactive in the absence of added phosphatidylcholine. The eluate fractions from DEAE-cellulose with 200mM-phosphate buffer also needed phosphatidylcholine for manifestation of inhibitory activity.

Mode of linkage of lipid with inhibitor protein and the effect of Triton X-100 on the linkage

The once-frozen-and-thawed suspension of mitochondria adjusted to 1mM-2-mercaptoethanol (prepared as described in the Materials and Methods section) was divided into two halves. One-half was left untreated and the other half was treated with Triton X-100 in final concn. of 1% (v/v). After 1 h at 0–4°C, the two suspensions were centrifuged at 105000g for 60 min. The residual fraction from the first sample was tested also in the presence of Triton X-100. A portion of each of the two 105000g supernatant fractions was dialysed against 1mM-2-mercaptoethanol for 6 h at 0–4°C and tested separately in the form of suspension and centrifuged extract and residue. The major part was used for precipitation with (NH₄)₂SO₄ at 40% saturation and the precipitate was tested in the presence and absence of phosphatidylcholine, after suspending it in 1mM-2-mercaptoethanol. The results obtained are recorded in Table 3.

The inhibitory activity in the detergent-treated sample passed totally into the supernatant, whereas in the absence of detergent only 50% of the inhibitory activity was recovered in the supernatant. The residue in the latter case contained the rest of the activity and this was increased by 46% on treatment with Triton X-100. When the two supernatant fractions were dialysed, the inhibitory activity was recovered totally in the supernatants on centrifugation at 15000g for 60 min. All these analyses were carried out in the absence of phosphatidylcholine. When the 105000g supernatants were fractionated with (NH₄)₂SO₄, the precipitate from the extract prepared in the absence of Triton X-100 was fully active without exogenously added phosphatidylcholine. However, the corresponding precipitate from the Triton X-100-treated material was active only when phosphatidylcholine was supplemented.

Effect of NaCl on the linkage of isolated inhibitor with phosphatidylcholine

A partially purified preparation of inhibitor, corresponding to fraction IV, containing 0.725mg of protein in 2.0ml of 1% (w/v) NaCl, was mixed with 2mg of phosphatidylcholine in 2.0ml of anhydrous ethanol. The suspension was dialysed against 150vol. of water, containing 1mM-2-mercaptoethanol for 6 h at 0–4°C. The resulting suspension was centrifuged at 15000g for 1 h and the residue was extracted with 1% NaCl.

The removal of NaCl by dialysis precipitated the inhibitor, which was fully active. When this material was extracted with 1% NaCl, the inhibitor needed

Table 3. *Elucidation of the role of lipid and the mechanism of action of Triton X-100*

The once-frozen-and-thawed mitochondrial suspension was fractionated for guanine deaminase inhibitor, without and with prior treatment with Triton X-100, as described in the text. The sample of guanine deaminase used in the assays contained 0.031 unit of activity and 62 µg of protein.

Fraction	Triton X-100 absent		Triton X-100 present	
	Inhibitor activity (units)	Protein (mg)	Inhibitor activity (units)	Protein (mg)
5000g residue	5.75	189	5.75	189
105000g supernatant	2.88	96.2	6.90	98.3
105000g residue	3.22	90.2	Nil	90.0
(105000g residue treated with Triton)	4.69	91.1		
Supernatant dialysed				
(a) Whole suspension	2.79	90.0	6.64	97.8
(b) Supernatant	2.79	88.9	6.64	96.9
40%-satd.-(NH ₄) ₂ SO ₄ precipitate from original supernatant				
(a) Without phosphatidylcholine	2.38	31.8	Nil	32.2
(b) With phosphatidylcholine	2.38	31.8	4.64	32.2

fresh supplementation with phosphatidylcholine for manifestation of its activity.

U.v. absorption of inhibitor fraction

A 300-fold purified sample, containing 34 μ g of protein/ml, and 200mM-phosphate buffer, pH8.0, gave a typical protein spectrum with E_{260}/E_{280} ratio of 1:2.5.

Influence of temperature on the inhibitor preparation

Portions of the inhibitor preparation were kept, without and with added phosphatidylcholine, for 5min at temperatures ranging from 30° to 80°C and chilled in ice for 10min. The tubes were then warmed to 30°C, phosphatidylcholine was added to those tubes that did not contain it and after another 3min at 30°C, enzyme preparation was added and the residual enzyme activity was analysed at the end of 3 min of preincubation.

The inhibitor was heat-labile, its activity being totally destroyed on heating for 5min at 80°C. Phosphatidylcholine gave some protection to the inhibitor. In the absence of phosphatidylcholine there was a 44% decrease in the inhibitory activity on heat-treatment at 60°C, whereas when phosphatidylcholine was present the inhibitory activity remained unaltered. Also when phosphatidylcholine was present, heating at 70°C led to destruction of 44% of inhibitory activity, whereas total destruction occurred in its absence.

Effect of precipitation of inhibitor with trichloroacetic acid

A portion of fraction IV (Table 1, NaCl extract of precipitate separating during dialysis), containing 250 μ g of protein with 1.28 units of inhibitor activity, was mixed with an equal volume of 10% (w/v) trichloroacetic acid. The resulting suspension was stored for 4h in an ice bath and the precipitate was collected by centrifugation at 700g for 70min. The sediment was washed three times by centrifugation with 5ml each time of anhydrous ether to remove trichloroacetic acid and the final product dissolved in 1% NaCl in 1mM-2-mercaptoethanol. The supernatant from the precipitate was freed of trichloroacetic acid by shaking five times with 5ml each of ether. The inhibitor activity was estimated both in the trichloroacetic acid-precipitable fraction and the supernatant.

The inhibitory activity passed into the fraction precipitable with trichloroacetic acid; the supernatant was completely devoid of activity. Since the precipitable fraction contained 40% or less of protein, an enrichment of inhibitor occurred in the process.

Effect of digestion of inhibitor with trypsin

Purified inhibitor preparation (2ml, containing 10 μ g of protein and 0.515 unit of inhibitor activity) was mixed with 2mg of trypsin (type III, from bovine pancreas; Sigma Chemical Co.) in 1ml of 100mM-phosphate buffer, pH7.5, and kept at 30°C for 2h. After acidification with 3ml of 10% (w/v) trichloroacetic acid, 1mg of bovine serum albumin was added to the tube. In a simultaneous control run trypsin was added after the acid and albumin. The tubes were left at 0–4°C for 1h and centrifuged at 700g for 10min. The precipitate was washed three times with 5ml batches of ether and dissolved in 2ml of 1% NaCl in 1mM-2-mercaptoethanol and assayed for inhibitory activity.

There was 78% recovery of inhibitory activity when trypsin was added after acidification with trichloroacetic acid; digestion with trypsin for 2h resulted in total loss of inhibitory activity precipitable by acid.

Discussion

The procedure adopted for purification, involving $(\text{NH}_4)_2\text{SO}_4$ fractional precipitation, dialysis and DEAE-cellulose column chromatography, pointed to guanine deaminase inhibitor being protein in nature. The peak of absorption of the inhibitor at 278–280nm, its thermolability and precipitability with trichloroacetic acid, were in consonance with its protein nature. More conclusive proof was provided by its trypsin-lability. It was recently reported from this laboratory that guanine deaminase inhibitor was inducible in rat liver mitochondria on administration of guanine and that the induction was blocked by inhibitors of protein synthesis (Sitaramaya *et al.*, 1974).

In respect of compartmentation of inhibitor from its enzyme, guanine deaminase inhibitor resembles protein methylase inhibitor, which, however, is essentially microsomal (Kim & Paik, 1971). A compartmentation is known also in the case of chitin synthetase inhibitor of yeast (Cabib & Keller, 1971); the inhibitor in this case is soluble and the enzyme particulate. On the other hand, ATPase inhibitor and the enzyme are both located in the mitochondria (Pullman & Monroy, 1963).

The occurrence of the inhibitor implies a role *in vivo*. As the untreated mitochondrial fraction was itself inhibitory to the enzyme in the supernatant, it was apparent that the enzyme had ready access to inhibitor. Submitochondrial fractionation revealed that the inhibitor was associated with the outer-membrane fraction. Such a ready accessibility of inhibitor to enzyme would permit regulation of guanine deaminase inhibitor activity *in vivo*. An alternative mechanism of control of enzyme activity, namely, a reversible release of inhibitor protein into

cytosol, is suggested by the finding of the specific solubilization of outer-membrane-associated brain mitochondrial hexokinase by glucose 6-phosphate (Craven & Basford, 1972). By using liver homogenates, Rose & Warms (1967) and Wilson (1968) found that the presence of various metabolites could influence the proportion of soluble and particulate hexokinase.

A number of membrane-associated enzymes are known to be influenced in activity by phospholipids. The lipid requirement may be either absolute, as for β -hydroxybutyrate dehydrogenase (Sekuzu *et al.*, 1963), or, as with particulate mitochondrial ATPase, may involve activation of an existing activity (Palatini *et al.*, 1972). The present investigations have revealed the apparently absolute phospholipid dependence of the inhibitor once isolated from mitochondria. Although no claim is made as to the purity of the phospholipids tested as supplements in the assay system, it appeared that the requirement was specifically for phosphatidylcholine. Colbeau *et al.* (1971) showed that phosphatidylcholine constitutes the major component of the phospholipids of the outer membrane of rat liver mitochondria (50% of the total phospholipid phosphorus). When treatment with Triton X-100 was omitted from the procedure for preparation of mitochondrial extract, the inhibitor was precipitated by $(\text{NH}_4)_2\text{SO}_4$ in a fully active form, presumably in the form of a phosphatidylcholine-protein complex. The need for exogenous phospholipid could be demonstrated only when protein was dissociated from the complex with lipid by the action of Triton X-100 and isolated by $(\text{NH}_4)_2\text{SO}_4$ fractionation and extraction with NaCl. These data appeared to establish that phosphatidylcholine functioned by activating the inhibitor protein and not by sensitizing guanine deaminase to an otherwise non-inhibitory protein.

We have previously isolated guanine deaminase inhibitor from rat brain and liver (Kumar *et al.*, 1967). The partially purified material was active without phospholipid supplement. In the light of the present data, it seems that the protein preparations contained residual phospholipid.

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