Plant Physiol. (1984) 76, 654–657
0032-0889/84/76/0654/04/$01.00/0

γ-Guanidinobutyraldehyde Dehydrogenase of *Vicia faba* Leaves

Received for publication February 28, 1984 and in revised form May 23, 1984

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ABSTRACT

γ-Guanidinobutyraldehyde dehydrogenase was purified 27-fold in 40% yield from extracts of *Vicia faba* leaves. High specificity exist only for γ-guanidinobutyraldehyde and γ-aminobutyraldehyde; the K_m value was 3.4 micromolar for γ-guanidinobutyraldehyde, 25 micromolar for γ-aminobutyraldehyde, and 84 micromolar (case of γ-guanidinobutyraldehyde) for NAD, respectively. The enzyme had a molecular weight of approximately 83,000. Optimal pH and temperature for activity were 9.5 and 45°C, respectively. The enzyme was inhibited strongly by *p*-chloromercuribenzoate, *N*-ethylmaleimide, and zincon (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene).

We have previously reported the purification and properties of the L-arginine decarboxylase (reaction: L-arginine → agmatine + CO_2) and diamine oxidase (reaction: agmatine + H_2O + O_2 → γ-GBAld[1] + H_2O_2 + NH_3) from *Vicia faba* leaves (10–12). Although γ-GBAld dehydrogenase has been found in *Pseudomonas putida* (15), and also γ-ABAld dehydrogenase has been found in *Pseudomonas fluorescens* (8) and *Pseudomonas* species (3), no report seems to have been made from higher plants. This paper describes for the first time from plant source the purification and properties of the NAD-dependent dehydrogenase which participates in the oxidation of γ-GBAld and γ-ABAld from the leaves of *Vicia faba*.

MATERIALS AND METHODS

Plant Materials. Seeds of broad bean (*Vicia faba* L. cv Wase) were germinated in moist vermiculite at 25°C for 4 d in the dark. Seedlings were transferred to plastic trays containing one-half Hoagland solution. They were grown under continuous light (about 4000 lux at plant level) at 25°C for 15 to 18 d. The harvested leaves were surface-sterilized in 0.1% benzalkonium chloride solution, rinsed with deionized H_2O, and used for the experiment.

Chemicals. γ-Guanidinobutyraldehyde diethylacetal carbonate was prepared according to the method of Hasse and Schührer (7) and recrystallized from water-acetone. γ-Aminobutyraldehyde diethylacetal was obtained from Aldrich Chemical Co. Glutaric semialdehyde was prepared by the procedure described by Adams and Chang (1). γ-GBAld and γ-ABAld were prepared by the treatment of each acetal (0.2 mmol) with 0.15 ml of 1 N HCl; the mixtures were incubated in plugged test tubes for 10 min at 60°C. After cooling the test tubes in a ice box, the mixtures were diluted to each 5 ml with deionized H_2O (final concentration of each aldehyde: 20 mM) and used as stock solution. γ-Guanidinobutyric acid, glyceraldehyde, succinic semialdehyde, α-aminoadipic acid, *N*-ethylmaleimide, and reduced glutathione were purchased from Sigma. Sephadex G-25, Sephadex G-150, Sephadex G-200, and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals. NAD and NADP were purchased from P-L Biochemical Inc. All other chemicals used were of analytical reagent grade.

Enzyme Assay. Enzyme activity was routinely assayed by measuring the increase in *A* at 340 nm of a reaction mixture. The standard assay mixture contained 200 μmol glycine buffer (pH 9.2), 1.2 μmol γ-GBAld, 60 μmol NAD, and enzyme solution (protein content, 0.1 mg) in a total volume of 3.0 ml. The assay was initiated by the addition of substrate and incubated at 32°C for 10 min. Enzyme activity is presented as the amount of formed NADH (nmol/min) in the standard assay system.

Protein Determination. Protein was determined by the Lowry procedure (9), with BSA as the standard.

Differential Centrifugation. The leaves (10 g) were blended in a mortar with 3 volumes (v/w) of 0.1 M K-phosphate buffer (pH 7.5) containing 0.25 M sucrose and 2 mM 2-ME. The macerate was subjected to differential centrifugation.

Stoichiometry Studies. The reaction mixture per 12 ml contained the following compounds: 12 μmol γ-GBAld, 24 μmol NAD, enzyme from step 5 (see below), and 200 μmol PPi-HCl buffer (pH 9.2). Incubation was carried out in test tubes for 1 or 2 h at 32°C. Formed NADH was determined by reading the *A* at 340 nm in a cuvette of 1-cm light path with a spectrophotometer. For the determination of γ-GBAld and γ-GBA, the reaction mixture (10 ml) was treated with 2 ml of 10% TCA containing 20 μmol HCl, then centrifuged for 10 min at 10,000 *g* to remove the precipitate. The precipitate was washed once with 5 ml of 2% TCA solution. The remaining γ-GBAld and formed γ-GBA in the above-combined TCA solution were separated from TCA by the fractionation with an equal volume of diethylether (three times). After neutralization, the aqueous solution was applied to a small column (1 × 10 cm) of cation exchange resin AG 50W × 2 (200–400 mesh, H-form; Bio-Rad Laboratories). After washing the column with 20 ml of 0.6 M HCl, γ-GBAld and γ-GBA were eluted from the column with 25 ml of 1.2 M HCl. The acidic elute was evaporated to dryness under reduced pressure at 60°C, and the residue was dissolved in 2 ml of 10 mM HCl. Recovery of authentic γ-GBAld and γ-GBA by this method was about 93% and 98%, respectively. γ-GBAld was determined by the following procedure; 0.5 ml of the sample solution mentioned above was added to the mixture of 0.5 ml of 20 mM phenylhydrazine hydrochloride solution and 0.5 ml of 0.1 M K-phosphate buffer (pH 7.0). After incubation for 20 min at 35°C, the mixture was adjusted to 20 ml with deionized H_2O and the γ-GBAld was estimated by reading the *A* at 274.5 nm caused by phenylhydrazone of γ-GBAld. γ-GBA did not interfere with color development of the standard γ-GBAld solution. γ-GBA was determined by general colorimetric procedure for guanidino compounds

[1] Abbreviation: γ-GBAld, γ-Guanidinobutyraldehyde; γ-ABAld, γ-aminobutyraldehyde; γ-GBA, γ-guanidinobutyric acid; 2-ME, 2-mercaptoethanol; N-EM, *N*-ethylmaleimide.

Table I. *Purification of γ-GBAld Dehydrogenase from Vicia faba Leaves*

Step	Total Protein	Total Activity (NADH Formed)	Specific Activity (NADH Formed)	Purification	Yield
	mg	*nmol/min*	*nmol/min·mg protein*	*–fold*	*%*
1. Crude extract	594.4	1,077.0	1.81	1.0	100
2. Protamine sulfate treatment	452.7	1,224.1	2.70	1.5	114
3. 45–65% Ammonium sulfate precipitation	72.84	983.0	13.49	7.5	91
4. DEAE-Sephadex chromatography	28.46	767.9	26.98	14.9	71
5. Sephadex G-150 chromatography	8.78	436.6	49.73	27.5	41

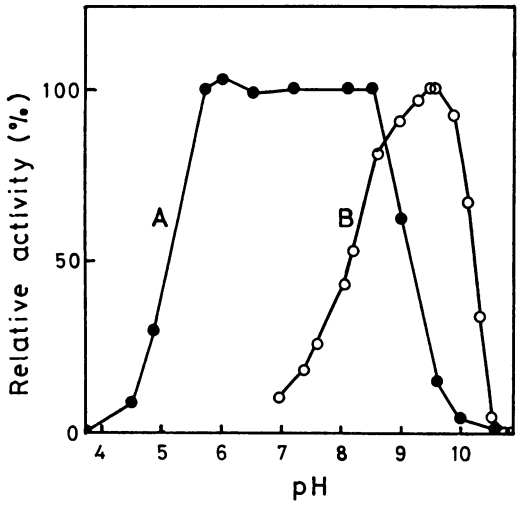

FIG. 1. A, pH stability of *Vicia* γ-GBAld dehydrogenase. B, Effect of pH on *Vicia* γ-GBAld dehydrogenase activity. Assay conditions are given in the text. The following buffer solutions were used: K-phosphate buffer for pH 4.5 to 8.2; glycine-NaOH buffer for pH 8.5 to 10.9.

(14). The estimated value was corrected for the presence of γ-GBAld by subtracting the absorbance.

Determination of Mol Wt. The mol wt of the enzyme was estimated on a Sephadex G-200 column according to the method of Andrews (2). The column was calibrated with Cyt *c* (mol wt 12,400), BSA (mol wt 67,000), γ-globulin (mol wt 165,000), and ovalbumin (mol wt 45,000) as markers of known mol wt.

RESULTS

Purification of Enzyme. A summary of the enzyme purification procedure is given in Table I.

Step 1: Crude Extract. The leaves (60 g) of 15 to 18-d-old seedlings were blended in a chilled Waring Blendor with 180 ml of cold 0.1 M K-phosphate buffer (pH 7.5) containing 5 mM 2-ME. The homogenate was filtered through cheesecloth and clarified by centrifugation (10,000 *g*, 15 min).

Step 2: Protamine Sulfate Treatment. The supernatant from step 1 was stirred with protamine sulfate (10 mg/10 g fresh weight) for 5 min and then the mixture was centrifuged at 10,000 *g* for 7 min.

Step 3: $(NH_4)_2SO_4$ Precipitation. The supernatant of step 2 was fractionated stepwise with $(NH_4)_2SO_4$, and the fraction obtained between 45% and 65% saturation was collected by centrifugation. The pellet containing the enzyme was dissolved in 7 ml of 20 mM glycine buffer (pH 8.5) containing 5 mM 2-ME (glycine-ME buffer). The enzyme fraction was freed of $(NH_4)_2SO_4$ by passing the solution through a Sephadex G-25 column (2.5 × 50 cm) equilibrated with the glycine-ME buffer. The active fractions (35 ml) were collected.

Step 4: Column Chromatography of DEAE-Sephadex A-50. The enzyme solution from step 3 was applied to a DEAE-Sephadex A-50 column (1 × 10 cm) equilibrated with the glycine-ME buffer. The column was washed with 60 ml glycine-ME buffer containing 0.1 M KCl, followed by 20 ml of the same buffer containing 0.15 M KCl. Then, the enzyme was eluted with 25 ml of the glycine-ME buffer containing 0.2 M KCl.

Step 5: Sephadex G-150 Filtration. Active fractions (25 ml) of step 4 was placed in dialysis tube and it was concentrated by contact with solid PEG (mol wt 20,000). The concentrated fraction (1 ml) was subjected to a Sephadex G-150 column (1.2 × 80 cm) equilibrated with 20 mM glycine-ME buffer containing 50 mM KCl (pH 8.5), the enzyme was eluted with the same buffer (at a rate of 7 ml/h). The active fractions were combined (12 ml) and used as the partially purified enzyme.

Differential Centrifugation Study. Enzyme activity was found mainly in the supernatant fraction (100,000 *g*, 30 min; 92% of the total activity).

Effect of pH and Temperature. The enzyme exhibited a single pH optimum at 9.5, activity rapidly declining above pH 8.6 and declining less rapidly below pH 5.7. The enzyme was most stable

Table II. *Stoichiometry for Conversion of γ-GBAld to γ-GBA*

Experimental conditions are given in the text.

Experiment	Added		Condition		Change		
	NAD	γ-GBAld	Enzyme	Incubation time	γ-GBAld	γ-GBA	NADH
	μmol		*mg*	*h*		*μmol*	
1	24	12	1.2	2	−9.96	+9.72	+10.63
2	24	12	1.5	1	−8.52	+8.04	+8.64

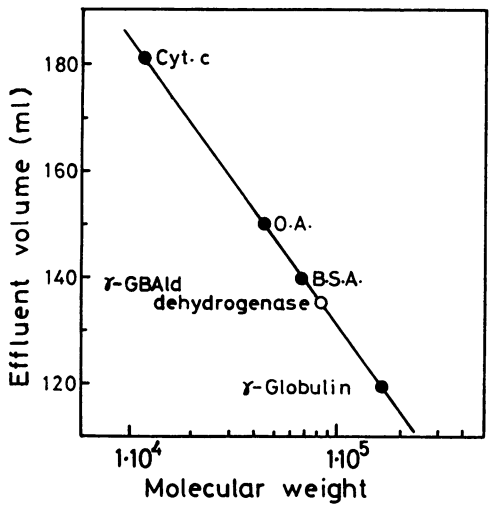

FIG. 2. Determination of mol wt of *Vicia* γ-GBAld dehydrogenase by means of Sephadex G-200 gel filtration. A Sephadex G-200 column (1.6 × 100 cm) was equilibrated with 0.1 M K-phosphate buffer (pH 7.5) containing 0.1 M KCl, after which it was calibrated with Cyt *c* (mol wt 12,000), ovalbumin (O.A: mol wt 45,000), BSA (mol wt 67,000), and γ-globulin (mol wt 165,000).

Table III. *Substrate Specificity of the NAD-Dependent γ-GBAld Dehydrogenase from Vicia faba Leaves*

Substrate	Concn.	Relative Activity
	mM	%
γ-GBAld	0.04	100
γ-GBAld	0.10	85
γ-GBAld	0.20	84
γ-ABAld	0.04	106
γ-ABAld	0.20	144
Propionaldehyde	0.10	0
Propionaldehyde	1.00	1.6
n-Butyraldehyde	0.10	0
n-Butyraldehyde	1.00	0.8
Acetaldehyde	0.10	1.3
Acetaldehyde	1.00	1.8
Benzaldehyde	0.10	0.3
Benzaldehyde	1.00	1.7
DL-Glyceraldehyde	0.10	1.4
DL-Glyceraldehyde	1.00	1.7
Succinic semialdehyde	0.10	0
Succinic semialdehyde	1.00	2.2
Glutaric semialdehyde	0.10	0

between pH 5.7 and 8.6; losing 25% of its activity in 24 h at 0°C. At pH 9.0 and 10.0, activity losses were 63% and 96%, respectively, under the same conditions (Fig. 1). Optimum temperature was 45°C. The enzyme activity was stable between 0°C and 20°C. Exposure of the enzyme to 30°C and 50°C for 10 min resulted in 4% and 62% loss of activity, respectively: above 60°C, however, the enzyme was rapidly inactivated.

Stoichiometry. The stoichiometry of the γ-GBAld dehydrogenase reaction was obtained by measuring the disappearance of γ-GBAld, and the appearance of γ-GBA and NADH. Approximately 1 mol each of γ-GBA and NADH were produced from 1 mol each of γ-GBAld and NAD by the enzyme from step 5 of the purification procedure (Table II).

Mol Wt. γ-GBAld dehydrogenase from *Vicia* leaves had an approximate mol wt of 83,000 by comparing their elution volumes from gel filtration of Sephadex G-200 with four proteins of known mol wt (Fig. 2).

Table IV. *Effect of Various Compounds on the Activity of γ-GBAld Dehydrogenase from Vicia faba Leaves*

	Concn.	Activity[a]
	mM	%
p-Chloromercuribenzoate	0.10	6
N-Ethylmaleimide	0.10	5
N-Ethylmaleimide	1.00	0
Na-Diethyldithiocarbamate	0.10	94
Na-Diethyldithiocarbamate	1.00	73
o-Phenanthroline	0.10	55
o-Phenanthroline	1.00	29
Zincon[b]	0.005	58
Zincon	0.05	17
Zincon	0.10	11
Ferron	0.05	97
Ferron	0.10	72
KCN	0.10	97

[a] Values show the activity after preincubation with inhibitors for 10 min at 32°C.

[b] 2-Carboxy-2′-hydroxy-5′-sulfoformazylbenzene.

Table V. *Reactivation by DTT and 2-Mercaptoethanol of Vicia γ-GBAld Dehydrogenase Inactivated by the Storage*

	Concn.	Specific Activity (NADH Formed)	
	mM	*nmol/min·mg protein*[a]	%
Native enzyme		32.8	100
Stored enzyme (13 d)[b]		2.9	9
+DTT	1	24.2	74
+DTT	2	22.6	69
+2-Mercaptoethanol	2	15.2	46
+2-Mercaptoethanol	4	16.4	50
+Glutathione	1	4.9	15
+Glutathione	2	6.2	19
+Cysteine	1	3.3	10
+Cysteine	2	4.9	15

[a] Standard assay conditions were used.

[b] Enzyme (step 5, see text) was stored at 4°C.

Substrate Specificity. *Vicia* γ-GBAld dehydrogenase was found to be substrate-specific. The preparation seems to oxidize only γ-GBAld and γ-ABAld. Other aldehydes did not allow the formation of NADH in the standard assay conditions (Table III). NADP does not replace NAD.

Kinetic Properties. The effects of the concentration of γ-GBAld, γ-ABAld, and NAD on the reaction velocity were determined. The K_m values for three substrates were calculated from the linear portion of the Lineweaver-Burk plots and found to be 3.4 μM (V_{max} 42 nmol/min·mg protein) for γ-GBAld, 25 μM (V_{max} 60 nmol/min·mg protein) for γ-ABAld, and 84 μM (case of γ-GBAld) for NAD.

Effect of Inhibitors. Table IV shows the effects of various substances on the enzyme activity. The sulfhydryl reagents such as *p*-chloromercuribenzoate and N-EM at a concentration of 0.1 mM did inhibit strongly the enzyme activity. The enzyme was

Table VI. *Protection and Reactivation by DTT of γ-GBAld Dehydrogenase Activity Against Inhibition by N-EM*

After 5 min preincubation of the enzyme with each of A and B, the reaction was initiated by the addition of C. Final concentration of each DTT and N-EM was 1 mM. Other conditions: standard assay method was used.

Order of Addition					Relative
A	→	B	→	C	Activity
					%
				γ-GBAld	100
DTT				γ-GBAld	120
N-EM				γ-GBAld	0
DTT		N-EM		γ-GBAld	111
N-EM		DTT		γ-GBAld	6
				γ-GBAld + N-EM[a]	24

[a] The mixture of γ-GBAld and *N*-ethylmaleimide.

also inhibited by several metal-binding reagents such as diethyldithiocarbamate, *o*-phenanthroline, zincon, and ferron. Among these reagents, the most strong inhibition was obtained by zincon. On the other hand, it has been confirmed that tetraethylthiuram disulfide (disulfiram) is competitive with NAD in the bovine liver NAD-linked aldehyde dehydrogenase (4). *Vicia* γ-GBAld dehydrogenase was inhibited with disulfiram and resulted in inhibition of 50% at 0.07 mM (in this case, 0.07 mM NAD was used). However, the value of the inhibition was not changed by concentrations greater than 0.07 mM of the reagent.

Effect of Sulfhydryl Compound and Sulfhydryl Reagents. The final preparation of the enzyme (Table I) was not stable: storage of enzyme for 1 and 13 d at 4°C resulted in about 25% and 90% loss of the activity, respectively. However, the inactivated enzyme was reactivated about 70% and 50% by adding DTT and 2-ME, respectively. Reduced glutathione and cysteine were less effective (Table V). When the enzyme was preincubated with N-EM, the inhibition was extensive; however, when preincubated with DTT before adding N-EM, it was protected. The N-EM-inhibited enzyme was reactivated partially by adding DTT (Table VI).

DISCUSSION

In metabolic pathways of arginine catabolism, the enzyme arginase that catalyzes formation of ornithine and urea from arginine has been commonly found in a number of higher plants (*e.g.* 13). However, α-keto-δ-guanidinovaleric acid has been identified in white spruce (6), and the feeding experiments with [^{14}C] arginine have been shown the formation of α-keto-δ-guanidinovaleric acid and γ-GBA in white spruce buds (5). These results suggest that the formation of γ-GBA from arginine could arise by transamination to form α-keto-δ-guanidinovaleric acid which could be oxidatively decarboxylated to form γ-GBA (13). We have described the enzymes which are concerned with the formation of agmatine and γ-GBAld in *Vicia* leaves (10–12), and γ-GBAld was identified by both thin-layer and paper chromatography as a product of agmatine oxidation by immobilized diamine oxidase (unpublished data). γ-GBAld dehydrogenase has been detected in *Pseudomonas putida* (15). Although γ-ABAld dehydrogenase has been found in *Pseudomonas fluorescens* (8) and *Pseudomonas* species (3), until now, these specific aldehyde dehydrogenases had not yet been found and characterized from higher plants. The purification obtained by the method described herein was 27-fold. The enzyme has very high substrate specificity, to oxidize only γ-GBAld and γ-ABAld. Glutaric semialdehyde, succinic semialdehyde, benzaldehyde, and other aliphatic aldehydes did not allow the formation of NADH in the standard assay system. With the Sephadex G-200 gel filtration method, the mol wt of the enzyme was found to be approximately 83,000. The K_m value was 3.4 μM for γ-GBAld and 25 μM for γ-ABAld. The *Vicia* enzyme is strongly inhibited by typical sulfhydryl reagents such as *p*-chloromercuribenzoate and N-EM. In addition, storage of the enzyme for about 2 weeks at 4°C resulted in 90% loss of the activity, but enzyme was reactivated 70% and 50% by adding DTT and 2-EM, respectively (Table V). These results suggest that *Vicia* dehydrogenase is a thiol enzyme. Present work also shows that the enzyme is sensitive to zincon and *o*-phenanthroline (Table IV). Therefore, it was postulated that the enzyme is a metalloprotein: however, it is not at present clear whether zinc is involved or not. The formation of aldehyde intermediates such as γ-GBAld from agmatine and γ-ABAld from putrescine by sequential reactions of arginine decarboxylation (10) and amine oxidation (11, 12) and then the NAD-dependent oxidation of these aldehydes to, respectively, guanidino- and amino-butyrates in *Vicia* plants make these pathways more efficient energetically in arginine catabolism.

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