Plant Cell Physiol. 38(3): 327–335 (1997)

An NADP-Glutamate Dehydrogenase from the Green Alga *Bryopsis maxima*. Purification and Properties

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NADP-glutamate dehydrogenase (EC 1.4.1.4; NADP-GDH) was purified to electrophoretic homogeneity from the multinuclear-unicellular green marine alga in Siphonales, *Bryopsis maxima*, and its properties were examined. M_r of the undenatured enzyme was 280 kDa, and the enzyme is thought to be a hexamer of 46 kDa subunit protein. Optimum pHs for the reductive amination and oxidative deamination were 7.5 and 8.2–9.0 respectively. The enzyme displayed NADPH/NADH-specific activities with a ratio of 18 : 1. Apparent K_m values for 2-oxoglutarate, ammonia, NADPH, glutamate and NADP$^+$ were 3.0, 2.2, 0.03, 3.2 and 0.01 mM respectively. The enzymochemical characteristics of the GDH were studied and compared to those of other species. The *B. maxima* GDH was insensitive to 5 mM Ca^{2+} and to 1 mM EDTA in contrast to higher plant NAD-GDHs. Chemical modifications with DTNB and *p*CMBS suggested that cysteine residues are essential for the enzymatic activity as in other species GDHs. The GDH was not affected by 1 mM purine nucleotides, suggesting that the enzyme is not allosteric, in contrast to animal NAD(P)-GDHs and fungal NAD-GDHs.

Key words: Ammonia assimilation — *Bryopsis maxima* — Glutamate dehydrogenase (EC 1.4.1.4) — GS-GOGAT cycle — NADP.

Inorganic nitrogen compounds which are particularly important as nutrients for plants are converted to ammonia prior to their ultimate incorporation into amino acids. There are several reactions in which ammonia participates. The glutamate dehydrogenases (GDH; NAD-GDH, EC 1.4.1.2, NAD(P)-GDH, EC 1.4.1.3 and NADP-GDH, EC 1.4.1.4) and the sequential reactions of glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; NADP-GOGAT, EC 1.4.1.13, NAD-GOGAT, EC 1.4.1.14 and Fd-GOGAT, EC 1.4.7.1), (GS-GOGAT cycle) have been studied in detail (Miflin and Lea 1977, Joy 1988). GDH catalyzes the reversible reductive amination of 2-oxoglutarate to form glutamate.

$$\text{2-oxoglutarate} + \text{NAD(P)H} + NH_4^+ + H^+ \leftrightarrows \text{L-glutamate} + \text{NAD(P)}^+ + H_2O$$

GS catalyzes the incorporation of ammonium into glutamate, forming glutamine which then reacts with 2-oxoglutarate to produce glutamate in a reaction catalyzed by GOGAT.

GDH is an ubiquitous enzyme, present in the three kingdoms of the eukaryotes, eubacteria and archaebacteria (Woese et al. 1990, Benachenhou and Baldacci 1991). GDH was considered as a key enzyme in ammonia assimilation, but since 1974 the major route of ammonia assimilation has generally been accepted to be the GS-GOGAT cycle in higher plants (Lea and Miflin 1974, Joy 1988) and also in certain algae (Cullimore and Smis 1981, Haxen and Lewis 1981, Davison and Stewart 1984). This is based on the following observations: (1) GS has a higher affinity for ammonia than GDH; K_m values for ammonia of GDH are much higher than that of GS (Miflin and Lea 1977). (2) Time course studies using ^{13}N- or ^{15}N-labeled NH_4^+ and NO_3^- (Yoneyama and Kumazawa 1974, Skokut et al. 1978, Rhodes et al. 1989, Thorpe et al. 1989, Stewart et al. 1995) show inorganic nitrogen to be incorporated first into the amido group of glutamine rather than glutamate in higher plants. (3) MSX (a potent inhibitor of GS) can completely prevent ammonia assimilation (Stewart and Rhodes 1976, Fentem et al. 1983, Rhodes et al. 1986). (4) Several higher plant mutants, which are deficient in chloroplast-localized GS and GOGAT, tend to be lethal under photorespiratory conditions and the GS-deficient mutants exhibit rapid accumulation of free ammonia under photorespiratory conditions (Wallsgrove et al. 1987, Givan et al. 1988, Joy 1988, Lea et al. 1989). GDH is thus considered to operate in the direction of the oxidative deamination of glutamate to provide 2-oxoglutarate for the TCA-cycle (Robinson et al. 1992, Moyano et al. 1995). This catabolic reaction of GDH is also assumed to be involved in the regulation of carbon and nitrogen metabolism under certain environmental or

Abbreviations: *p*CMBS, *p*-chloromercuribenzen sulfonate; DTNB, 5-5′-dithio-*bis*-2-nitrobenzoic acid; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; MSX, L-methionine-D,L-sulphoximine; MTT, 3-(4,5-dimethylthiozolyl-2)-2,5-diphenyltetrazolium bromide; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride.

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developmental conditions (Oaks and Hirel 1985, Srivastava and Singh 1987).

Other reports show that algal GDH may be essential for amination reactions. GDH enzymes from *Chlorella sorokiniana* (Tischner 1974, Bascomb and Schmidt 1987) and the siphonous green alga *Caulerpa simpliciuscula* (Gayler and Morgan 1976, McKenzie et al. 1979) show high affinities for ammonia. Further, *Chlorella autotrophica* (Ahmad and Hellebust 1984) and *Chlamydomonas reinhardtii* (Muñoz-Blanco et al. 1989) can grow in the presence of MSX. The role of GDH in ammonia assimilation in algal cells is still controversial and it may be that both GDH and GS-GOGAT cycle can support ammonia assimilation.

The uncertainty may be due to environmental or developmental conditions differentially inducing these nitrogen assimilating enzymes (Bascomb and Schmidt 1987). There may be species specificity for these enzymes suggesting that various types of algae, such as macro algae, should be studied in greater detail. GDH activity of the marine macro alga, *B. maxima*, was reported using 25–75% ammonium sulfate fraction of the cell extract (Nishizawa et al. 1978). In the present paper, *B. maxima* GDH was purified and its physicochemical and enzymological properties studied for comparison with those of other species GDHs to elucidate the physiological role of GDH in this alga.

Materials and Methods

Reagents—NADPH, NADH and $NADP^+$ were purchased from Oriental Yeast Co., LTD. (Tokyo, Japan). PMSF and *p*CMBS were obtained from Sigma Chemical Co. (MO, U.S.A.). DE-52 and DEAE-Toyopearl 650S were products of Whatman International Ltd. (Maidstone, England) and Tosoh (Tokyo, Japan) respectively. Red Sepharose CL-6B and Superose 6 were procured from Pharmacia (Upssala, Sweden). All reagents were of analytical grade. Miracloth was purchased from Calbiochem-Novabiochem Corporation (CA, U.S.A.).

Plant materials—Algal thalli of *B. maxima* were collected at Kimigahama in Chiba prefecture on the Pacific coast of Japan. They were immediately brought back to laboratory to use for extraction of enzyme.

Assay procedures—(1) GDH assay: GDH activity was assayed with slight modification of the method of Israel et al. (1977). Aminating and deaminating enzyme activity was determined based on the absorption change at 340 nm according to NADPH (or NADH) oxidation or $NADP^+$ reduction. Assay mixture for amination reaction contained 150 mM Tris-HCl (pH 7.5), 5.0 mM 2-oxoglutarate, 0.06 mM NADPH (or NADH) and 50 mM $(NH_4)_2SO_4$. Assay mixture for deamination reaction contained 150 mM Tris-HCl (pH 8.6), 50 mM L-glutamate and 0.1 mM $NADP^+$. These reactions were initiated by the addition of the 0.1 ml enzyme solution to the 1.0 ml assay mixture at 25°C. The reaction rates were calculated using an extinction coefficient of $6.22 \times 103\ M^{-1}\ cm^{-1}$ at 340 nm. One unit of enzymatic activity was defined as that which oxidized 1 μmol NADPH (or NADH) or reduced 1 μmol of $NADP^+$ per min. Phosphatase activity was not detected in the purified GDH fraction with this assay method.

(2) GS assay: GS activity was measured by synthetic assay as described by Canovas et al. (1984). The assay mixture (0.65 ml) contained 100 mM Tris-HCl (pH 7.5), 70 mM HCl-hydroxylamine (neutralized just before use), 200 mM sodium glutamate, 7 mM ATP and 50 mM $MgSO_4$. Following incubation with 0.1 ml enzyme solution for 60 min at 37°C, the mixture was treated with 0.25 ml of a (1 : 1 : 1) mixture of 10% $FeCl_3 \cdot 6H_2O$ (in 0.2 M HCl), 24% trichloroacetic acid and 50% HCl. Absorbance of the supernatant was measured at 540 nm, after centrifugation for 5 min at $10,000 \times g$. One unit of enzymatic activity was defined as the formation of 1 μmol of γ-glutamyl hydroxamate per min.

Protein concentration—Protein concentration was measured colorimetrically according to the method of Smith et al. (1985) using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis—(1) SDS-PAGE and peptide staining: SDS-PAGE was performed by the method of Laemmli (1970). The gel consisted of 5% polyacrylamide stacking gel and 12% polyacrylamide separation gel. The reservoir buffer used was 25 mM Tris-HCl and 192 mM glycine containing 0.1% SDS. Samples were incubated in 2% SDS and 5% mercaptoethanol for 30 min at room temperature. The electrophoresis was performed at 4°C. Initial running was performed at a current of 15 mA. The running current was raised to 30 mA after the samples had entered the separation gel.

Peptides in SDS-PAGE gel were detected by silver staining (Morrissey 1981). The gel was prefixed in 50% methanol and 10% acetic acid for 30 min, followed by 5% methanol and 7% acetic acid for 30 min. The gel was fixed further in 10% glutaraldehyde for 30 min and then washed for 30 min with several changes of distilled water and soaked in 5 $\mu g\ ml^{-1}$ dithiothreitol (DTT) for 30 min. The DTT solution was subsequently discarded and replaced with 0.1% silver nitrate for 30 min. The gel was rinsed rapidly with distilled water and then rinsed rapidly twice with the developer solution (1.0 M sodium carbonate decahydrate and 0.3 M formaldehyde). The gel was kept in the solution until the desired level of staining was attained. Staining was terminated by the addition of a sufficient 2.3 M citric acid solution to bring the developer solution to neutral pH. The gel was rinsed with distilled water several times. A photograph was taken immediately.

(2) Non-denaturing gel electrophoresis (native PAGE): Native PAGE was conducted on the crude extract and the purified enzyme by a modification of Laemmli (1970). The gel consisted of stacking gel in 3% acrylamide and separation gel in 6.25% acrylamide in the absence of SDS. The buffer used was 25 mM Tris-HCl and 192 mM glycine. Electrophoresis, performed at 4°C, was started at 15 mA and continued until the samples entered the stacking gel. The intensity of current then raised to 30 mA. On completion of electrophoresis, NADP-GDH activity was detected by staining with the tetrazolium (MTT) according to Cammaerts and Jacobs (1983) with modification. The gel was incubated in a staining solution of 50 ml containing 150 mM Tris-HCl (pH 8.6), 0.1 mM MTT, 0.1 mM phenazine methosulfate, 65 mM L-glutamate and 0.4 mM $NADP^+$ in a reciprocal shaking water bath at 25°C until sufficient color development. The reaction was terminated by rinsing the gel with distilled water. The gel was photographed immediately.

Purification of NADP-GDH—Step 1: Algal thalli (600 g) were washed with filtered sea water to remove macroscopic contaminants, chopped into small (about 5 mm) segments and squeezed through four layers of gauze. The extract from thalli was poured into 120 ml of 200 mM Tris-HCl (pH 8.0) at 4°C. The suspension was passed through one layer of Miracloth to remove small thallus fragments and used as a starting material for further

purification. The following purification procedures were all carried out at 0 to 4°C.

Step 2: Final concentrations of 0.01% Triton X-100, 0.1 mM PMSF and 4 mM 6-aminohexanoic acid were added to the starting material, homogenized (LSC homogenizer LH-21, Yamato, Japan) and centrifuged at 9,000×*g* for 20 min.

Step 3: DE-52 column chromatography: The supernatant obtained in Step 2 was readjusted to pH 8.0 by 0.1 M NaOH solution and applied onto a column of DE-52 (6.5×20 cm) equilibrated with 50 mM Tris-HCl (pH 8.0). Elution was conducted with a linear gradient of 0 to 0.8 M NaCl in the same buffer.

Step 4: DEAE-Toyopearl 650S column chromatography: Fractions with GDH activity in Step 3 were dialyzed against 50 mM Tris-HCl (pH 8.0). The dialyzate was applied onto a column of DEAE-Toyopearl 650S (3.5×13.5 cm) that had been equilibrated with 50 mM Tris-HCl (pH 8.0). Elution was carried out with a linear gradient of 0 to 0.6 M NaCl in the same buffer.

Step 5: Superose 6 column chromatography: Fractions with GDH activity obtained in Step 4 were combined and concentrated in a dialysis tube covered with PEG 20000. The concentrated sample was applied onto a column of Superose 6 (1.6×48.5 cm, FPLC system; Pharmacia) previously equilibrated with 50 mM Tris-HCl (pH 7.5) containing 4 mM 6-amino-*n*-caproic acid. The elution was carried out with the same buffer.

Step 6: Red Sepharose CL-6B column chromatography: Fractions with GDH activity obtained in Step 5 were combined and applied to a Red Sepharose CL-6B substrate-affinity column (1.8×15.0 cm) that had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 4 mM 6-amino-*n*-caproic acid. The column was washed with 100 ml of the same buffer. The enzyme was eluted with 100 ml of the same buffer with 1 mM NADPH. The enzyme fractions obtained were dialyzed against 50 mM Tris-HCl (pH 7.5) to remove NADPH. This purified enzyme preparation was used for enzymochemical studies.

The enzyme was further purified by the native PAGE described previously using 50 mM Tris-HCl and 50 mM Tricine buffer instead of 25 mM Tris-HCl and 192 mM glycine according to Ploug et al. (1989) to remove trace amounts of possible contaminating proteins and to ensure maximal purity for amino acid composition and N-terminal amino acid analyses. $NADP^+$-GDH activity was detected colorimetrically at the completion of electrophoresis and the region corresponding to the enzymatic activity was excised. The purified NADP-GDH protein was eluted by electrophoresis from the gel using the 50 mM Tris-HCl, 50 mM Tricine and 0.1% SDS buffer at 100 V for 4 h at 4°C. Eluted solution was dialyzed against deionized water using dialysis membrane and concentrated with PEG 20000.

Determinations of the molecular mass and subunit—M_r of the native enzyme was estimated by gel filtration through a column of Superose 6 equilibrated with 50 mM Tris-HCl (pH 7.5) and eluted with the same buffer. M_r of the denatured protein was estimated as follows. After native PAGE, $NADP^+$-GDH activity was detected by the MTT method and the active band was excised and incubated in 70 mM Tris-HCl (pH 8.8) containing 3% SDS, 6% mercaptoethanol and 1.7 M sucrose for 30 min at room temperature and kept overnight at −20°C. The gel was loaded directly onto the SDS-PAGE and M_r of the subunit was estimated.

Amino acid analysis—The enzyme was hydrolyzed in 6 M HCl under an N_2 atmosphere for 24 h and dried in vacua. Amino acid analysis was performed using the method of Bidlingmeyer et al. (1989) with a Pico-Tag™ amino acid analysis system (Waters Corp., MA, U.S.A.). Phenylthiocarbamoyl amino acids were separated by reversed phase HPLC with a Pico-Tag™ column (0.4×150 mm) equilibrated with the solvent [0.14 M sodium acetate buffer (pH 6.4) : acetonitril=940 : 60, v/v] and eluted with a concave gradient between the solvent and 60% acetonitrile.

N-terminal amino acid analysis—The enzyme purified by native PAGE was blotted onto a polyvinylidene difluoride (PVDF) membrane using ProSpin™ Sample Preparation Cartridge (Applied Biosystems, CA, U.S.A.). N-terminal amino acid sequence was determined with a gas-phase sequencer (model 491A/120A; Applied Biosystems) according to Hewick et al. (1981).

Chemical modifications (inactivation by thiol-modifying reagents)—Modifications of cysteine residues were made essentially according to Syed et al. (1994). The enzyme solutions were incubated separately with either 0.1 mM *p*CMBS or 2 mM DTNB in 150 mM Tris-HCl (pH 7.5) at 25°C for 3.5 min. Samples were periodically withdrawn and their NADPH-GDH activities were assayed.

Results and Discussion

Purification of NADP-GDH—Purification procedure is summarized in Table 1. *B. maxima* NADP-GDH was purified about 584-fold, in recovery of ca. 7.1% from the crude extract. The purified NADP-GDH gave a single band both on SDS-PAGE (M_r 46 kDa) and on native PAGE (Fig. 1).

Properties of the NADP-GDH protein

Molecular mass and subunit—The M_r of the native enzyme determined by Superose 6 gel filtration was 280 kDa, implying that the native enzyme consists of identical six sub-

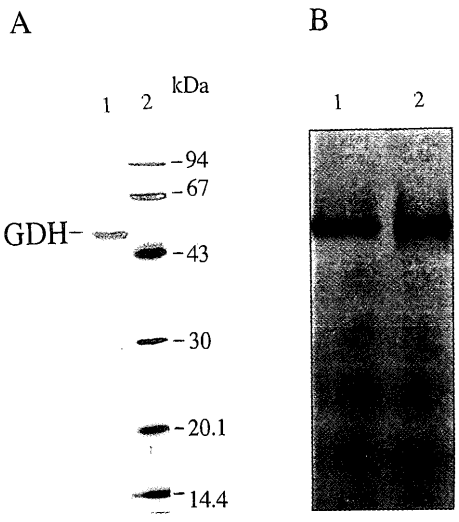

Fig. 1 (A) SDS-PAGE of purified *B. maxima* NADP-GDH. Lane 1, NADP-GDH; lane 2, the molecular mass of marker proteins. The gel was stained with silver. The molecular mass marker proteins are phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). (B) Native PAGE of crude extract and purified NADP-GDH. Lane 1, crude extract; lane 2, purified NADP-GDH. The enzyme was visualized by activity staining.

Table 1 Purification of NADP-GDH from *B. maxima*

Step	Total activity (units)	Total protein (mg)	Specific activity (units mg^{-1})	Purification (-fold)	Recovery (%)
1. Crude extract	28.2	900	0.0313	1	100
2. DE-52	22.3	142	0.157	5.02	79.1
3. DEAE-Toyopearl	18.2	10.8	1.69	53.8	64.5
4. Superose 6	8.76	3.21	2.73	87.2	31.1
5. Red Sepharose CL-6B	2.01	0.11	18.3	584	7.1

units of M_r 46 kDa.

GDH isozymes have been separated by native PAGE in higher plants (Cammaerts and Jacobs 1983, Loulakakis and Roubelakis-Angelakis 1992, Schlee et al. 1994, Sakakibara et al. 1995) and several algae (Bascomb and Schmidt 1987, Muñoz-Blanco et al. 1989). However, only one band was observed on the native PAGE gel stained for $NADP^+$-GDH activity in *B. maxima* (Fig. 1B). *B. maxima* NADPH-GDH activity was observed in a single peak on DEAE-Toyopearl 650S column chromatography (data not shown) in contrast to the report of Moyano et al. (1992) that the elution pattern of *Chlamydomonas reinhardtii* NAD(P)-GDH from an anion-exchange (DEAE-Sephacel) column showed three peaks. Isozymes could not be confirmed both on native PAGE and by ion-exchange column chromatography in freshly harvested material under the current experi-

Table 2 Amino acid composition of *B. maxima* NADP-GDH in comparison of the compositons of other species hexamerous GDHs

Amino acid	Residues/subunit						
	B. maxima		*Chlorella sorokiniana*[b]	*Eschericha coli*[c]	*Sulfolbos solfataricus*[d]	*Neurospora crassa*[e]	*Bovine liver*[f]
	Experimental value	Nearest integer[a]					
Asp	24.6	25	53	41	42.8	41	50
Glu	33.6	34	55	54	38.3	55	45
Ser	40.9	41	30	29	13.4	29	30
Gly	82.1	82	54	51	53.0	56	47
His	8.1	8	6	9	2.9	9	14
Arg	18.5	19	28	18	17.9	16	30
Thr	20.2	20	21	22	17.1	14	28
Ala	33.4	33	50	45	41.2	54	37
Pro	30.1	30	23	16	10.1	15	21
Thr	15.8	16	24	12	11.4	15	18
Val	24.7	25	38	34	33.4	35	34
Met	8.9	9	15	10	13.2	9	13
1/2 Cys	7.1	7	6	6	3.8	6	6
Ile	20.2	20	23	15	25.7	17	38
Leu	21.9	22	39	31	34.6	40	31
Phe	22.0	22	15	15	8.9	16	23
Lys	34.1	34	38	22	38.1	30	33
Trp	n.d.	n.d.	5	4	3.6	7	3

[a] Calculated assuming Phe residues as 22.0. The amount of protein used for analysis was 0.3 μg.
[b] Gronostajski et al. (1978)
[c] Veronese et al. (1975)
[d] Schinkinger et al. (1991)
[e] Blumenthal and Smith (1973)
[f] Julliard and Smith (1979)
n.d. not determined.

mental conditions.

Amino acid composition—The amino acid composition of the NADP-GDH is shown in Table 2 together with the compositions of the hexamerous enzymes from *Chlorella sorokiniana* NADP-GDH (Gronostajski et al. 1978), *Escherichia coli* NADP-GDH (Veronese et al. 1975), *Sulfodobus solfataricus* NAD(P)-GDH (Schinkinger et al. 1991), *Neurospora crassa* NADP-GDH (Blumenthal and Smith 1973), and bovine liver NAD(P)-GDH (Julliard and Smith 1979). The numbers of Asp and Glu residues in *B. maxima* GDH are less than those in other species, whereas the number of Gly residues in *B. maxima* GDH is more than that in other species.

N-terminal amino acid sequence—The amino acid sequence of the N-terminal region was determined up to the 20 residues by Edman degradation.

NH_2-Ala-Asp-Val-Gly-Thr-Val-Thr-Val-Arg-Gly-Leu-Gly-Asp-Arg-X-X-X-Pro-Val-Lys- (X; not determined)

Searches for homologies were performed using the program BLAST with the libraries NBRF-PIR, GenBank CDS Translation, PBD Protein sequence, and SWISS-PROT. No homologous GDH was found. This result is accordance with the observation of Baker et al. (1992) that the similarity between hexameric GDHs is low for the N-terminal 50 residues.

Table 3 Properties of *B. maxima* NADP-GDH

M_r (kDa) (native)	280
M_r (kDa) (subunit)	46
Number of subunits	6
pH Optimum for amination	7.5
pH Optimum for deamination	8.6
pH Stability[a]	5.2–8.0
Heat Stability[b] (°C)	≤45
K_m (mM) ammonia	2.2
K_m (mM) NADPH	0.03
K_m (mM) 2-oxoglutarate	3.0
K_m (mM) $NADP^+$	0.01
K_m (mM) glutamate	3.2
K_m (mM) of GS for ammonia	0.31

[a] The enzyme was incubated at various pH for 1 h at 25°C. Aliquots were withdrawn, adjusted to pH 7.5, and assayed for NADPH-GDH activities. Buffer used: 0.15 M glycine-HCl (pH 1.3–3.2), 0.15 M acetic acid-NaOH (pH 4.2–6.6), 0.15 M Tris-HCl (pH 7.2–8.6), 0.15 M 3-cyclohexylaminopropanesulfonic acid (CAPS-HCl, pH 9.6–10.6) and 0.15 M KCl-NaOH (pH 12.0).
[b] The enzyme was incubated at various temperatures in 0.1 M Tris-HCl (pH 7.5). Aliquots were withdrawn, quickly chilled in ice water, and NADPH-GDH activities assayed at pH 25°C. The remaining activity is expressed relative to the initial rate.

Coenzyme specificity—The rate of amination with NADPH was 18 times higher than that with NADH at optimal pH of 7.5.

pH-optima of amination and deamination—Properties of NADP-GDH are summarized in Table 3. The pH optima for amination and deamination were determined. The highest amination activity was taken as 100%. Optimum pH of the amination was 7.5, whereas the deamination showed a broad peak at pH 8.6. Deamination activity was approximately 40% of the amination rate. These properties are similar to other plant NAD-GDHs (Yamaya et al. 1984, Itagaki et al. 1988).

Stability—The pH stability of NADP-GDH was examined. The enzyme was preincubated in buffers at various pH for 1 h at 25°C, but the NADPH-GDH activity was assayed at pH 7.5. The enzyme was stable between pH 5.2 and 8.0, but a sharp decrease of the activity was noted below pH 4.0 and above pH 10.0.

The heat-stability of NADP-GDH was examined. The enzyme was incubated at various temperatures. NADPH-GDH activity was assayed at 25°C. The activity of the enzyme was stable for 60 min incubation up to 45°C. It decreased to 50% within 10 min at 50°C and quickly diminished to 10% within 5 min at 55°C.

Effect of divalent cations and EDTA on the activity—Higher plant NAD-GDHs are thought to be metalloenzymes and affected by divalent metal cations (Yoneyama et al. 1974, Kindt et al. 1980, Yamaya et al. 1984, Srivastava and Singh 1987, Das et al. 1989, Itagaki et al. 1990). In green algae, the NAD-specific GDH activities of the three dual coenzyme-specific GDH isozymes from *Chlamydomonas reinhardtii* were stimulated by 1 mM Ca^{2+}, and was inhibited or unaffected by several other divalent cations

Table 4 Effect of divalent cations and EDTA on *B. maxima* NADPH-GDH activity

Addition (1 mM)	Activity (%)
None	100
Ca^{2+}[a]	100
Cd^{2+}	98
Mg^{2+}	84
Mn^{2+}	81
Zn^{2+}	36
Hg^{2+}	0
EDTA	100

[a] 5 mM.
Enzyme was added to the reaction mixture to a final concentration of 6.0 munits ml^{-1} and the mixture was incubated for 10 min at 25°C, pH 7.5 before the reaction was initiated. The activity without additions was taken as 100%. Redistilled water further purified by Milli-Q Jr. (Millpore, Japan) was used throughout the experiments.

(Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ba^{2+}) and strongly inhibited by chelating agents including EDTA (Moyano et al. 1992). On the other hand, both NAD-specific and NADP-specific GDHs from *Stichococcus bacillaris* naeg. (eubacteria) were unaffected by Ca^{2+} or EDTA (Ahmad and Hellebust 1986).

The effect of divalent cations on *B. maxima* NADP-GDH were examined (Table 4). No significant effects were seen by the addition of Ca^{2+}, Mn^{2+}, Mg^{2+} and EDTA. Either species specificity or difference coenzyme dependency may explain these findings. The activity was inhibited completely by Hg^{2+} and strongly by Zn^{2+}. Additional study on the various plant enzymes needs to be conducted to clarify the mechanism of action of these metals.

Effect of inhibitors and purine nucleotides on the activity—Table 5 summarizes the effects of inhibitors and purine nucleotides on the activity of *B. maxima* NADP-GDH.

These inhibitors were found to affect NAD-GDHs from *Lemna minor* (Ehmke and Hartmann 1976), *Lupinus luteus* (Stone and Copeland 1982) and *Chlamydomonas reinhardtii* (Moyano et al. 1992). *B. maxima* GDH was essentially not affected by sodium oxalate, pyridoxal 5′-phosphate, nitrilotriacetic acid and *o*-phenanthroline but was slightly inhibited by sodium citrate.

NAD-GDH in *Lupinus* nodules was shown to have allosteric sites (Pahlich and Gerlitz 1980, Stone and Copeland 1982). Animal NAD(P)-GDHs and fungal NAD-GDHs may possibly be allosteric enzymes since they are modulated by small molecular weight ligands, such as purine nucleotides (LéJohn 1971, Van Laere 1988, Cho et al. 1995). The aminating activity of *B. maxima* NADP-GDH was not affected by 1.0 mM AMP, ADP, ATP or GTP with or without 1.0 mM Ca^{2+}. These results indicate that the allosteric regulation by purine nucleotides does not occur in the *B. maxima* NADP-GDH in contrast to higher plant, fungal and animal GDHs.

Chemical modifications of NADP-GDH—GDHs from plants (Yamasaki and Suzuki 1969, Chou and Splittstoesser 1972, Errel et al. 1973, Fawole and Boulter 1977, Puranik and Srivastava 1986, Moyano et al. 1992), animal (Surendra and Colman 1986) and fungi (Syed et al. 1994) have been modulated by -SH binding reagents. The effect of DTNB and *p*CMBS on *B. maxima* NADP-GDH were examined to determine the possible involvement of free -SHs at the active sites of the enzyme (Fig. 2). NADPH-GDH activity was completely inhibited by 2 mM DTNB in 1 min and by 0.1 mM *p*CMBS in 3.5 min. The inhibition by DTNB was completely abolished by adding 2 mM 2-mercaptoethanol. These results and the strong inhibition by Hg^{2+} (Table 4) suggest that *B. maxima* GDH contains free -SH group(s) at the active center as in the case of other species GDHs.

Examinations of GDHs from plants (Stone et al. 1979), animals (Chen and Engel 1974) and fungi (Lilley and Engel 1992) clearly have indicated that one or more lysyl residues are involved in the active sites. An attempt was made to modify the lysine residues in *B. maxima* NADP-GDH with pyridoxal 5′-phosphate (Chen and Engel 1974). Enzyme solution was incubated with 0.1 mM pyridoxal 5′-phosphate in 150 mM Tris-HCl (pH 7.5) at 25°C. Samples were periodically withdrawn and their NADPH-GDH activities were assayed. Pyridoxal 5′-phosphate did not affect *B. maxima* NADPH-GDH activity at the concentration of 0.1 mM for 30 min at 25°C (data not shown) or at 1 mM (Table 5). The *B. maxima* GDH would thus appear to be

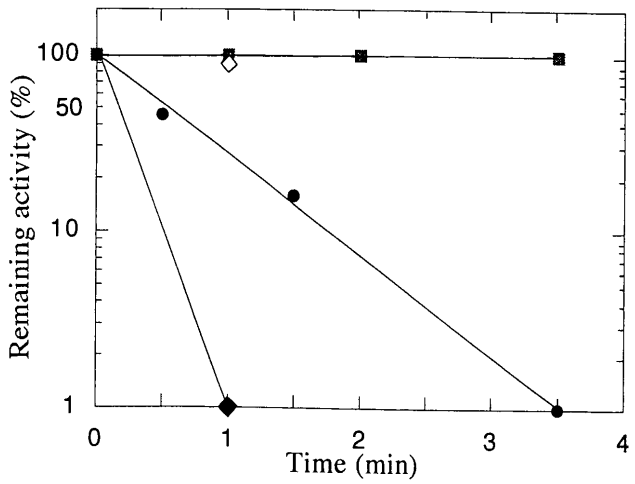

Fig. 2 Time courses of inactivation of *B. maxima* NADP-GDH by DTNB and *p*CMBS. The enzyme was treated with thiol-modifying reagent (DTNB or *p*CMBS) in 136 mM Tris-HCl (pH 7.5) at 25°C. Aliquots were periodically withdrawn. NADPH activities were assayed. –■–, no addition of the thiol-modifying reagent; –◆–, 2 mM DTNB; –●–, 0.1 mM *p*CMBS; –◇–, Addition of 2 mM 2-mercaptoethanol after 1 min incubation with 2 mM DTNB. Pseudo-first-order plots of decline in NADPH-GDH activity are shown.

Table 5 Effect of inhibitors and nucleotides on *B. maxima* NADPH-GDH activity

Inhibitor (1 mM)	Activity (%)	Nucleotide (1 mM)	Activity (%)
None	100	None	100
Sodium citrate	89	AMP	93
Sodium oxalate	89	ADP	98
Nitrilotriacetic acid	93	ATP	98
Pyridoxal 5′-phosphate	100	GTP	80
o-Phenanthroline	102		

Enzyme was added to the reaction mixture just before the reaction to a final concentration of 6.0 units ml^{-1} and the mixture was incubated for 5 min at 25°C, pH 7.5. The activity without additions was taken as 100%.

dissimilar to higher plant, animal and fungal GDHs with respect to the active sites.

Affinities toward coenzymes and substrates—Apparent K_m values for coenzymes and substrates were determined (Table 3). Coenzyme affinities for both amination and deamination reactions were almost identical (0.03 and 0.01 mM respectively), as were substrate affinities for both reactions (3.0 and 3.2 mM respectively). These results suggest that *B. maxima* NADP-GDH catalyzes both reactions.

K_m values of GDH for ammonia (as ammonium sulphate) and GS for ammonia were not significantly different (2.2 and 0.31 mM respectively). The incubation of *B. maxima* cells for 22 h with 5 mM MSX (a strong inhibitor of GS) failed to cause GDH activity to increase (data not shown) in contrast to that noted by Muñoz-Blanco et al. (1989) in *Chlamydomonas reinhardtii*.

Various characteristics of *B. maxima* NADP-GDH have been clarified in this paper. Its kinetic properties are similar to those reported by Nishizawa et al. (1978). *B. maxima* GDH is an -SH enzyme and not inhibited by EDTA nor activated by either Ca^{2+} or purine nucleotides. These results are different from NAD-GDH activities of NAD(P)-GDH isozymes of *Chlamydomonas reinhardtii* which are inhibited by pyridoxal 5′-phosphate and EDTA and stimulated by Ca^{2+} (Moyano et al. 1992). Higher plant NAD-GDHs are considered to be -SH dependent, and metalloenzymes, and fungal and animal GDHs are considered to be -SH and allosteric enzymes (Hudson and Daniel 1993). Eubacteria *Stichoccus bacillaris* naeg. (Ahnad and Hallubust 1986) has both the NAD-GDH and NADP-GDH activities which are insensitive to Ca^{2+} and EDTA. These differences are probably result from an evolutionary response to their different growing environments.

The localization of GDH and GS in *B. maxima* cells is presently being studied. GDH appears in chloroplasts, mitochondria and cytosol, whereas GS localized in chloroplasts and cytosol. The relative activity of NADP-GDH is four times larger than that of GS in chloroplasts. Thus there is a possibility that GDH catalyzes the amination reaction in this alga as well as GS. Detailed localization of GDH and its fractionation under various incubation conditions is now under investigation.

We thank Dr. N. Inokuchi of Department of Microbiology, College of Pharmacy, Nihon University, for carrying out the amino acid analysis. This research was supported in part by a Grant-in-Aid for Scientific Research (No. 07640871) from the Ministry of Education, Science and Culture, Japan.

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(Received August 12, 1996; Accepted January 7, 1997)