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An NAD-specific glutamate dehydrogenase from cyanobacteria

Identification and properties

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The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 presents a hexameric NAD-specific glutamate dehydrogenase with a molecular mass of 295 kDa. The enzyme differs from the NADP-glutamate dehydrogenase found in the same strain and is coded by a different gene. NAD-glutamate dehydrogenase shows a high coenzyme specificity, catalyzes preferentially glutamate formation and presents K_m values for ammonium, NADH and 2-oxoglutarate of 4.5 mM, 50 μM and 1.8 mM respectively. An animating role for the enzyme is discussed.

NAD-glutamate dehydrogenase; Ammonium assimilation; Cyanobacterium; (*Synechocystis* sp. PCC 6803)

1. INTRODUCTION

Glutamate dehydrogenases (EC 1.4.1.2–4) catalyze either the reductive amination of 2-oxoglutarate to glutamate using reduced pyridine nucleotides as electron donors, or the oxidative deamination of glutamate through the reverse reaction. NADP-dependent glutamate dehydrogenases (NADP-GDH) have been demonstrated to play a role in nitrogen assimilation, especially in yeast and other fungi, as well as in some prokaryotes [1,2], while NAD-dependent enzymes (NAD-GDH) have been proposed to play a catabolic function in eukaryotes [1–3]. Higher eucaryotic enzymes with coenzyme ambiguity (NAD(P)-GDH) show complex regulatory and functional behaviours [1,2]. So far very little is known about NAD-GDH in prokaryotes. In cyanobacteria its occurrence has been cited [4] and its association with thylakoid membranes has been postulated [5], but nothing is known about its kinetic, structural and regulatory properties, information which is needed to propose a metabolic function. In this article we report for the first time the partial purification and detailed characterization of a cyanobacterial NAD-GDH, from the unicellular species *Synechocystis* sp. PCC 6803, suggesting an aminating role for it.

Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; PMS, phenazine methosulphate; PMSF, phenylmethylsulfonyl fluoride.

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2. MATERIALS AND METHODS

2.1. *Strains and growth of cells*

Synechocystis sp. PCC 6803 and the strain SCh1 were grown at 35°C on BG11 medium as in [6]. Low CO_2 means cultures grown under air in an illuminated orbital shaker ±2% glucose. High CO_2 corresponds to cultures bubbled with 5% CO_2 in air. Ammonia means replacing $NaNO_3$ by 15 mM NH_4Cl buffered with 30 mM TES-NaOH (pH 7.5). SCh1 is a mutant strain derived from *Synechocystis* sp. PCC 6803 by targeting inactivation of the *gdhA* gene, whose detailed characterization will be given elsewhere (Chávez, S., Florencio, F.J. and Candau, P., unpublished).

2.2. *Enzyme assays and protein determination*

NAD-dependent glutamate dehydrogenase aminating activity was determined at 30°C in a reaction mixture containing 85 mM Tris-HCl buffer (pH 8.5), 5 mM 2-oxoglutarate, 50 mM NH_4Cl and 0.2 mM NADH. The reaction was started by the addition of ammonium chloride and followed spectrophotometrically at 340 nm. Control experiments were made to correct for ammonium-independent oxidation of NADH. Deaminating activity was followed spectrophotometrically at 340 nm in a reaction mixture containing 85 mM Tris-HCl buffer (pH 9.5), 50 mM L-glutamate and 2 mM NAD^+. The reaction was started by the addition of L-glutamate and carried out at 30°C. NADP-dependent glutamate dehydrogenase aminating and deaminating activities were assayed as previously described [7], except that 10 mM $CaCl_2$ was included in the reaction mixture. Cell-free extracts for assays were obtained as in [6] by freezing the cells in liquid air and crushing them in a mortar in 50 mM HEPES-NaOH buffer (pH 7.0), containing 10 mM $CaCl_2$ (hereafter referred to as standard buffer) supplemented with 1 mM PMSF, followed by centrifugation at 10 000 × *g*, 5 min, to eliminate cell debris. Protein was determined as in [8] using ovalbumin as standard.

2.3. *Purification of enzymes*

NADP-GDH was purified to electrophoretic homogeneity as in [7]. NAD-GDH purification was carried out as follows: cell-free extracts were obtained by sonic disruption of high CO_2 ammonia-grown cells followed by centrifugation for 15 min at 9000 × *g*. Ammonium sulphate was added with stirring to the supernatant up to 35% saturation. After 10 min, the protein solution was centrifuged for 15 min at

10 000 × g and the pellet was discarded. The supernatant was dialyzed twice against 5 l of standard buffer. After centrifugation for 15 min at 10 000 × g the supernatant was adsorbed onto a DE-52 DEAE-cellulose column (2.5 × 25 cm) equilibrated in standard buffer. After washing the bed with 3 column volumes of standard buffer, the enzyme was eluted with a 0.05–0.5 M KCl linear gradient (300 ml) in standard buffer. Fractions with high NAD-GDH activity, eluting at 0.15 to 0.20 M KCl, were combined, diluted 4-fold with standard buffer and applied to a Reactive Red-120 agarose column (2.5 × 10 cm) equilibrated in standard buffer. The column was washed with 3 bed-volumes of buffer and the enzyme eluted by using a 0 to 0.35 M KCl gradient (150 ml) in standard buffer. The active fractions were collected, diluted 3-fold and applied to another Reactive Red-120 agarose column (1 × 8 cm) equilibrated in standard buffer. The column was washed with 3 bed-volumes of buffer and the enzyme eluted with a 0 to 0.35 M KCl gradient (25 ml) in standard buffer. Fractions with high activity were pooled, concentrated by using a Centricon 30 (Amicon, USA) and applied to a Sephacryl S-300 column (1.6 × 40 cm) equilibrated in standard buffer plus 0.15 M NaCl. Active fractions were collected, concentrated as above, analyzed by SDS-PAGE and then pooled.

2.4. *Other techniques*

Discontinuous SDS-PAGE was carried out as in [9], using 12% acrylamide slab gels. Native electrophoresis was performed in 6.25% acrylamide gels. Gels were stained for NAD- and NADP-GDH deaminating activities by immersion in the corresponding reaction mixture containing 0.5 mg/ml MTT and 0.1 mg/ml PMS.

The molecular mass of the native protein and the Stokes radius were determined by gel filtration using the standards cited in [7].

The isoelectric point was determined by column chromatofocusing in the pH range of 7.5–4.5, carried out in a Polybuffer Exchanger 94 column (1 × 20 cm) according to the instructions of the manufacturer (Pharmacia Fine Chemicals, Sweden).

3. RESULTS AND DISCUSSION

In *Synechocystis*, NADP-dependent glutamate dehydrogenase activity has been proposed to be an alternative route for ammonia incorporation into carbon skeletons [7]. In fact, in ammonium growth conditions, GS activity is only about 10% of the control value in nitrate containing medium, and NADP-GDH shows activity levels similar to those of GOGAT. NADP-GDH from *Synechocystis* was purified and characterized, and showed a great substrate specificity, being NAD unable to sustain any significant activity of the purified enzyme [7]. However, a low but significant NAD-dependent glutamate dehydrogenase can be detected in crude extracts of *Synechocystis* (Table I). In order to characterize this activity, we proceeded to purify it. The results from a typical purification are summarized in Table I. The final preparation showed a specific activity of 5.9 U/mg protein, after being increased more than 400-fold. It is worth noting that NAD and NADP specific GDHs eluted in different peaks in ion-exchange chromatography. In spite of being a partially pure preparation, molecular weight determination in SDS-PAGE was possible comparing electrophoretic patterns of different fractions eluted from the S-300 gel filtration column; only a clear single band of 48.5 kDa correlated to NAD-GDH activity. Gel filtration data gave a molecular mass of 295 ± 5 kDa for the native enzyme and, therefore, the enzyme appeared to be composed of 6 identical subunits. As far as we know this is the first time that subunit number is determined for a cyanobacterial NAD-GDH and coincides with the only other determined in prokaryotes [10].

The purified enzyme was able to catalyze the reaction in either direction, although at different rates. The enzyme showed a very strict substrate specificity. No reaction took place in the absence of NADH, 2-oxoglutarate or ammonium, in the aminating direction, or without glutamate or NAD^+ in the reverse reaction. Neither oxalacetate nor pyruvate were capable of substituting for 2-oxoglutarate, and neither aspartic acid nor alanine sustained the reaction in the absence of glutamate. NADPH could substitute for NADH as electron donor for the reaction, although at a much lower rate (NADH/NADPH activity ratio = 42). Again, in the deaminating direction, $NADP^+$ did not sustain the activity ($NAD^+/NADP^+$ activity ratio = 45). Under our assay conditions, the ratio between aminating and deaminating activities using NAD(H) ranged from 17.0 at pH 7.5, to 1.3 at the less physiological pH 10.0. Optimal pHs for either one were 8.5–9.0 for glutamate formation and one unit higher for the deaminating activity, values relevantly higher than those found for NADP-GDH [7]. K_m values determined at these optimal pHs are shown in Table II, together with other parameters of the enzyme. The structural and kinetic features of the enzyme resemble those of the NAD-GDH from *Clostridium* [2,10], the NAD(P)-GDHs from some bacteria as *Sulfolobus* [11], or the NADP-GDHs from enterobacteria [12] or

Table I

Purification of NAD-GDH from *Synechocystis* sp. PCC 6803

Purification step	Volume (ml)	Activity (U)	Protein (mg)	Spect. act. (mU/mg protein)	Purification (fold)	Yield (%)
Crude extract	123	11.9	861	14	1	100
$(NH_4)_2SO_4$ supernatant	203	12.0	550	22	1.6	101
DE-52 eluate	59	14.4	177	81	6	121
Red-agarose 1 eluate	33	8.1	15.5	520	38	68
Red-agarose 2 eluate	17	4.8	4.1	1170	85	40
S-300 eluate	3	1.2	0.21	5900	424	10

Starting material: 60 g fresh wt.

Bacillus [13]. Other NAD-GDH from prokaryotes have a lower molecular weight, as in *Thiobacillus* [2], or different kinetic properties, as in *Halobacterium* [14]. The enzyme is also very different from the NAD-GDHs of yeasts and other fungi, which are tetrameric with a higher K_m for ammonium, a lower K_m for glutamate [1,2] and with a clear catabolic function [3]. Among photosynthetic eukaryotes, only the soybean mitochondrial GDH is known to have similar features and, even in this case, ammonium K_m is slightly higher [15]. Other plant NAD-GDHs are not as specific for NADH as in the *Synechocystis* one, show higher K_m or have a tetrameric configuration [1,16].

As shown in Fig. 1, NAD- and NADP-GDHs present different electrophoretic mobility in native conditions. This fact, taken together with the differential elution of the two activities from DEAE-cellulose and the different properties of the 2 enzymes, clearly indicates the existence in *Synechocystis* of 2 proteins carrying out GDH activity. To exclude the possibility of NAD-GDH to be a degradation product or a postranslational modification of NADP-GDH, the mutant SCh1, lacking NADP-GDH (0.0 vs 10.1 mU/mg protein in the wild type) was used. No significant changes in NAD-GDH activities were found in the mutant (2.4 mU/mg protein vs 2.2 in the wild type), demonstrating that the two activities are coded by different genes, and that NAD-GDH activity level is not influenced by NADP-GDH.

To investigate if the low values of NAD-GDH were due to regulatory repression, we determined the enzyme activity in a wide range of physiological conditions: high or low CO_2, presence of absence of glucose, carbon starvation, light or darkness, nitrate or ammonium as nitrogen source, and nitrogen starvation. In all cases NAD-GDH activity values ranged between 0.5 and 2.5 mU/mg protein. None of the factors tested induced any significant change in the activity levels. Only the carbon source seemed to influence the activity, being 3 times higher in low CO_2 conditions, but, even in this case, the NAD-GDH activity reached no more than 25% of the NADP-GDH level [6]. In addition, the activity did not present any relevant variation along the growth profile of the culture, either in low or high CO_2-containing medium, or in photoheterotrophic conditions (data not shown). The absence of regulation of NAD-GDH in photosynthetic microorganisms has already been shown in *Chlorella sorokiniana* which presents a very fine ammonia- and light-regulated pattern of NADP-GDH activity, but lacks any kind of regulation of its NAD-GDH [17,18]. The regulation of this enzyme in higher plants is complex and no clear conclusion can be stated [16].

From the above characteristics, an ammonium assimilatory role, or at least an aminating ammonia-detoxifying function, can be suggested for this NAD-GDH, as has been proposed in *Synechococcus*, where the expression of the *E. coli gdh A* gene increased the ammonium tolerance [19]. Association of GDH with thylakoid membranes in cyanobacteria [5] supports a role for GDH preventing ammonia uncoupling. On the other hand, the occurrence of an hypothetical catabolic GDH, similar to that of yeast and other fungi [3], is not likely in cyanobacteria, where the Krebs cycle is incomplete [20]. In fact no 2-oxoglutarate dehy-

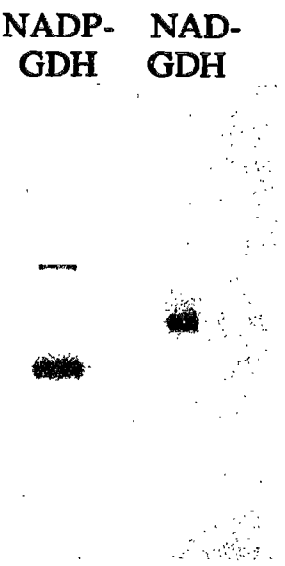

Fig. 1. Non denaturing polyacrylamide gel analysis of purified NADP- and NAD-GDH from *Synechocystis*. After electrophoresis, the gel was sliced and each track was stained with the corresponding reaction mixture. The slowest band showing NADP-GDH activity is presumed to reflect the formation of aggregates of high molecular weight.

Table II

Structural and kinetics parameters of *Synechocystis* PCC 6803 NAD-GDH

Parameter	Value
Molecular mass	
Native enzyme (kDa)	295
Subunit (kDa)	48.5
Stokes radius (nm)	5.9
Heat inactivation (°C)[a]	65
Isoelectric point	5.1
Optimal pH	
Amination	8.5-9.0
Deamination	9.5-10.0
K_m	
2-Oxoglutarate (mM)[b]	1.8
Ammonium (mM)	4.5
Glutamate (mM)	16.1
NAD (μM)	190
NADH (μM)	50

[a]Temperature at which 50% of the enzyme activity remained after 20 min of incubation

[b]Inhibition by substrate; K_m value calculated considering this fact

drogenase activity has been found in *Synechocystis* (data not shown), so the only known pathway to metabolize the 2-oxoglutarate generated by glutamate deamination, ought to be amination again, which would imply the existence of a futile cycle.

The presence of 2 different GDH enzymes with anabolic features in *Synechocystis* points to a second ammonia assimilating pathway, besides the GS-GOGAT cycle, in this cyanobacterium.

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REFERENCES

[1] Stewart, G.R., Mann, A.F. and Fentem, P.A. (1980) in: The Biochemistry of Plants (Stumpf, K. and Conn, E.E. eds) vol. 5, pp. 271–327, Academic Press, New York, London.
[2] Smith, E.L., Austen, B.M., Blumenthal, K.M. and Nyc, J.F. (1975) in: The Enzymes (Boyer, P.D. ed.) vol. 11a, pp. 293–367, Academic Press, New York, San Francisco.
[3] Miller, S.M. and Magasanik, B. (1990) J. Bacteriol. 172, 4927–4935.
[4] Neilson, A.H. and Doudoroff, M. (1973) Arch. Microbiol. 89, 15–22.
[5] Sallal, A-K.J. and Nimer, N.A. (1990) FEMS Microbiol. Lett. 67, 215–220.
[6] Florencio, F.J., Chávez, S., Muro-Pastor, M.I., Reyes, J.C., Marqués, S., Mérida, A. and Candau, P. (1991) in: Trends in Photosynthesis Research (Barber, J., Guerrero, M.G. and Medrano, H. eds) Intercept Ltd., England, in press.
[7] Florencio, F.J., Marqués, S. and Candau, P. (1987) FEBS Lett. 223, 37–41.
[8] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Rice, D.W., Baker, P.J., Farrants, G.W. and Hornby, D.P. (1987) Biochem. J. 242, 789–795.
[11] Consalvi, V., Chiaraluce, R., Politi, L., Gambacorta, A., De Rosa, M. and Scandurra, R. (1991) Eur. J. Biochem. 196, 459–467.
[12] Sakamoto, N., Kotre, A.M. and Savageau, M.A. (1975) J. Bacteriol. 124, 775–783.
[13] Hemmilä, I.A. and Mäntsälä, P.I. (1978) Biochem. J. 173, 45–52.
[14] Bonete, M.J., Camacho, M.L. and Cadenas, E. (1986) Int. J. Biochem. 18, 785–789.
[15] McKenzie, E.A., Copeland, L. and Lees, E.M. (1981) Arch. Biochem. Biophys. 212, 298–305.
[16] Srivastava, H.S. and Singh, R.P. (1987) Phytochemistry 26, 597–610.
[17] Molin, W.T., Cunningham, T.P., Bascomb, N.F., White, L.H. and Schmidt, R.R. (1981) Plant Physiol. 67, 1250–1254.
[18] Bascomb, N.F. and Schmidt, R.R. (1987) Plant Physiol. 83, 75–84.
[19] Lightfoot, D.A., Baron, A.J. and Wootton, J.C. (1988) Plant Mol. Biol. 11, 335–344.
[20] Pearce, J., Leach, C.K. and Carr, N.G. (1969) J. Gen. Microbiol. 55, 371–378.