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PURIFICATION AND PROPERTIES OF NADP$^+$-DEPENDENT GLUTAMATE DEHYDROGENASE FROM *NITROBACTER HAMBURGENSIS* STRAIN *X*14

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Abstract—NADP$^+$-specific glutamate dehydrogenase (L-glutamate: NADP$^+$ oxidoreductase, EC 1.4.1.4) has been purified to homogeneity from *Nitrobacter hamburgensis* strain *X*14. The native enzyme has M_r of 310000 and is composed of 6 identical subunits with M_r of 48000. The pH optimum for the amination reaction was 7.5, and that for deamination was 8.5. The K_m values for NH_4^+ and 2-oxoglutarate were 9 and 5 mM, respectively. Substrate activation of the amination reaction was observed with NADPH.

INTRODUCTION

Nitrobacter hamburgensis strain *X*14 is a gram-negative, nitrifying bacterium isolated by Sundermeyer and Bock [1]. This novel species can grow under autotrophic, heterotrophic and mixotrophic conditions. The study of intermediary carbon metabolism in this bacterium revealed that it has an incomplete citric acid cycle when grown under autotrophic conditions [2]. However, very little information is available on the nitrogen metabolism of this bacterium.

Glutamate dehydrogenase (GDH) [L-glutamate: NAD(P) oxidoreductase] occupies a pivotal position in nitrogen metabolism and carbon pathways. It catalyses the oxidative amination of 2-oxoglutarate to glutamate as well as the reductive deamination of glutamate [3]. NADP$^+$-dependent GDH has been purified and characterized in ammonia-oxidizing bacterium *Nitrosomonas europaea* [4]. It was shown that *Nitrobacter agilis* contains NAD$^+$- and NADP$^+$-dependent GDH activities [5, 6]. In this paper we report on the purification and the molecular characteristics of NADP$^+$-dependent GDH from *N. hamburgensis*.

RESULTS

Purification of NADP$^+$-dependent GDH

Crude extracts of cells grown under mixotrophic conditions had high activities of NADP$^+$-dependent glutamate dehydrogenases (fractions S_{30} and S_{110} in Table 1). The separation of NADP$^+$-dependent GDH was achieved by affinity chromatography and finally an electrophoretically homogeneous enzyme was obtained (Table 1). Washed cells (5 g wet wt) suspended in 50 mM Tris–HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol were disrupted by two passages through a French pressure cell at 4° (1300 kg/cm^2) and centrifuged at 30000 *g* for 20 min in a Sorvall RC-2B centrifuge. The supernatant (S_{30}) was then centrifuged at 110000 *g* for 1 hr at 4° in a Beckman L8-70 centrifuge. The high speed supernatant (S_{110}) was loaded onto a 2′-5′-ADP Sepharose 4B column (0.8 × 11 cm) pre-equilibrated with 50 mM Tris–HCl containing 5 mM 2-mercaptoethanol, pH 7.5. The column was then washed with the same buffer until the absorbance at 280 nm of the effluent was close to zero. The enzyme was eluted with 10 ml of 2 mM NADPH in the same buffer. Active fractions were pooled, dialysed overnight against the buffer and concentrated on an Amicon PM-10 membrane and then loaded onto a Sepharose 6B column (2 × 70 cm) equilibrated with Tris/2-mercaptoethanol buffer. The enzyme was eluted with the same buffer (flow rate 12 ml/hr). Pooled fractions containing the enzyme were concentrated as described above.

Molecular weight and subunits of NADP$^+$-dependent GDH

The apparent M_r of NADP$^+$-dependent GDH from *N. hamburgensis* was 310000 as determined by a gel filtration technique (Sepharose 6B). The separation of the enzyme by SDS polyacrylamide gel gave rise to a single protein band with M_r of 48000. Thus it is likely that the enzyme is composed of six identical subunits.

Properties of purified NADP$^+$-dependent GDH

The pH optimum of the amination reaction of the enzyme was 7.5, whereas that of deamination reaction was 8.5. A double reciprocal plot of the rate of NADPH oxidation against substrate concentration gave an apparent K_m value of 9 mM for NH_4Cl (Fig. 1A) and 5 mM for 2-oxoglutarate (Fig. 1B). The NADPH oxidation rate increased rapidly with increasing concentration of NADPH up to 15 μM and slowly thereafter. Double reciprocal plots of these data (Fig. 2) produced two distinct apparent K_m values of 40 μM above 15 μM NADPH and 13.3 μM below 15 μM NADPH. These data however can also be interpreted as negative cooperativities.

Table 1. Purification of NADP$^+$-GDH from *Nitrobacter hamburgensis*

Fractions	Total protein (mg)	Total activity (units)*	Specific activity (units/mg protein)	Purification (fold)	Yield %
1. S_{30}	234	28	0.12	1	100
2. S_{110}	107	24	0.22	1.8	86
3. Affinity chromatography (2',5'-ADP Sepharose 4B)	3.1	18	5.8	48	64
4. Gel filtration	1.2	11	9.2	77	39

*One enzyme unit is defined as μmol. NAD(P)H oxidized per min.

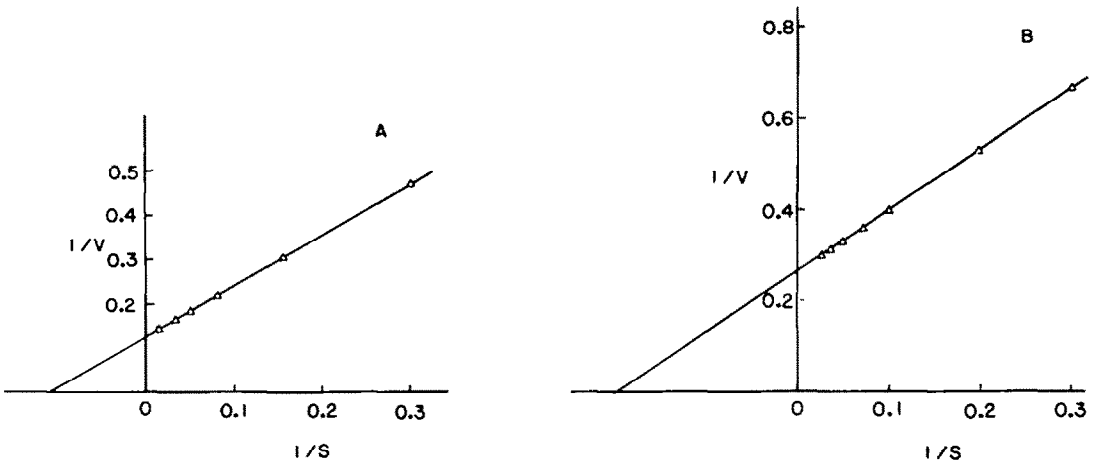

Fig. 1. Effects of various concentrations of NH_4^+ and 2-oxoglutarate on the amination reaction of purified NADP$^+$-GDH (Fraction 4, Table 1). Enzyme activity assayed as described in Experimental, except that the amounts of (A) NH_4Cl and (B) 2-oxoglutarate were varied. 1/v (nmol. NADPH oxidized/min)$^{-1}$. (A) 1/s, [NH_4Cl (mM)]$^{-1}$. (B) 1/s [2-oxoglutamate (mM)]$^{-1}$.

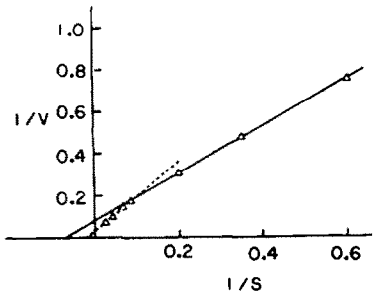

Fig. 2. Effects of various concentrations of NADPH on the amination reaction of purified NADP$^+$-GDH (Fraction 4, Table 1). Enzyme activity assayed as described in Experimental, except that the amounts of NADPH were varied. 1/v, (nmol. NADPH oxidized/min)$^{-1}$. 1/s (NADPH μM)$^{-1}$.

Some carboxylic acids are known to inhibit NADP$^+$-dependent GDH from *Nitrosomonas europaea* and *Nitrobacter agilis* [4, 6, 6a]. The amination reaction of the enzyme was inhibited by *ca* 30% in the presence of 20 mM fumarate, malate or glyoxylate. The inhibition by oxalacetate was 60% at 20 mM.

DISCUSSION

Purified NADP$^+$-dependent GDH from *N. hamburgensis* has an estimated M_r of 310000, which is similar to those of the enzyme from *Escherichia coli* (300000) [7], *Neurospora* (NADP$^+$-specific 288000) [8] and bovine liver (320000) [9], but it is higher than that of the enzyme from *Thiobacillus novellus* (NAD$^+$- and NADP$^+$-specific forms; 120000) [10]. The M_r of NADP$^+$–GDH subunit from *N. hamburgensis* is 48000, as determined by SDS polyacrylamide gel electrophoresis. This indicates that the enzyme is a hexamer and is thus similar to GDH from bovine liver [9], *Neurospora* [8] and *E. coli* [7].

The pH optimum of 7.5 for amination reaction (glutamate formation) of the NADP$^+$–GDH is in the range of the pH optimum (7.4–7.6) for cell growth of *N. hamburgensis* [11], whereas, the pH optimum for deamination reaction (glutamate oxidation) was 8.5. The NADP$^+$-dependent GDH can therefore function in either direction: i.e. amination of 2-oxoglutarate to glutamate or deamination of glutamate to 2-oxoglutarate. The amination rate, however, is twice as great as that of deamination; whereas the NAD$^+$-dependent GDH functions mainly in the direction of glutamate. Thus the multiple forms of GDH in *N. hamburgensis* are likely to play an important role in glutamate formation.

The amination reaction of NADP$^+$–GDH from *N. hamburgensis* was stimulated by NADPH and two apparent K_m values were recorded for the nucleotide but the data may also indicate negative cooperativities. It is likely that this effect is associated with changes in aggregation of the enzyme [3]. Similar substrate stimulation effects [12] were observed for GDH from bovine liver [13] and *Nitrobacter agilis* [6, 6a].

EXPERIMENTAL

Materials. NAD$^+$, NADH, NADP$^+$, NADPH, Tris, sodium glutamate, 2-oxoglutarate and all carboxylic acids were from Sigma. 2′,5′-ADP Sepharose 4B, Sepharose 6B and M_r calibration kits for various protein markers were from Pharmacia. All other chemicals were the best grade available.

Growth conditions. A culture of *Nitrobacter hamburgensis* strain *X*14 was kindly supplied by Dr Edberhard Bock, Institut für Allgemeine Botanik der Universität Hamburg, Abteilung für Mikrobiologie, F.R.G. Batch cultures were grown at 30° for 36 hr in a mixotrophic medium as described previously [2, 11]. Cells were washed twice with 50 mM Tris–HCl buffer (pH 7.5) and finally suspended in 50 mM Tris–HCl, 5 mM 2-mercaptoethanol.

Enzyme assay. Activity of GDH was determined as described in ref. [4] either from the rate of oxidation of NAD(P)H (amination reaction) or that of NAD(P) reductive deamination reaction at 340 nm at 30°. For the amination reaction the assay mixture in a total vol. of 3 ml contained (mM): 2-oxoglutarate, 20; NH_4Cl, 240; NAD(P)H, 0.33; Tris–HCl buffer (pH 7.5), 50; and an appropriate quantity of the enzyme preparation. For the deamination reaction, the assay mixture in a final vol. of 3 ml contained (mM): sodium glutamate, 17; NAD(P), 0.33; Tris–HCl buffer (pH 8.5), 50; and an appropriate quantity of the enzyme preparation. Both reactions were carried out at 30° in 1 cm quartz cuvettes. The amination and deamination reactions were started by adding 2-oxoglutarate and glutamate respectively. The reaction rates were corrected for endogenous oxidation/reduction of NAD(P)H/NAD(P).

Determination of M_r. The M_r of the native enzyme was determined by gel filtration on a Sepharose 6B column (1.6 × 100 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) according to the method of ref. [14]. The column was calibrated with aldolase (158 000), catalase (232 000), ferritin (440 000) and thyroglobulin (669 000) as marker proteins. The determination of subunit M_r was done by discontinuous gel electrophoresis in the presence of 0.1% (w/v) SDS using Tris–glycine buffer (pH 8.3) according to the methods of refs [15] and [16]. The gels were calibrated with the following protein standards: phosphorylase *b* (94 000), albumin (670 000), ovalbumin (43 000), carbonic anhydrase (30 000) and trypsin inhibitor (20 000).

Protein was determined by the method of ref. [17] using bovine serum albumin as a standard protein.

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