Properties of the cold-labile NAD⁺-specific glutamate dehydrogenase from *Bacillus cereus* DSM 31

THOMAS JAHNS* and HEINRICH KALTWASSER

Universität des Saarlandes, Lehrstuhl für Mikrobiologie, 13.3 Im Stadtwald, D-W-6600 Saarbrücken, Federal Republic of Germany

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Nicotinamide-adenine-dinucleotide-specific glutamate dehydrogenase (NAD-GDH; EC 1.4.1.3) from *Bacillus cereus* DSM 31 was enriched 260-fold. The molecular mass was determined by gel filtration to be 270 kDa (± 25 kDa). The enzyme was highly specific for the coenzyme NAD(H) and catalysed both the formation and the oxidation of glutamate. Apparent K_m values of 7.7 mM for glutamate and 0.56 mM for NAD⁺ during oxidative deamination were measured. Both in crude cell-free extracts and in enriched preparations the enzyme was extremely unstable, especially at low temperatures. The loss of activity in the cold was found to be due to the dissociation of the holoenzyme into catalytically inactive subunits of molecular mass 48 kDa (± 5 kDa), indicating that the native enzyme has a hexameric structure. The activity was restored under certain conditions, and no instability of the enzyme in the cold was observed in undisrupted cells.

Introduction

Glutamate dehydrogenases (GDH) catalyse the inter-conversion of 2-oxoglutarate and L-glutamate:

NAD⁺-specific GDH (NAD-GDH; EC 1.4.1.3) is generally believed to play a catabolic role in the degradation of glutamate, whereas NADP⁺-GDH primarily has the anabolic function of synthesizing glutamate. GDH that can use both NAD and NADP as its coenzyme has been found in eukaryotic systems as well as in *Mycoplasma* (*Acholeplasma*) *laidlawii* (Yarrison *et al.*, 1972), *Bacillus subtilis* (Kimura *et al.*, 1977), *Azospirillum brasilense* (Maulik & Ghosh, 1986) and *Bacteroides thetaiotaomicron* (Glass & Hylemon, 1980). NADP-GDH from prokaryotes has been purified from, *inter alia* *Escherichia coli* (Sakamoto *et al.*, 1975), *Salmonella typhimurium* (Coulton & Kapoor, 1973), *Bacillus licheniformis* (Phibbs & Bernlohr, 1971) and *Bacillus megaterium* (Hemmilä & Mäntsälä, 1978), while NAD-GDH has been found in *Clostridium* SB₄ (Winnacker & Barker, 1970), *Pseudomonas aeruginosa* (Joannou & Brown, 1992), cyanobacteria (Chávez & Candau, 1991)

and *Rhodospirillum rubrum* (Bachofen & Neeracher, 1968). There are conflicting reports on the occurrence of NAD-GDH in *Bacillus subtilis* (Kane *et al.*, 1981; Phibbs & Bernlohr, 1971); in *B. cereus*, traces of NAD-GDH activity were measured by McCormick & Halvorson (1963), while the same species was described as lacking the enzyme by Phibbs & Bernlohr (1971). Recently, NAD-GDH which was unstable at low temperatures (0 °C) has been detected in several species of *Bacillus*, including *B. cereus*, *B. subtilis* and other *Bacillus* species formerly described as lacking this enzyme (Jahns, 1992). The reason for the conflicting reports on the presence or absence of NAD-GDH in *Bacillus* strains may be the extremely rapid loss of activity of the enzyme in the cold (Jahns, 1992). In this communication the enrichment of a cold-labile NAD-GDH from *B. cereus* DSM 31 is described. Some biochemical and physical properties, the molecular mechanism of cold inactivation and possibilities of stabilizing the enzyme were investigated.

Methods

Bacterial strains and growth conditions. *Bacillus cereus* DSM 31 was grown aerobically under vigorous agitation in 0.8% nutrient broth to the early stationary growth phase, harvested by centrifugation (10000 g for 20 min at 4 °C), and washed twice in 50 mM-Tris/HCl, pH 7.7, 3 mM-mercaptoethanol (buffer A).

In one set of experiments, the specific activities of NAD-GDH were measured in cell-free extracts of *B. cereus* DSM 31 grown in media of different composition. The basal medium (BM) used for these experiments contained 0.02% each of Casamino acids and yeast

* Author for correspondence. Tel. 0681 3022745; fax 06897 798181.

Abbreviations: NAD-GDH, nicotinamide-adenine-dinucleotide-specific glutamate dehydrogenase.

extract, 25 mM- Na_2HPO_4 , 11 mM- KH_2PO_4 , 0.8 mM- MgSO_4 , 0.1 mM- CaCl_2 and 0.1 % (v/v) trace element solution SL6 (Claus *et al.*, 1983). The compounds indicated in the experiments were added to this medium.

Preparation of cell-free extracts. Cells were disintegrated by ultrasonification of cell suspensions containing about 0.2 g wet weight per ml buffer A and 20 % (v/v) glycerol. Ultrasonic treatment lasted a total of 30 s ml^{-1} , applied in periods of 10 s, using a Branson sonifier B12 at a maximum output of 50 W, and was followed by centrifugation for 30 min at 30000 g in order to obtain cell-free extracts. The temperature was kept between 20 and 25 °C throughout all stages of cell disruption and centrifugation. Modifications to this procedure are indicated in the experiments.

Enzyme assays and protein determination. Reductive aminating activity was measured at 30 °C in 50 mM-Tris/HCl pH 7.7, 200 mM-NaCl, containing 100 mM-ammonium chloride, 0.2 mM-NADH and 1 to 5 μl cell-free extract or enriched enzyme in appropriate dilution; the reaction was started by the addition of 5 mM-2-oxoglutarate. The oxidative deaminating activity was determined in 100 mM-glycine/NaCl/NaOH pH 9.4, containing 2 mM-NAD⁺ and 50 mM-L-glutamate; the reaction was started by the addition of cell-free extract (10 to 50 μl). Enzyme assays were performed immediately after the preparation of cell-free extracts; modifications are indicated in the experiments. NAD-GDH activity calculated from the decrease (reductive amination) or increase (oxidative deamination) in absorbance, followed spectrophotometrically at 340 nm, was corrected for non-specific NADH-oxidase activity determined for each assay. One unit of enzyme activity was defined as the amount of enzyme that catalysed the oxidation of 1 μmol NADH or the reduction of 1 μmol NAD⁺ in 1 min under the conditions described. Protein was measured by the Lowry method or by that of Schmidt *et al.* (1963), with the modification that only 10 % of the indicated amount of potassium iodide was used; bovine serum albumin served as standard.

Enzyme enrichment. The enzyme enrichment, except heat treatment, was carried out at between 18 and 22 °C. NAD-GDH was enriched as follows.

(i) **Heat treatment.** Cell-free extracts obtained in the presence of 20 % (v/v) glycerol by sonic disruption of cells grown in 0.8 % nutrient broth were heated with continuous stirring at 65 °C for 10 min; the solution was centrifuged for 15 min at 30000 g and the pellet was discarded.

(ii) **Ammonium sulphate fractionation.** The supernatant was brought up to approximately the initial volume with buffer A containing 20 % (v/v) glycerol, and solid ammonium sulphate was added to 40 % saturation with stirring. After 10 min, precipitated material was removed by centrifugation (15 min at 30000 g) and discarded. Solid ammonium sulphate was then added to 60 % saturation to the supernatant which was subsequently stirred for 15 min. The pellet obtained by centrifugation (15 min at 30000 g) was dissolved in a small amount of buffer A containing 20 % (v/v) glycerol.

(iii) **Column chromatography.** Gel filtration was performed on a Sephacryl S400 high-resolution column (99 cm $\times$ 2 cm²) in buffer A containing 200 mM-NaCl; the fractions exhibiting the highest specific activity were pooled, diluted 10-fold in 50 mM-potassium phosphate pH 7.7, 3 mM-mercaptoethanol, 1 mM-EDTA (buffer B) and applied to a column (5 cm $\times$ 2 cm²) packed with DEAE-cellulose and equilibrated with buffer B. The column was eluted with 10 ml aliquots of buffer B containing NaCl at increasing molarities, applied in steps of 10 mM in the range between 20 and 120 mM-NaCl. Fractions exhibiting the highest NAD-GDH activity were pooled and subjected to a second run through the same column.

Molecular mass determinations. The molecular mass of the native NAD-GDH was determined by gel filtration according to Andrews (1964), carried out at 20 °C on a Sephacryl S400 high-resolution

column (99 cm $\times$ 2 cm²) with buffer A containing 200 mM-NaCl. Thyroglobulin (669 kDa), ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa), all purchased from Boehringer Mannheim, were used to calibrate the column. The molecular mass of the cold-inactivated NAD-GDH was determined by the same method, except that the column was run at 4 °C.

Reproducibility of results. The tables and the figures show representative results from single experiments, which were repeated at least once. Experiments for the determination of the K_m values of NAD-GDH were repeated at least three times; linear correlations were fitted by regression analysis, yielding values for r of between 0.990 and 0.998. The other results are mean values from at least two independent experiments; SD values did not exceed ± 20 % of the mean values and are not indicated.

Results

Instability of NAD-GDH from Bacillus cereus DSM 31

Rapid loss of NAD-GDH activity occurred during cell disintegration at low temperatures. No activity was detected in cell-free extracts prepared at 0 to 4 °C, whereas high activities were measured when the cell-free extracts were prepared between 20 and 25 °C and assayed immediately. Loss of enzyme activity in the cold was also observed when Tris/HCl in buffer A was replaced by equimolar concentrations of sodium phosphate or imidazole during preparation of the extracts. Glycerol present at 20 % (v/v) during the preparation of cell-free extracts at 0 to 4 °C stabilized the NAD-GDH, resulting in activities similar to those obtained after cell disintegration at 20 to 25 °C (results not shown).

When extracts exhibiting high NAD-GDH activities were incubated at 0 °C for 30 min in the presence of only 5 % (v/v) glycerol and subsequently assayed, no NAD-GDH activity was detectable, whereas after incubation of the same enzyme preparation at 30 °C, only a slight loss of NAD-GDH activity was measured. In the absence of glycerol, a significant loss of NAD-GDH activity occurred upon incubation at temperatures of 15 °C and below (Fig. 1). High concentrations of glycerol protected the enzyme from loss of activity: in the presence of 20 % (v/v) glycerol, about 70 % of the initial enzyme activity was retained upon incubation for 120 min at 0 °C, and no loss of NAD-GDH activity was observed in the presence of 50 % (v/v) glycerol. Furthermore, 20 % (v/v) glycerol in the presence of 20 mM-NaCl protected the enzyme completely from cold inactivation, demonstrating a stabilizing effect of NaCl. In the absence of glycerol, however, no protective effect of NaCl (20 to 200 mM) during incubation in the cold was observed (results not shown). The substrates of NAD-GDH, ammonium ions, glutamate or 2-oxoglutarate at concentrations of 50 mM, 50 mM and 5 mM respectively did not prevent the loss of NAD-GDH activity in the cold. The protein concentration did not affect the loss of

Fig. 1. Stability of NAD-GDH from *B. cereus* DSM 31 at different temperatures in the presence and absence of glycerol. Cell-free extract (15 mg protein ml⁻¹) prepared in buffer A containing 200 mM-NaCl at 20 to 25 °C was incubated for 30 min at the temperatures indicated without glycerol (○) or with 20% (v/v) glycerol (●), brought to 30 °C and immediately assayed for NAD-GDH activity in the reductive amination assay.

enzyme activity in the cold: an identical decrease in enzyme activity was observed in extracts with protein concentrations between 1.5 and 25 mg protein ml⁻¹, and the addition of 20 mg ml⁻¹ bovine serum albumin or ovalbumin to the cell-free extracts had no stabilizing effect on NAD-GDH in the cold.

The loss of NAD-GDH activity in the cold was essentially irreversible in buffer A: no reactivation of the enzyme was observed upon incubation of the cold-inactivated cell-free extract at 30 °C for 5 min. However, when 200 mM-NaCl was present during cold incubation, a complete reactivation of NAD-GDH was observed upon rewarming the extracts; this reactivation was not observed when NaCl was replaced by equimolar concentrations of KCl, sucrose, glucose or 5% (w/v) polyethylene glycol. Only a minor loss of NAD-GDH activity was observed when whole cells were stored at 0 °C for several days prior to the preparation of cell-free extracts.

The composition of the buffer turned out to be crucial to the stability of the enzyme at 30 °C. When cell-free extracts prepared at 20 to 25 °C in the presence of 20% (v/v) glycerol were diluted 20-fold in different buffers at pH 7.7, the enzyme was found to be stable in 50 mM-potassium phosphate, whereas a complete loss of enzyme activity was observed within 60 min at 30 °C in buffer A. The presence of 50 mM-NaCl or 5% (v/v) glycerol in buffer A partially prevented the loss of activity; in the presence of 20% (v/v) glycerol and 200 mM-NaCl, KCl or MgCl₂, no loss of activity occurred at 30 °C.

Regulation of NAD-GDH formation by the composition of the growth medium

Prior to the enrichment of NAD-GDH from *B. cereus* DSM 31, the influence of the growth medium on the specific enzyme activities was determined. Low specific activities were observed in cells grown in BM containing 0.1% (w/v) ammonium chloride and 0.5% (w/v) glucose, whereas higher activities (about fivefold) were measured in basal media containing 0.8% (w/v) nutrient broth, or 0.5% (w/v) of either glutamate, glutamine or Casamino acids (results not shown). Cells grown in nutrient broth were therefore used for the enrichment of NAD-GDH.

Enrichment of NAD-GDH

Since NAD-GDH lost its activity at low temperatures or in the absence of glycerol or NaCl, enrichment of the enzyme was carried out at room temperature (18 to 22 °C) in the presence of 20% glycerol and/or NaCl. From cell-free extracts NAD-GDH was enriched 260-fold with a yield of 6% (Table 1). For column chromatography, only fractions exhibiting the highest NAD-GDH activities were used for subsequent enrichment; this explained the loss of NAD-GDH activity

Table 1. Purification of NAD-GDH from *Bacillus cereus* DSM 31

Purification step	Volume (ml)	Protein (mg)	Total activity (U)	Protein (mg ml ⁻¹)	Specific activity* [U (g protein) ⁻¹]	Yield (%)	Purification (-fold)
1. Crude extract	18.0	147.6	162.4	8.2	1100	100	1
2. Heat treatment	18.9	72.0	172.1	3.8	2390	106	2
3. (NH ₄) ₂ SO ₄ precipitation	1.0	9.5	68.2	9.4	7200	42	7
4. Sephacryl S400HR	16.0	1.9	30.9	0.12	16210	19	15
5. DEAE-cellulose (second run)	3.0	0.03	9.7	0.01	286400	6	260

* Measured in the reductive amination assay.

during gel filtration and ion-exchange chromatography. After gel filtration on a Sephacryl S400 high-resolution column, the molecular mass of the native enzyme was determined to be 270 kDa (± 25 kDa).

Dependence on pH and kinetic parameters

Enzyme activity at different pH values was determined in cell-free extracts (8.5 mg protein ml⁻¹, prepared at 20 to 25 °C in buffer A containing 200 mM-NaCl and 1 %, v/v, glycerol). Maximum activities were measured at pH 7.7 for the reductive amination of 2-oxoglutarate and at pH 9.4 for the oxidative deamination of glutamate.

The kinetic parameters for the different substrates of enriched NAD-GDH were determined from Lineweaver-Burk plots. Measuring oxidative deamination, Michaelis-Menten kinetics were observed, and K_m values of 0.56 mM-NAD⁺ (at concentrations between 0.05 and 5 mM-NAD⁺) and 7.4 mM-glutamate (at concentrations between 0.25 and 50 mM-glutamate) were determined; no enzyme activity was observed when NAD⁺ was replaced by NADP⁺, or glutamate by alanine or leucine. Several attempts to determine exactly the kinetic constants for the reductive amination failed, since NAD-GDH was strongly inhibited by ammonium at high concentrations. Maximal activities were observed at 200 mM-ammonium; at 500 mM-ammonium, only about 50 % of this activity was measured. Double-reciprocal plots of velocity against concentration between 5 and 200 mM-ammonium yielded a K_m value of 96 mM-ammonium. At 200 mM-ammonium, a K_m value of 0.033 mM for NADH was determined from linear Lineweaver-Burk plots. In the case of 2-oxoglutarate, however, sigmoidal saturation curves were obtained in plots of velocity against substrate concentration. This indicated that cooperative interactions between the subunits of the enzyme may occur. No activity was observed with 2-oxoisocaproate or pyruvate as substrates, indicating that NAD-GDH activity was not due to leucine or alanine dehydrogenase activity, both present in crude extracts of *B. cereus* DSM 31 (results not shown). NAD-GDH catalysed the reductive amination of 2-oxoglutarate in preference to the oxidation of glutamate; at the respective pH optima of 7.7 and 9.4 the rate of amination was about six times that of deamination. No inhibition or activation of NAD-GDH was measured in the presence of 0.5 M-AMP, ADP, ATP, cyclic AMP, GMP, GDP or GTP.

Mechanism of cold inactivation of NAD-GDH

The structural changes leading to the loss of enzyme activity in the cold were examined by gel filtration. When an enriched preparation of NAD-GDH in buffer A

Fig. 2. Estimation of molecular masses of native and cold-inactivated NAD-GDH from *B. cereus* DSM 31 by gel filtration. The Sephacryl S400 high-resolution column was calibrated at 20 °C with thyroglobulin (669 kDa; 1), ferritin (450 kDa; 2), catalase (240 kDa; 3) aldolase (158 kDa; 4), bovine serum albumin (68 kDa; 5), ovalbumin (45 kDa; 6) and chymotrypsinogen A (25 kDa; 7).

containing 200 mM-NaCl was applied to an S400 high-resolution column and run at 20 °C in the presence of 200 mM-NaCl, a single protein peak was eluted which corresponded to a molecular mass of 270 kDa (± 25 kDa; Fig. 2). When the same enriched preparation of NAD-GDH was first incubated for 60 min at 0 °C and then subjected to gel filtration at 4 °C in the presence of 200 mM-NaCl, the protein was eluted in a single peak that corresponded to a molecular mass of 48 kDa (± 5 kDa; Fig. 2). When these low-molecular-mass fractions were brought to 30 °C for 5 min and subsequently assayed for NAD-GDH activity, 60 % of the specific activity initially applied to the column was measured. This indicated that a partial reactivation of the enzyme had occurred. Resubmission of the reactivated fraction to gel filtration at 20 °C in the presence of 200 mM-NaCl resulted in the elution of NAD-GDH activity in the fraction corresponding to a molecular mass of 270 kDa. These results suggest that upon incubation in the cold, NAD-GDH from *B. cereus* DSM 31 dissociates into catalytically inactive subunits of molecular mass approximately 48 kDa, and indicate that the active holoenzyme has a hexameric structure.

Discussion

In a recent study, NAD-GDH was detected in different *Bacillus* species, including several strains of *B. cereus*; interestingly, the enzyme exhibited extreme instability in the cold (Jahns, 1992). Since cell-free extracts are usually prepared in the cold, the contradictoriness of reports on the presence of this enzyme in species of *Bacillus* (Kane *et al.*, 1981; McCormick & Halvorson, 1963; Phibbs & Bernlohr, 1971) may be explained by this instability. The results presented in this paper demonstrate that: (a) high

specific activities of NAD-GDH are present in cells of *B. cereus* DSM 31; (b) the enzyme is extremely unstable under several conditions; and (c) the enzyme resembles known NAD-GDHs from other bacteria in several respects. The molecular mass of 270 kDa is close to that observed in other bacteria, e.g. *Clostridium* sp. (275 kDa; Winnacker & Barker, 1970) and *Synechocystis* sp. PCC 6803 (295 kDa; Chávez & Candau, 1991); in the latter case the holoenzyme was proposed to have a hexameric structure. Similar molecular masses and, if examined, the same subunit composition were described for the NADP-GDH of *B. megaterium* (270 kDa; Hemmilä & Mäntsälä, 1978), *Salmonella typhimurium* (280 kDa; Coulton & Kapoor, 1973), *Escherichia coli* (300 kDa; Sakamoto *et al.*, 1975), *Candida utilis* (276 kDa; Naumann *et al.*, 1976) and the GDH with dual coenzyme specificity of *B. subtilis* PCI 219 (250 to 270 kDa; Kimura *et al.*, 1977), *Mycoplasma (Acholeplasma) laidlawii* (250 kDa; Yarrison *et al.*, 1972) and *Azospirillum brasilense* (285 kDa; Maulik & Ghosh, 1986).

The kinetic parameters of NAD-GDH from *B. cereus* DSM 31 are within the range of those measured for the enzyme of *B. pasteurii* (Jahns, 1992) and *Synechocystis* sp. PCC 6803 (Chávez & Candau, 1991), with the exception of the high affinity of the NAD-GDH of the latter organism for ammonium (K_m value 4.5 mM); in this cyanobacterium the enzyme was suggested to have an assimilatory function. The NAD-GDH of *B. cereus* DSM 31 exhibited a low affinity for ammonium, with an apparent K_m value of 96 mM; moreover, high specific NAD-GDH activities were observed when cells were grown on nutrient broth or Casamino acids, or media containing glutamine or glutamate at high concentrations. These observations suggest that NAD-GDH of *B. cereus* DSM 31 is involved in catabolic oxidation of glutamate. In several *Bacillus* species, high intracellular concentrations of glutamate and low concentrations of ammonium have been observed (Schreier *et al.*, 1982; Kanamori *et al.*, 1987), indicating that the function of NAD-GDH *in vivo* is the oxidative deamination of glutamate. Furthermore, only low specific NAD-GDH activities were observed when *B. cereus* DSM 31 was grown in a medium containing ammonium and glucose, also suggesting that NAD-GDH is not responsible for assimilation of ammonium. Compared with *B. cereus* DSM 31, significantly higher affinities for its substrates were observed for the enzyme of *Clostridium* SB₄ (Winnacker & Barker, 1970). An inhibition of NAD-GDH by high ammonium concentrations, as observed for the enzyme of *B. cereus* DSM 31, was reported for the GDH with dual coenzyme specificity of *B. subtilis* PCI 219 (Kimura *et al.*, 1977).

The most interesting property of the NAD-GDH from *B. cereus* DSM 31 was its rapid loss of activity under

several conditions, especially at low temperatures (Fig. 1). Cold lability has been described for a number of enzymes of both eukaryotic and prokaryotic origin, including GDH (Fincham, 1957; Maulik & Ghosh, 1986; Naumann *et al.*, 1976). A reversible conformational change was proposed in the case of *Candida utilis*, where a temperature rise from 5 to 20 °C led to an activation of the enzyme (Naumann *et al.*, 1976). NAD(P)-GDH from *Azospirillum brasilense* lost about 80% of its activity within 60 min at 0 °C; glycerol stabilized the enzyme (Maulik & Ghosh, 1986). The molecular mechanism of this inactivation was not studied in this organism; however, the results of cold lability observed in *A. brasilense* are similar to those of *B. cereus* DSM 31 and other *Bacillus* species (Jahns, 1992). In previous work (Kimura *et al.*, 1977), NAD-GDH of *B. subtilis* was described to be unstable under several conditions, and glycerol was used to stabilize the enzyme during isolation from this bacterium (Kane *et al.*, 1981; Kimura *et al.*, 1977). Glycerol forms strong hydrogen bonds with water, thus slowing down the motion of water molecules and reducing water activity (Scopes, 1982).

Gel filtration experiments suggested that in preparations of NAD-GDH from *B. cereus* DSM 31, the loss of activity in the cold was due to the dissociation of the hexameric holoenzyme into its catalytically inactive monomeric subunits (Fig. 2), and that the reactivation of NAD-GDH by incubation at 30 °C was due to the reassociation of the subunits to the catalytically active hexameric holoenzyme. Such changes in protein structure are commonly observed for cold-labile enzymes. Dissociation of an oligomeric holoenzyme into its subunits upon incubation at 0 °C has been described for yeast glyceraldehyde-3-phosphate dehydrogenase (Bartholmes & Jaenicke, 1978), mitochondrial chicken liver pyruvate carboxylase (Irias *et al.*, 1969) and steer liver argininosuccinase (Havir *et al.*, 1965). Another mechanism of cold inactivation, the formation of catalytically inactive aggregates, was described for 17 β -hydroxysteroid dehydrogenase from human placenta (Jarabak *et al.*, 1966).

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