Arch Microbiol (1997) 168 : 282–289

ORIGINAL PAPER

Jens Boch · Gabriele Nau-Wagner · Susanne Kneip
Erhard Bremer

Glycine betaine aldehyde dehydrogenase from *Bacillus subtilis*: characterization of an enzyme required for the synthesis of the osmoprotectant glycine betaine

Received: 2 May 1997 / Accepted: 2 July 1997

Abstract Production of the compatible solute glycine betaine from its precursors choline or glycine betaine aldehyde confers a considerable level of tolerance against high osmolarity stress to the soil bacterium *Bacillus subtilis*. The glycine betaine aldehyde dehydrogenase GbsA is an integral part of the osmoregulatory glycine betaine synthesis pathway. We strongly overproduced this enzyme in an *Escherichia coli* strain that expressed a plasmid-encoded *gbsA* gene under T7ϕ10 control. The recombinant GbsA protein was purified 23-fold to apparent homogeneity by fractionated ammonium sulfate precipitation, ion-exchange chromatography on Q-Sepharose, and subsequent hydrophobic interaction chromatography on phenyl-Sepharose. Molecular sieving through Superose 12 and sedimentation centrifugation through a glycerol gradient suggested that the native enzyme is a homodimer with 53.7-kDa subunits. The enzyme was specific for glycine betaine aldehyde and could use both NAD^+ and $NADP^+$ as cofactors, but NAD^+ was strongly preferred. A kinetic analysis of the GbsA-mediated oxidation of glycine betaine aldehyde to glycine betaine revealed K_m values of 125 μM and 143 μM for its substrates glycine betaine aldehyde and NAD^+, respectively. Low concentrations of salts stimulated the GbsA activity, and the enzyme was highly tolerant of high ionic conditions. Even in the presence of 2.4 M KCl, 88% of the initial enzymatic activity was maintained. *B. subtilis* synthesizes high levels of proline when grown at high osmolarity, and the presence of this amino acid strongly stimulated the GbsA activity in vitro. The enzyme was stimulated by moderate concentrations of glycine betaine, and its activity was highly tolerant against molar concentrations of this osmolyte. The high salt tolerance and its resistance to its own reaction product are essential features of the GbsA enzyme and ensure that *B. subtilis* can produce high levels of the compatible solute glycine betaine under conditions of high osmolarity stress.

Key words Osmoregulation · Compatible solutes · Glycine betaine synthesis · Aldehyde dehydrogenase · *Bacillus subtilis*

J. Boch[1] · G. Nau-Wagner · S. Kneip · E. Bremer (✉)
Philipps Universität, Department of Biology,
Laboratory for Microbiology, Karl-von-Frisch-Strasse,
D-35032 Marburg, Germany
Tel. + 49-6421-281529; Fax + 49-6421-288979
e-mail: bremer@mailer.uni-marburg.de

Present address:
[1] Washington University, Department of Biology,
One Brookings Drive, St. Louis, MO 63130, USA

Introduction

High osmolarity strongly interferes with the proliferation of bacteria by limiting the availability of water (Csonka and Hanson 1991; Galinski and Trüper 1994; Lucht and Bremer 1994). The soil-borne, gram-positive bacterium *Bacillus subtilis* frequently encounters periods of high osmolarity due to the drying of the upper layers of the soil (Miller and Wood 1996). To cope effectively with these stressful conditions, the bacteria induce the expression of a large stress regulon that prepares them for a variety of environmental challenges (Hecker et al. 1996). In addition, on exposure to high osmolarity, *B. subtilis* incites specific and highly co-ordinated physiological adaptation reactions to limit the loss of water from the bacterial cell and to maintain turgor within acceptable boundaries. These specific stress responses are essential for the growth of *B. subtilis* in media of high osmolarity (Whatmore et al. 1990; Whatmore and Reed 1990; Boch et al. 1994).

A rapid uptake of potassium ions via turgor-responsive transport systems is the primary response of the *B. subtilis* cells to a sudden increase in the osmolarity of the environment (Whatmore and Reed 1990). This initial reaction is followed by the accumulation of proline to high intracellular levels both via de novo synthesis (Whatmore et al. 1990) and through uptake from the environment by an osmoregulated transport system (von

Blohn et al. 1997). Proline is one of a small group of compounds, the compatible solutes, that are amassed by many bacterial, plant, animal, and human cells in response to high osmolarity (Csonka and Hanson 1991; Galinski and Trüper 1994). In contrast to ionic osmolytes, which are deleterious at high concentrations, compatible solutes can be accumulated to very high intracellular levels without disturbing essential cell functions and, thus, contribute to the restoration of turgor in high-osmolarity environments. In addition, they can protect the functioning and integrity of proteins and cell components at low water activity and do not interfere with protein-DNA interactions (Yancey 1994).

A third response of *B. subtilis* to osmotic stress is the accumulation of glycine betaine. This trimethylammonium compound is one of the most potent compatible solutes and is used as an osmoprotectant by a wide variety of prokaryotic and eukaryotic organisms. Since glycine betaine is synthesized by many plants (Rhodes and Hanson 1993), root exudates and decaying plant tissues are its likely sources in the soil (Goldmann et al. 1991). *B. subtilis* can scavenge glycine betaine directly from the environment via three high-affinity uptake systems that comprise both secondary and ABC transporters (Kempf and Bremer 1995; Lin and Hansen 1995; Kappes et al. 1996). In addition, it also can synthesize glycine betaine when the precursors choline or glycine betaine aldehyde are present in the growth medium (Boch et al. 1994). A genetic and physiological analysis of the osmoregulatory choline-glycine betaine pathway in *B. subtilis* has revealed that glycine betaine production occurs by a two-step oxidation process with glycine betaine aldehyde as the intermediate (Boch et al. 1994, 1996). Two enzymes act in concert for glycine betaine synthesis: a type-III alcohol dehydrogenase (GbsB), which oxidizes choline to glycine betaine aldehyde, and a glycine betaine aldehyde dehydrogenase (GbsA), which converts this intermediate into glycine betaine. The structural genes (*gbsAB*) for these enzymes are genetically organized in an operon, and their transcription is induced by the presence of choline in the growth medium (Boch et al. 1996).

B. subtilis shares the ability to oxidize choline to glycine betaine with a variety of microbial, plant, animal, and human cells (Csonka and Hanson 1991; Rhodes and Hanson 1993; Galinski and Trüper 1994; Miller and Wood 1996). Although the combination of enzymes that mediate this reaction can differ, a soluble, substrate-specific glycine betaine aldehyde dehydrogenase is frequently an integral part of the biosynthetic pathway. The bacterial enzymes involved in glycine betaine synthesis have recently gained considerable biotechnological attention in connection with attempts to genetically engineer plants with increased salt tolerance and resistance to drought (Holmström et al. 1994; Rathinasabapathi et al. 1994). In the present study, we focus on the osmoregulatory choline-glycine betaine pathway from *B. subtilis* and report the biochemical characterization of the glycine betaine aldehyde dehydrogenase GbsA.

Materials and methods

Bacterial strains, plasmids, growth conditions, and chemicals

The *Escherichia coli* strain PD141 (P. Dersch and E. Bremer, unpublished results) is a derivative of the Δ(*betTIBA*) strain MC4100 (Lamark et al. 1991) and carries in its chromosome the structural gene for the phage T7 RNA polymerase under *lacPO* control on the defective prophage λDE3 (Studier and Moffat 1986). Plasmid pJB009 (Boch et al. 1996) is a derivative of the low-copy-number expression vector pPD100 (Dersch et al. 1994) harboring the *gbsA*$^+$ gene under the control of the T7ϕ10 promoter. For the overexpression of the *gbsA* gene, the cells were grown in M9 minimal medium (Miller 1992) enriched with 0.2% casamino acids and 0.2% glucose as the carbon source. Glycine betaine, glycine betaine aldehyde, choline, carnitine, proline, trehalose, NAD^+, $NADP^+$, and a variety of aldehydes were purchased from Sigma (Deisenhofen, Germany), Biomol (Hamburg, Germany), and Fluka (Neu-Ulm, Germany). The osmoprotectant ectoine was obtained from Bi-Top (Witten, Germany), and β-alanine betaine (homobetaine) was synthesized from acrylic acid and methylamine (Le Berre and Delacroix 1973).

Expression of the *gbsA* gene under T7ϕ10 control

For the overproduction of the *B. subtilis* GbsA protein, a fresh overnight culture of the recombinant *E. coli* strain PD141 (pJB009) was diluted 100-fold into M9 minimal medium containing 0.2% casamino acids, 0.2% glucose, and chloramphenicol (30 μg ml^{-1}) to select for the *gbsA*$^+$ plasmid pJB009. The cultures (2-l) were grown at 37° C with aeration to mid-exponential phase until the cells reached an OD_{578} of 0.5. The T7ϕ10-mediated expression of the plasmid-encoded *gbsA* gene was initiated by inducing the expression of the chromosomal T7 RNA polymerase gene with isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration 1 mM) in strain PD141. After further growth of the cultures for 30 min, rifampicin was added to a final concentration of 100 μg ml^{-1} to inhibit the *E. coli* RNA polymerase. The cells were subsequently grown for an additional hour and were harvested by centrifugation at 6,000 × *g* for 15 min at 4° C. The cell pellet was resuspended in 5 ml 100 mM Hepes buffer (pH 7.9) containing 10 mM dithiothreitol (DTT).

Purification of the GbsA protein

For the isolation of the GbsA enzyme, we routinely used cells from 4-l cultures of strain PD141 (pJB009). The enzyme isolation procedures were all performed at 4° C or on ice. The bacterial cells were disrupted by passing them three times through a French pressure cell operating at 100 MPa. Unbroken cells and cellular debris were removed by low-speed centrifugation (27,000 × *g* for 10 min), and the cell-free extract was then further cleared from large membrane fragments by ultracentrifugation (100,000 × *g* for 30 min). Fractionated precipitation with ammonium sulfate was used as the initial step for the purification of GbsA. A saturated ammonium sulfate solution (4.1 M) was added to the cleared cell lysate to final concentrations of 35, 45, 55, and 75%, and the precipitated proteins were collected by centrifugation (27,000 × *g* for 10 min). The protein fraction precipitating between 55 and 75% saturation contained most of the GbsA enzyme activity. This fraction was solubilized in 4 ml 10 mM Hepes (pH 7.9) containing 10 mM DTT and 10% glycerol (buffer A). The protein solution was desalted on a Sephadex G-25 column (PD-10; Pharmacia LKB Biotechnology, Freiburg, Germany) that was equilibrated and run with buffer A. The eluted proteins were applied to a Q-Sepharose Fast Flow (1 × 13 cm) column equilibrated with buffer A, the column was washed with 40 ml 250 mM KCl in buffer A, and the proteins were eluted from this column by applying an 80-ml linear gradient of KCl (250–800 mM) in buffer A. The fractions showing GbsA activity eluted at a concentration of approximately 360 mM KCl. These

fractions were pooled, solid ammonium sulfate was added to a final concentration of 1.5 M, and the solution was applied to a phenyl-Sepharose (2.6 × 10 cm) column equilibrated with a buffer containing 10 mM potassium phosphate (pH 8.0), 10 mM DTT, and 1.5 M ammonium sulfate (buffer B). The column was washed with 100 ml buffer B, and the proteins were subsequently eluted with 200 ml of a declining gradient of ammonium sulfate (1.5–0 M) in buffer B. Fractions with GbsA activity eluted at a concentration of approximately 400 mM ammonium sulfate. These fractions were pooled, ammonium sulfate was added to a final concentration of 1.5 M, and the proteins were rechromatographed on the phenyl-Sepharose column. The purified GbsA enzyme was stored at –80° C.

Enzyme assay

The activity of the GbsA protein was determined spectrophotometrically at 30° C by measuring the reduction of NAD^+ to NADH at 340 nm in an LKB spectrophotometer (LKB Ultrospec 2000; Pharmacia, Freiburg, Germany). The standard assay contained 100 mM Tris-HCl (pH 8.0), 10 mM DTT, 1 mM NAD^+, 0.5 mM glycine betaine aldehyde, and various amounts of enzyme in a final reaction volume of 1 ml. The enzyme reaction was started by the addition of the GbsA protein, and the absorbance was measured at 12 s intervals over a period of 4 min. The reduction of NAD^+ in the reaction mixture in the absence of the substrate glycine betaine aldehyde was negligible. One unit of enzyme activity is defined as the amount of the GbsA protein required to convert 1 μmol of NAD^+ to NADH per min.

Protein determination and SDS-polyacrylamide gel electrophoresis

Protein concentrations were determined using the Protein Assay Kit II (BioRad, Munich, Germany) with bovine serum albumin as the standard. The purification of GbsA was monitored by electrophoresis in SDS-12% polyacrylamide gels (Laemmli 1970). The proteins were visualized by staining with Coomassie Brilliant Blue or silver nitrate. Scanning of SDS-polyacrylamide gels was performed with a laser densiometer (Ultrascan XL; LKB, Freiburg, Germany).

Molecular mass analysis

For the molecular mass analysis of native GbsA, we used fractions from the Q-Sepharose Fast Flow column that were enriched in GbsA activity. The relative molecular mass of the enzyme was estimated by gel-filtration chromatography on a Superose 12 column (1 × 30 cm) in a 100 mM Tris-HCl buffer (pH 8.0). β-Amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as reference proteins. The Stokes radius of the native GbsA enzyme was determined from the chromatographic properties of GbsA on the Superose 12 column and was calculated according to the method of Siegel and Monty (1966). The values for $-(\log K_{av})^{1/2}$ were calculated with the equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the GbsA enzyme, V_0 the column void volume, and V_t the total column volume. Blue dextran (2,000 kDa) was used to determine the V_0. The sedimentation coefficient ($S_{20,w}$) of GbsA was determined by centrifugation through a continuous 10–35% (v/v) glycerol gradient. Partially purified GbsA (25 μg) was centrifuged through the gradient at 220,000 × *g* in a Sorvall TST 60.4 swing-out rotor at 4° C for 20 h. After the centrifugation run, 0.2-ml fractions were collected from the top of the tube and assayed for GbsA enzyme activity. Cytochrome *c* (12 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and alcohol dehydrogenase (150 kDa) were combined and sedimented through a parallel gradient; their positions in the gradient were determined by SDS-polyacrylamide gel electrophoresis and plotted against their known $S_{20,w}$ values (Ilag et al. 1991).

Results

Purification of the GbsA enzyme

For the purification of the *B. subtilis* glycine betaine aldehyde dehydrogenase GbsA, we first overproduced the enzyme in the heterologous host *E. coli* using a plasmid (pJB009) that expresses the *gbsA*$^+$ gene under the control of the bacteriophage T7ϕ10 promoter (Boch et al. 1996). The *E. coli* strain used for *gbsA* overexpression, strain PD141, carries a chromosomal deletion of the entire *betTIBA* gene cluster (Lamark et al. 1991), thus avoiding a possible contamination of GbsA preparations with the *betB*-encoded *E. coli* glycine betaine aldehyde dehydrogenase. Induction of the T7 RNA polymerase/T7ϕ10 promoter system (Studier and Moffat 1986) by the addition of IPTG resulted in the production of the GbsA polypeptide to an extent that it represented approximately 17% of the soluble cell proteins (Fig. 1). The GbsA protein migrates on SDS-12% polyacrylamide gels as a species with an apparent molecular mass of 62 kDa (Fig. 1), although the molecular mass deduced from the DNA sequence of the *gbsA* structural gene is 53.7 kDa (Boch et al. 1996). Such a difference between the calculated molecular mass and that inferred from the mobility of the protein on SDS-polyacrylamide gels has previously also been observed for the glycine betaine aldehyde dehydrogenase from spinach (Weretilnyk and Hanson 1990). We purified the GbsA protein from cleared cell lysates of strain PD141 (pJB009) by fractionated ammonium sulfate precipitation and two subsequent chromatographic steps on anion exchange (Q-Sepharose) and hydrophobic interaction

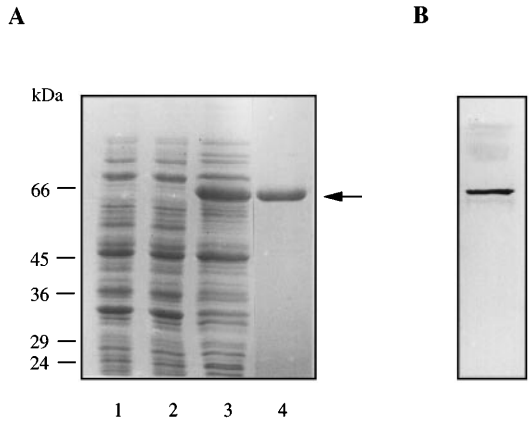

Fig.1 A, B Purification of the GbsA protein. **A** The proteins were electrophoretically separated on an SDS-12% polyacrylamide gel and were stained with Coomassie Brilliant Blue. *Lane 1* protein extract of strain PD141 (pPD100; vector) that was induced with 1 mM IPTG, *lane 2* protein extract from an uninduced culture of strain PD141 carrying the *gbsA*$^+$ plasmid pJB009, *lane 3* protein extracts from a culture of PD141 (pJB009) that was induced with 1 mM IPTG, and *lane 4* purified GbsA protein (1.5 μg). The molecular mass of marker proteins is indicated, and the location of the GbsA protein is marked with an *arrow*. **B** The purified GbsA protein (0.12 μg) was run on an SDS-12% polyacrylamide gel and stained with silver nitrate

Table 1 Purification of the glycine betaine aldehyde dehydrogenase GbsA of *Bacillus subtilis*. The *B. subtilis* GbsA protein was overproduced in a heterologous *Escherichia coli* host strain harboring a *gbsA*$^+$ plasmid under the control of the phage T7ϕ10-promoter/RNA polymerase expression system. Cell extract was prepared from 4 l of *E. coli* cells. One unit of enzyme activity is defined as the amount of protein required for the oxidation of 1 μM NAD^+ to NADH per min

Purification step	Activity (U)	Protein (mg)	Specific activity (U/mg protein)	Purification factor (-fold)	Yield (%)
Cell-free extract	131	185	0.4	–	100
Ammonium sulfate precipitation (55–75%)	115	97	1.2	1.7	73
Q-Sepharose	77	12	6.3	9.0	58
Phenyl-Sepharose	48	3	15.9	22.7	37

(phenyl-Sepharose) columns to apparent homogeneity (Fig. 1). The GbsA enzyme was purified approximately 23-fold, yielding a preparation with a specific activity of 16 units per mg of protein. The purified protein was stable in Tris-HCl, Hepes, or phosphate buffer in the presence of a reducing agent (10 mM DTT) and showed no loss of activity after incubation for 1 h at room temperature. It could be stored without glycerol at –80° C for several months without showing a detectable decrease in its enzyme activity. The results of a typical purification for the GbsA enzyme from 4 l of cell culture are summarized in Table 1.

Molecular mass of the GbsA enzyme

To deduce the subunit composition of the active GbsA enzyme, we determined the molecular mass of the partially purified protein under nondenaturing conditions by two independent methods: gel filtration on Superose 12 and rate zonal centrifugation in glycerol gradients. The GbsA protein was applied to an FPLC molecular sieving column that had been standarized with proteins of known molecular mass. Chromatography of GbsA on the Superose 12 column yielded a sharp activity peak in the elution position between the reference proteins bovine serum albumin (66 kDa) and alcohol dehydrogenase (150 kDa), corresponding to a molecular mass of 125 kDa (Fig. 2A). The chromatographic behavior of the GbsA protein on the Superose 12 column (Fig. 2B) also permitted us to deduce an apparent Stokes radius of 4.3 nm for the enzyme (Siegel and Monty 1966). GbsA activity in the glycerol gradients was also found in a position between the bovine serum albumin and alcohol dehydrogenase marker proteins (Fig. 2C). A sedimentation coefficient of $S_{20,w}$ of 6.0 for GbsA was deduced from a standard plot of the known $S_{20,w}$ values of the different reference proteins (Fig. 2D). This allowed us to calculate a native molecular mass of the GbsA protein of 109 kDa from its sedimentation coefficient (Martin and Ames 1961). Thus, both the gel-filtration chromatography

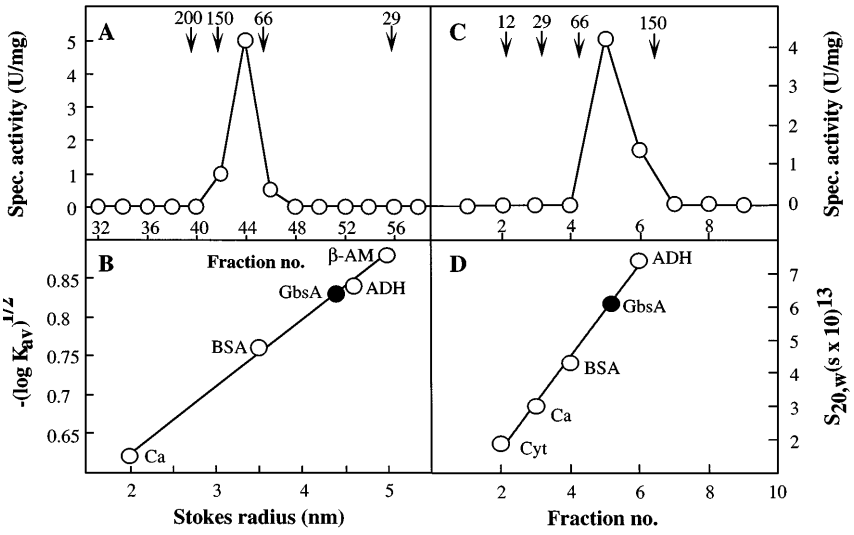

Fig. 2A–D Determination of the molecular mass and the Stokes radius of the GbsA protein. **A** Estimation of the native molecular mass by gel-filtration chromatography. Partially purified glycine betaine aldehyde dehydrogenase was chromatographed through a Superose 12 column. An aliquot of each fraction was assayed for glycine betaine aldehyde dehydrogenase activity. The following marker proteins were used to calibrate the column: β-amylase (*β-AM*; 200 kDa), alcohol dehydrogenase (*ADH*; 150 kDa), bovine serum albumin (BSA; 66 kDa), and carbonic anhydrase (Ca; 29 kDa). **B** Estimation of the Stokes radius of the glycine betaine aldehyde dehydrogenase from the data determined in **A**. The known Stokes radii were plotted against their $-(\log K_{av})^{1/2}$ (Siegel and Monty 1966). **C** Estimation of the molecular mass by sedimentation through glycerol gradients. Partially purified glycine betaine aldehyde dehydrogenase (25 μg) was centrifuged through a 10–35% glycerol gradient. A set of fractions was assayed for enzyme activity. The marker proteins cytochrome *c* (*Cyt*; 12 kDa), carbonic anhydrase (*Ca*; 29 kDa), bovine serum albumin (*BSA*; 66 kDa), and alcohol dehydrogenase (*ADH*; 150 kDa) were centrifuged through a parallel gradient, and their position in the gradient was subsequently determined on an SDS-12% polyacrylamide gel. **D** Estimation of the sedimentation coefficient ($S_{20,w}$) of the glycine betaine aldehyde dehydrogenase. The mobility of the proteins in the glycerol gradient is plotted against their known sedimentation coefficients ($S_{20,w}$)

and the centrifugation in glycerol gradients of GbsA gave comparable results for the molecular mass determination. We conclude from these data that the native enzyme is a homodimer composed of 53.7-kDa subunits.

Characterization and kinetic properties of the GbsA protein

The purified GbsA exhibited its greatest enzyme activity in the Tris-HCl buffer system [100 mM Tris-HCl (pH 8.0)]. The high activity of the *B. subtilis* enzyme in the Tris-HCl buffer contrasts with the inhibitory effect of this buffer system on the glycine betaine aldehyde dehydrogenase from *Amaranthus hypochondriacus* (Valenzuela-Soto and Muñoz-Clares 1994) and *E. coli* (Falkenberg and Strøm 1990). The pH-activity profile of GbsA showed a broad range of activity with an optimum at pH 8.0. We therefore used the Tris-HCl buffer system at a pH of 8.0 for all further determinations of the GbsA enzyme activity. GbsA showed specificity for glycine betaine aldehyde as substrate; no GbsA-catalyzed reduction of NAD^+ was observed with propionaldehyde, pivalaldehyde, isovaleraldehyde, acetaldehyde, or benzaldehyde in final concentrations of 0.5 or 50 mM (data not shown). The initial velocities for the enzymatic reaction of GbsA were determined at various concentrations of glycine betaine aldehyde and the cofactors NAD^+ or $NADP^+$. For these assays, the concentration of one substrate was varied, while that of the other compound was kept constant (Fig. 3). Substrate inhibition of enzymatic activity has been observed for various glycine betaine aldehyde dehydrogenases (Falkenberg and Strøm 1990; Valenzuela-Soto and Muñoz-Clares 1994). We found that GbsA activity was significantly inhibited when glycine betaine aldehyde levels exceeded 0.5 mM; we therefore used lower levels of this substrate for our kinetic experiments. We found that the GbsA protein could use both NAD^+ and $NADP^+$ as a cofactor for the enzyme reaction, but that NAD^+ was clearly preferred. The Michaelis constants (K_m) for glycine betaine aldehyde and the cofactors were determined from secondary plots of the intercepts of the Lineweaver-Burk plots (Fig. 3A, B) against reciprocal concentrations of the substrates (Fig. 3C, D). This derivation leads to K_m values independent of the concentration of the second substrate. From the experiments documented in Fig. 3, we calculated K_m values of 0.12 mM for glycine betaine aldehyde and 0.14 mM for NAD^+. The apparent K_m value for $NADP^+$ was approximately 1 mM (data not shown). The oxidation of glycine betaine aldehyde by the GbsA protein was effectively irreversible since no oxidation of NADH was observed with glycine betaine as substrate at concentrations of up to 10 mM.

Effect of salts and osmoprotectants on GbsA activity

B. subtilis accumulates large amounts of K^+ during water stress (Whatmore and Reed 1990). We therefore investigated the influence of this ion on the GbsA enzyme activity. KCl had an activating effect at moderate concentrations; the enzymatic activity of GbsA was stimulated to approximately 150% when 0.2 M KCl was added to the assay mixture (Fig. 4). Higher concentrations of this salt resulted in a progressive decline of this maximal activity. However, the GbsA enzyme was quite salt-tolerant since it retained 88% of its original activity even in the presence of 2.4 M KCl (Fig. 4). NaCl had a similar influence on the

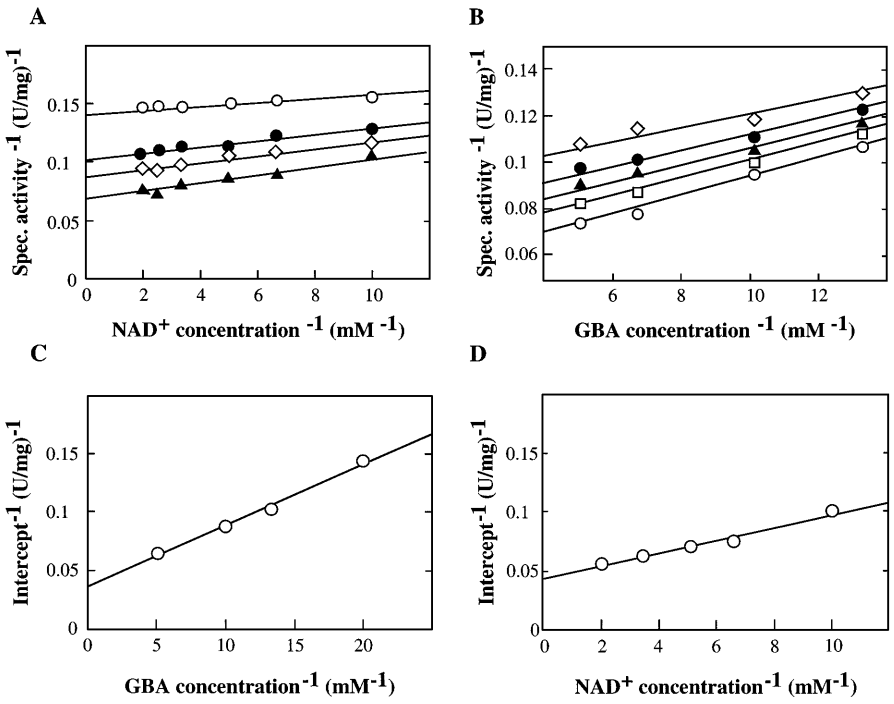

Fig. 3A–D Double-reciprocal plots of the enzymatic activity of GbsA. The initial velocities of the enzymatic reactions were determined with **A** NAD^+ as the variable substrate at various fixed concentrations of glycine betaine aldehyde (*GBA*): ○ 0.05 mM, ● 0.075 mM, ◇ 0.1 mM, and ▲ 0.2 mM. **B** Glycine betaine aldehyde was used as the variable substrate and various fixed concentrations of NAD^+ were employed: ◇ 0.1 mM, ● 0.15 mM, ▲ 0.2 mM, □ 0.3 mM, and ○ 1 mM). **C** and **D** show secondary plots of the intercepts against the reciprocal concentrations of the fixed substrate from **A** and **B**, respectively

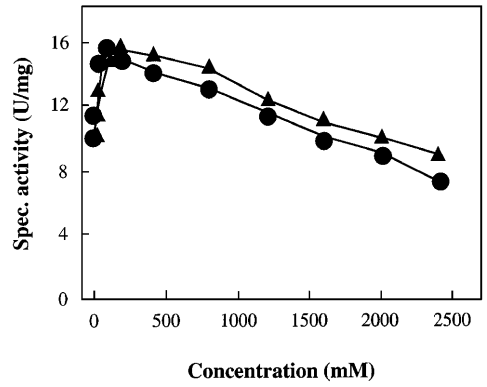

Fig. 4 Effect of salts on the activity of the GbsA protein. Enzyme assays were performed in 0.1 M Tris-HCl containing salts (▲ KCl, ● NaCl) in concentrations between 0 and 2.4 M. The enzyme activity was plotted against the concentration of salt in the assay

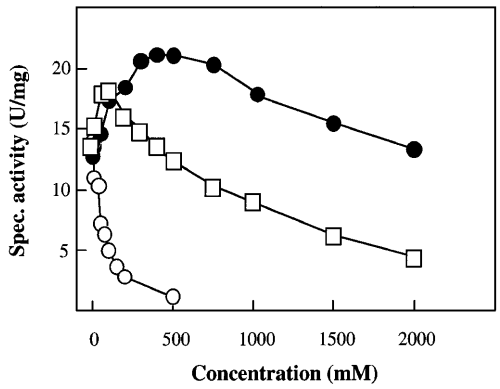

Fig. 5 Effect of amino acids and osmoprotectants on the activity of GbsA. Enzyme assays were performed in the presence of varying concentrations of ○ choline, □ glycine betaine, or ● proline. The specific activity of the enzyme under these conditions was plotted against the concentration of the added compound

GbsA activity (Fig. 4), suggesting that the nature of the cation had no strong effect.

Glycine betaine, the end product of the GbsA-mediated enzyme reaction, had a dual effect on the purified GbsA protein. At moderate concentrations, glycine betaine stimulated the GbsA enzymatic activity to approximately 140% of its original value, whereas at higher concentrations it inhibited the enzyme activity somewhat (Fig. 5). A *gbsA*$^+$ *B. subtilis* mutant defective in the *gbsB*-encoded type-III alcohol dehydrogenase was able to grow in high-osmolarity medium when glycine betaine aldehyde, but not choline, was added to the medium. These physiological data suggest that the GbsA protein cannot use choline as a substrate for glycine betaine production. Indeed, we did not detect any reduction of NAD^+ by the purified GbsA enzyme in the presence of choline. We found, however, that even low concentrations of choline strongly inhibited GbsA (Fig. 5). A kinetic analysis of the inhibitory effect of choline on the enzymatic function of GbsA showed that choline competitively inhibited GbsA with a K_i value of 7 mM (data not shown).

Table 2 Effects of various salts and osmoprotectants on the activity of the GbsA glycine betaine aldehyde dehydrogenase. The specific activity of the purified enzyme was determined under standard assay conditions [100 mM Tris-HCl (pH 8.0, 1 mM NAD^+, 0.5 mM glycine betaine aldehyde, and 10 mM DTT] containing 100, 500, or 1,500 mM of the various compounds. The enzyme activity is given in % with respect to the specific enzyme activity in a standard assay without additional substances (100%) (*nd* not determined)

Compound	100 mM	500 mM	1,500 mM
KCl	143	146	105
NaCl	151	138	100
Proline	136	165	121
Glycine betaine	138	93	46
Choline	36	8	nd
Homobetaine	111	128	156
Carnitine	4[a]	nd	nd
Ectoine	113	100	48
Trehalose	94	85	nd
Glycine	98	96	79

[a] At a concentration of 25 mM

High concentrations of proline are amassed by the *B. subtilis* cells through de novo synthesis when the cells are subjected to a sudden increase in the external osmolarity (Whatmore et al. 1990). We therefore tested the influence of this compatible solute on the activity of GbsA over a wide range of concentrations. Proline strongly increased the enzyme activity. The maximal effect was observed at concentrations of approximately 0.5 M when the GbsA-mediated oxidation of glycine betaine aldehyde reached 167% of its original value (Fig. 5). The enzyme was not inhibited even at a concentration of 2 M proline and exhibited 106% of its original activity (Fig. 5). We analyzed whether this was a specific effect of proline, or whether there was a general stimulation of the GbsA enzyme by osmoprotective compounds. The amino acid glycine neither stimulated nor inhibited GbsA activity to any significant extent up to concentrations of 1.5 M (Table 2). The compatible solute ectoine, a cyclic amino acid, and the sugar trehalose are known to stabilize proteins and cell components under high osmotic conditions (Csonka and Hanson 1991; Galinski and Trüper 1994; Yancey 1994). Neither compound had a stimulating effect on GbsA activity. In contrast, the osmoprotectant homobetaine, a structural analogue of glycine betaine with one additional methylene group in the main carbon chain, had a stimulating effect similar to glycine betaine (Table 2). L-Carnitine, which is also structurally related to glycine betaine and choline, almost completely inhibited the GbsA enzyme even at concentrations as low as 25 mM (Table 2). Hence, the activity of the GbsA enzyme is not stimulated in a general manner by the presence of compatible solutes, and it appears that proline and homobetaine have specific effects on the function of the GbsA protein.

Discussion

The osmoprotectant glycine betaine is accumulated by the soil bacterium *B. subtilis* cells as a metabolically inert stress compound (Boch et al. 1994). Its synthesis from its precursors choline or glycine betaine aldehyde confers a high degree of osmotic tolerance and, consequently, is an important facet of the specific adaptation reactions of *B. subtilis* to high osmolarity stress (Boch et al. 1996). We report in the present communication the purification and characterization of one of the enzymes from the osmoregulatory choline-glycine betaine pathway, the glycine betaine aldehyde dehydrogenase GbsA. Deletion of the structural gene (*gbsA*) for this enzyme from the *B. subtilis* chromosome completely abolishes the production of glycine betaine from the precursor glycine betaine aldehyde (Boch et al. 1996), attesting to the key role of GbsA for glycine betaine synthesis in vivo. The in vitro characterization of the purified GbsA protein now provides direct experimental evidence for its previously proposed enzymatic function (Boch et al. 1996). Our database searches revealed that the GbsA protein is a member of a large superfamily of specialized and nonspecialized aldehyde dehydrogenases from both prokaryotes and eukaryotes (Habenicht et al. 1994). Within this superfamily, the *B. subtilis* GbsA protein shows strong sequence identity (between 37 and 45%) to glycine betaine aldehyde dehydrogenases from plants (*Beta vulgaris*, *Spinacia oleraceae*, *Hordeum vulgare*, *Atriplex hortensis*, and *Sorghum bicolor*) and bacteria (*E. coli* and *Sinorhizobium meliloti*) with a known involvement in the adaptation to high osmolarity and drought.

Overproduction of the *B. subtilis* GbsA protein in the heterologous *E. coli* host permitted the development of a simple, three-step purification procedure for this enzyme. The kinetic parameters of GbsA for its substrate glycine betaine aldehyde (K_m = 125 μM) and its cofactor NAD^+ (K_m = 143 μM) are comparable to those of glycine betaine aldehyde dehydrogenases purified from bacterial, fungal, plant, animal, and human cells (Nagasawa et al. 1976; Mori et al. 1980; Pan et al. 1981; Weretilnyk and Hanson 1989; Falkenberg and Strøm 1990; Valenzuela-Soto and Muñoz-Clares 1994; Chern and Pietruszkor 1995). Two procedures, gel filtration and sedimentation centrifugation through a glycerol gradient, suggest that the GbsA enzyme constitutes a homodimer with 53.7-kDa subunits. It shares this native composition with the glycine betaine aldehyde dehydrogenases from the plants *S. oleracea* and *A. hypochondriacus* (Weretilnyk and Hanson 1990; Valenzuela-Soto and Muñoz-Clares 1994), whereas the corresponding enzymes from the gram-negative bacterium *E. coli*, the fungus *Cylindrocarpon didymum*, and human liver are tetramers (Mori et al. 1980; Valenzuela-Soto and Muñoz-Clares 1994; Chern and Pietruszkor 1995). The *B. subtilis* GbsA protein is specific for glycine betaine aldehyde, and this high substrate specificity is also manifested in the homologous enzymes participating in the osmoregulatory choline-glycine betaine pathways in *E. coli* (Falkenberg and Strøm 1990), *Pseudomonas aeruginosa* (Nagasawa et al. 1976), plants (Weretilnyk and Hanson 1989; Valenzuela-Soto and Muñoz-Clares 1994), and the horseshoe crab (Dragolovich and Pierce 1994). A high substrate specificity of these enzymes for the intermediate in glycine betaine synthesis is consistent with their highly specialized physiological function in the cellular defence against high osmolarity stress. In contrast, the glycine betaine aldehyde dehydrogenase isolated from human liver displays a broad substrate specificity, and this enzyme probably functions in this organ in the synthesis of glycine betaine as a methyl donor rather than as an osmoprotectant (Chern and Pietruszkor 1995).

In osmoregulating *B. subtilis* cells, the GbsA protein is initially faced with substantial intracellular concentrations of K^+ and proline (Whatmore et al. 1990; Whatmore and Reed 1990). Analysis of the purified GbsA protein revealed that this enzyme is highly tolerant towards K^+ (Fig. 4) and that proline has a strong stimulating influence on GbsA activity (Fig. 5). Even in the presence of 1.5 M proline, the enzyme displayed 121% of its original activity. Glycine betaine is accumulated in many osmoregulating microbial cells up to molar concentrations (Whatmore et al. 1990; Csonka and Hanson 1991; Galinski and Trüper 1994). Obviously, the *B. subtilis* GbsA protein must function in the presence of large amounts of its reaction product. The enzyme was fully active even at a glycine betaine concentration of 0.5 M and still exhibited 66% of its initial activity in the presence of 1 M glycine betaine (Fig. 5). It is apparent that the tolerance of the GbsA protein to high salt, proline, and its reaction product are key features for an enzyme participating in the production of the osmoprotectant glycine betaine in *B. subtilis*. These characteristics ensure that the glycine betaine aldehyde dehydrogenase GbsA can effectively function under those conditions that are prevalent in *B. subtilis* cells exposed to a high osmolarity environment.

Acknowledgements We appreciate the expert technical assistance of J. Gade and the help of V. Koogle in preparing the manuscript. We thank our colleagues W. Buckel and D. Jahn for their advice throughout this study and are grateful to B. Kempf for critical reading of the manuscript. Financial support was provided by the Deutsche Forschungsgemeinschaft (SFB-395) the Graduiertenkolleg Enzymchemie, and the Fonds der Chemischen Industrie.

References

Boch J, Kempf B, Bremer E (1994) Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. J Bacteriol 176:5364–5371

Boch J, Kempf B, Schmid R, Bremer E (1996) Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterization of the *gbsAB* genes. J Bacteriol 178:5121–5129

Chern M-K, Pietruszkor R (1995) Human aldehyde dehydrogenase E3 isoenzyme is a betaine aldehyde dehydrogenase. Biochem Biophys Res Commun 213:561–568

Csonka LN, Hanson AD (1991) Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol 45:569–606

Dersch P, Fsihi H, Bremer E (1994) Low-copy-number T7 vectors for selective gene expression and efficient protein overproduction in *Escherichia coli*. FEMS Microbiol Lett 123:19–26

Dragolovich J, Pierce SK (1994) Characterization of partially purified betaine aldehyde dehydrogenase from horseshoe crab (*Limulus polyphemus*) cardiac mitochondria. J Exp Zool 270: 417–425

Falkenberg P, Strøm AR (1990) Purification and characterization of osmoregulatory betaine aldehyde dehydrogenase of *Escherichia coli*. Biochim Biophys Acta 1034:253–259

Galinski EA, Trüper HG (1994) Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol Rev 15:95–108

Goldmann A, Boivin C, Fleury V, Message B, Lecoeur L, Maille M, Tepfer D (1991) Betaine use by rhizosphere bacteria: genes essential for trigonelline, stachydrine, and carnitine catabolism in *Rhizobium meliloti* are located on pSym in the symbiotic region. Mol Plant Microbe Interact 4:571–578

Habenicht A, Hellman U, Cerff R (1994) Non-phosphorylating GAPDH of higher plants is a member of the aldehyde dehydrogenase superfamily with no sequence homology to phosphorylating GAPDH. J Mol Biol 237:165–171

Hecker M, Schumann W, Völker U (1996) Heat-shock and general stress response in *Bacillus subtilis*. Mol Microbiol 19:417–428

Holmström K-O, Welin B, Mandal A, Kristiansdottir I, Teeri TH, Lamark T, Strøm AR, Palva ET (1994) Production of the *Escherichia coli* betaine-aldehyde dehydrogenase, an enzyme required for the synthesis of the osmoprotectant glycine betaine, in transgenic plants. Plant J 6:749–758

Ilag LL, Jahn D, Eggertsson G, Söll D (1991) The *Escherichia coli hemL* gene encodes glutamate 1-semialdehyde aminotransferase. J Bacteriol 173:3408–3413

Kappes R, Kempf B, Bremer E (1996) Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. J Bacteriol 178:5071–5079

Kempf B, Bremer E (1995) OpuA: an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in *Bacillus subtilis*. J Biol Chem 270:16701–16713

Laemmli UK (1970) Cleavage of structural proteins during assembly of bacteriophage T4. Nature 227:680–685

Lamark T, Kaasen I, Eshoo MW, Falkenberg P, McDougall J, Strøm AR (1991) DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. Mol Microbiol 5:1049–1064

Le Berre A, Delacroix A (1973) L'addition des sels d'amines tertiaires aux composes ethylenique electrophiles. Bull Soc Chim 7–8:2404–2408

Lin Y, Hansen JN (1995) Characterization of a chimeric *proU* operon in a subtilin-producing mutant of *Bacillus subtilis* 168. J Bacteriol 177:6874–6880

Lucht JM, Bremer E (1994) Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. FEMS Microbiol Lett 14:3–20

Martin RG, Ames BN (1961) A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J Biol Chem 236:1372–1379

Miller JH (1992) A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Miller KJ, Wood JM (1996) Osmoadaptation by rhizosphere bacteria. Annu Rev Microbiol 50:101–136

Mori N, Kawakami B, Hyakutome K, Tani Y, Yamada H (1980) Characterization of betaine aldehyde dehydrogenase from *Cylindrocarpon didymum* M-1. Agric Biol Chem 44:3015–3016

Nagasawa T, Kawabata Y, Tani Y, Ogata K (1976) Purification and characterization of betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa* A-16. Agric Biol Chem 40:1743–1749

Pan S, Moreau AR, Yu C, Huang AHC (1981) Betaine accumulation and betaine-aldehyde dehydrogenase. Plant Physiol 67: 1105–1108

Rathinasabapathi B, McCue KF, Gage DA, Hanson AD (1994) Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. Planta 193:155–162

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol 44:357–384

Siegel LM, Monty KJ (1966) Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases. Biochim Biophys Acta 112:346–362

Studier FW, Moffat BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189:113–130

Valenzuela-Soto EM, Muñoz-Clares RA (1994) Purification and properties of betaine aldehyde dehydrogenase extracted from detached leaves of *Amaranthus hypochondriacus* L. subjected to water deficit. J Plant Physiol 143:145–152

von Blohn C, Kempf B, Kappes RM, Bremer E (1997) Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B. Mol Microbiol 25:175–187

Weretilnyk EA, Hanson AD (1989) Betaine aldehyde dehydrogenase from spinach leaves: purification, in vitro translation of the mRNA, and regulation by salinity. Arch Biochem Biophys 271:53–63

Weretilnyk EA, Hanson AD (1990) Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. Proc Natl Acad Sci USA 87:2745–2749

Whatmore AM, Reed RH (1990) Determination of turgor pressure in *Bacillus subtilis*: a possible role for K^+ in turgor regulation. J Gen Microbiol 136:2521–2526

Whatmore AM, Chudek JA, Reed RH (1990) The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. J Gen Microbiol 136:2527–2535

Yancey PH (1994) Compatible and counteracting solutes. In: Strange K (ed) Cellular and molecular physiology of cell volume regulation. CRC Press, Boca Raton, FL, USA, pp 81–109