Interaction of the Membrane-bound GlnK-AmtB Complex with the Master Regulator of Nitrogen Metabolism TnrA in Bacillus subtilis*\(^\text{S}\)

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Many bacteria use a GlnK-AmtB complex to control nitrogen assimilation. In this study, we report on biochemical properties of the PII homologue GlnK in Bacillus subtilis. By using biochemical and genetic analysis, we show that the formation of the GlnK-AmtB complex depends on the presence of ATP and TnrA. The trimeric GlnK protein forms a tight complex with the membrane-bound ammonium transporter AmtB (NrgA), from which it can be relieved by millimolar concentrations of ATP. The GlnK subfamily proteins are encoded by genes arranged in conjunction with genes encoding the ammonium permease AmtB. The trimeric PII proteins may bind up to three ATP and three 2-oxoglutarate molecules, which are the effectors of PII signal transduction proteins occurring in bacteria, Archaea, and plants. They are the product of the nitrogen-assimilation enzymes GlnB and NifL, are central players in sensing and transmitting signals of the cellular nitrogen status (reviewed in Ref. 1).

Most bacteria can assimilate nitrogen from simple organic and/or inorganic nitrogen compounds. The utilization of the various sources as well as the activity of the nitrogen assimilation enzymes is strictly regulated in almost all bacteria. Proteins of the PII family, consisting of the subfamilies GlnB, GlnK and NifL, are central players in sensing and transmitting signals of the cellular nitrogen status (reviewed in Ref. 1).

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\(^\text{1}\) The on-line version of this article (available at http://www.jbc.org) contains Construction of Plasmids and detailed Purification Protocols and Refs. 1–6.

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\(^\text{1}\) The abbreviations used are: GS, glutamine synthetase; ITC, isothermal titration calorimetry; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; TOF, time of flight; SMM, Spizizen minimal medium; 2-OG, 2-oxoglutarate; ESI, electrospray ionization.

P\(_\text{II}\) proteins are widespread and highly conserved signal transduction proteins occurring in bacteria, Archaea, and plants and play pivotal roles in controlling nitrogen assimilation metabolism. This study reports on biochemical properties of the P\(_\text{II}\) homologue GlnK (originally termed NrgB) in Bacillus subtilis (BsGlnK). Like other P\(_\text{II}\) proteins, the native BsGlnK protein has a trimeric structure and readily binds ATP in the absence of divalent cations, whereas 2-oxoglutarate is only weakly bound. In contrast to other P\(_\text{II}\)-like proteins, Mg\(^{2+}\) severely affects its ATP-binding properties. BsGlnK forms a tight complex with the membrane-bound ammonium transporter AmtB (NrgA), from which it can be relieved by millimolar concentrations of ATP. Immunoprecipitation and co-localization experiments identified a novel interaction between the BsGlnK-AmtB complex and the major transcription factor of nitrogen metabolism, TnrA. In vitro in the absence of ATP, TnrA is completely tethered to membrane (AmtB)-bound GlnK, whereas in extracts from BsGlnK- or AmtB-deficient cells, TnrA is entirely soluble. The presence of 4 mM ATP leads to concomitant solubilization of BsGlnK and TnrA. This ATP-dependent membrane re-localization of TnrA by BsGlnK/AmtB may present a novel mechanism to control the global nitrogen-responsive transcription regulator TnrA in B. subtilis under certain physiological conditions.

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Interaction of GlnK and TnrA in B. subtilis

$P_h$ regulates the key enzyme of arginine biosynthesis, N-acetylglutamate kinase, by complex formation (20). The $P_h$-parologue GlnK controls ammonium transport by direct interaction with the high affinity transporter AmtB (21). Recently, it could be demonstrated that AmtB-bound $P_h$ is able to sequester the regulatory enzyme dinitrogenase reductase glycohydrolase (DraG) to the membrane following ammonium shock (22).

In the Gram-positive soil bacterium Bacillus subtilis, transcriptional regulation in response to the nitrogen regime involves other factors than those identified in proteobacteria, corynebacteria, or cyanobacteria. Two different transcription factors, GlnR and TnrA, are involved in transcriptional nitrogen control and in the response to the cellular nitrogen status (for review, see Ref. 23). Both transcriptional regulators belong to the MerR family of DNA-binding regulatory proteins (24, 25). GlnR represses gene expression under nitrogen excess conditions. There are only three known targets for GlnR in the B. subtilis genome, the glnRA operon, consisting of its own and the glutamine synthetase gene (25), the tnrA gene (26), and the ureABC operon, encoding urease (27). By contrast, TnrA activates gene expression under nitrogen-limiting conditions, in particular the genes for ammonium uptake (amtBglnK, originally termed nrgAnrgB; see Ref. 28), for assimilatory nitrate reduction (nasDEF; see Ref. 29), its own gene (tnrA; see Ref. 23), and other genes of nitrogen assimilation (30). Moreover, TnrA acts as a repressor for the genes gltAB and glnRA (31) as well as some other genes (30). Concerning the mechanism of control of TnrA activity, it could be shown that feedback-inhibited GS directly interacts with TnrA and blocks its DNA binding activity under conditions of good ammonium supply (32).

Compared with other bacterial systems, the only conserved nitrogen regulatory factor in B. subtilis is a homologue of the P$_h$ family. It belongs to the GlnK subfamily (BsGlnK), based on the fact that its gene forms an operon with an $amtB$ homologue (28). The BsGlnK protein differs from other P$_h$ proteins by the lack of potential modification sites in the T-loop (Ser-49 or Tyr-51). Indeed, it has been suggested that BsGlnK might not be modified by covalent modification (28, 33). Recently, BsGlnK was shown to bind to the cytoplasmic membrane in an AmtB-dependent manner (33), suggesting that it regulates the ammonium transporter like other GlnK-proteins do (13, 21).

Taking into consideration the apparent unique properties of nitrogen control mechanisms in B. subtilis (23), the function of P$_h$ signaling in this organism was poorly understood. This work aimed at the elucidation of the signaling properties of P$_h$ in B. subtilis by characterizing its biochemical properties and by identifying potential interaction partners. During our studies, we identified interaction of the transcriptional regulator TnrA with GlnK and characterized its dependence on GlnK-AmtB complex formation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—B. subtilis strains used in this study, strain 168 (wild type), the AmtB-deficient strain GP254, and BsGlnK-deficient mutant GP 253, were described previously (33). E. coli strains XL1 and BL21(DE3) (34) were used for cloning experiments and protein expression, respectively. B. subtilis was grown in Spizizen minimal medium (SMM) (35) containing glucose (0.5% (w/v)) as carbon source. Sources of nitrogen were added as indicated. Tryptophan was added to a final concentration of 50 mg liter$^{-1}$. E. coli was grown in Luria-Bertani (LB) medium, and transformants were selected on plates containing ampicillin (100 $\mu$g ml$^{-1}$). LB plates were prepared by addition of 15 g of agar-agar (Carl Roth) per liter of medium.

**DNA Methods**—DNA isolation, restriction analysis, and cloning procedures were performed according to standard procedures (36). Restriction enzymes were from New England Biolabs or MBI Fermentas; oligonucleotides were purchased from MWG Biotech. DNA sequence analysis was performed on an ABI Prism 310 genetic analyzer (Applied Biosystems, PerkinElmer Life Sciences) according to the manufacturer’s instructions.

**Overproduction and Purification of Proteins**—BsGlnK, BsGlnK-ST, TnrA, and GS-ST—Construction of plasmids for overproduction of proteins used in this study and detailed purification protocols are given in the Supplemental Material.

**ATP Binding to BsGlnK**—Determination of ATP binding in the presence or absence of 2-oxoglutarate was initially performed by the ultrafiltration method described previously (8). UV cross-linking of $[^\gamma-32P]ATP$ (Amersham Biosciences) or $[^8-14C]ADP$ (Moravek Biochemicals, Inc.) to BsGlnK was carried out as described (20). Briefly, 7.5 $\mu$g of BsGlnK was incubated in 25 $\mu$L of 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.005% Nonidet P-40, and 5 $\mu$L of bovine serum albumin with $[^\gamma-32P]ATP$ (0.1 $\mu$Ci/100 $\mu$L) or $[^8-14C]ADP$ (0.05 $\mu$Ci/50 $\mu$L) in the presence or absence of divalent cations and various amounts of 2-oxoglutarate as indicated. After 10 min on ice in the dark, the samples were exposed for 20 min to UV light (UV-lamp 254 nm, 4 watts; Heraeus) at a distance of 1 cm. The samples were then mixed with 9 $\mu$L of 4 $\times$ SDS-loading buffer, heated for 4 min to 95 °C, and separated by 15% SDS-PAGE. The gels were stained with Coomassie Blue G-250, dried, and exposed to phosphor-screens (Bio-Rad) for 72 h in the case of $^{32}$P labeling and for 1 week in the case of $^{14}$C labeling. The phosphor-screens were scanned with the Phosphorimager system (Personal Imager FX; Bio-Rad) at 200 $\mu$m resolution.

**Isothermal Titration Calorimetry (ITC)**—ITC experiments were performed on a VP-ITC microcalorimeter (MicroCal, LLC) in 50 mM HEPES-NaOH, pH 7.5, 50 mM KCl at 30 °C with BsGlnK (72–78 $\mu$m monomer concentration) in the cell (cell volume = 1.4285 ml) and ATP (2 $\mu$m) or 2-oxoglutarate (20 mM) in the syringe. Depending on the experiment, ATP or 2-oxoglutarate was titrated into BsGlnK by injecting 100 times 2 $\mu$L or 50 times 4 $\mu$L, respectively, with stirring at 450 rpm. Data for ATP binding to BsGlnK were fitted to a single site binding equation as well as to a model assuming three independent binding sites using the MicroCal ORIGIN software.

**Immunoblot Analysis**—Purified nontagged BsGlnK (550 $\mu$L) and TnrA (250 $\mu$L) and Strep-tagged GS (630 $\mu$L) were used to generate polyclonal antibodies in rabbits (Pineda Antikörperf-Service). For immunoblot analysis, B. subtilis cell-free extracts were separated on 15% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by semi-dry electroblotting. Antibodies were visualized by using anti-rabbit IgG-POD secondary antibodies (Sigma).
and the Lumilight detection system (Roche Diagnostics). For control experiments, the presence of GS was assayed using polyclonal antibodies directed against B. subtilis GS.

Non-denaturing PAGE Analysis of BsGlnK—To reveal a possible modification of BsGlnK, the electrophoretic mobility of the native BsGlnK protein was determined as described previously by immunoblot analysis of non-denaturing gels (37).

Preparation of Membrane Fractions—Overnight cultures of the appropriate B. subtilis cells, which had been grown with 20 mM NaNO₃ as nitrogen sources, were diluted to an OD₆₀₀ of 0.1 with Spizizen’s minimal medium (20 mM NaNO₃ final concentration). Cells were harvested at late exponential phase of growth at an OD₆₀₀ of about 0.8 by centrifugation (14,000 rpm, 10 min, 4°C), resuspended in 500 μl of disruption buffer A and broken in the RiboLyser (Hybaid). The cellular debris was removed by centrifugation (3,500 × g, 2 min, 4°C), and the fractions of the cell-free extract were separated by ultracentrifugation (100,000 × g, 1 h, 4°C). The supernatant was divided into equal parts in an upper (S1) and a lower (S2) fraction. The sediment (P1) was resuspended in the initial volume of buffer A, and a part of P1 was centrifuged again as before. The supernatant was fractionated once more in an equal upper (S3) and lower (S4) part, and the pellet was resuspended in buffer A in the same volume of P1 as it was used for the second centrifugation step. The fractions were analyzed by Western blot analysis using antibodies against BsGlnK or TnrA, respectively. GS-specific antibodies were used as a control.

Immunoprecipitations—For immunoprecipitation experiments, cultures of B. subtilis were grown in SMM with 20 mM NaNO₃ to an OD₆₀₀ of 1.5 by centrifugation, resuspended in buffer I (50 mM HEPES-NaOH, pH 7.0, 50 mM KCl, 100 mM EDTA, 2 mM MgCl₂, 1 mM benzamidine), and broken by using the RiboLyser. After centrifugation (3,500 × g, 2 min, 4°C) to remove debris and unbroken cells, the supernatant was subjected to a 1-h ultracentrifugation step (100,000 × g, 4°C). The pellet was washed once with buffer I and finally resuspended in the same buffer. 1.5 mg of the total protein amount of the cellular fractions was used for immunoprecipitation. The extracts were diluted with detergent-containing buffer (NET buffer I: 50 mM Tris-Cl, pH 7.0, 150 mM NaCl, 0.1% (v/v) nonionic detergent Nonidet P-40, 1 mM EDTA) (36) to a total volume of 1.5 ml, and following a 15-min incubation at 24°C, the sample was briefly centrifuged (14,000 rpm, 30 s) to remove debris. After addition of 20 μl of polyclonal antibodies against GlnK or TnrA, respectively, and a 1-h incubation at 24°C, 20 μl of protein A-agarose was added. An incubation of 1 h at 4°C followed. After centrifugation (14,000 rpm, 30 s, 4°C), the sediment was washed twice with NET buffer I, once with NET buffer II (NET buffer I with 500 mM NaCl), and once with buffer IP (10 mM Tris-Cl, pH 7.5, 0.1% (v/v) Nonidet P-40). The pellet was resuspended in 100 μl of 1X SDS sample buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% (w/v) SDS, 10% (v/v) glycerol, 1% (w/v) bromphenol blue) and heated for 5 min at 95°C. The immunoprecipitations were analyzed both by immunoblot analysis and by Coomassie Blue G-250 staining of the gels.

Matrix-assisted Laser Desorption Ionization—Mass Spectrometry (MALDI-MS)—MALDI-TOF-MS was carried out in cooperation with the Department of Analytic Chemistry (D. Kirsch, University of Giessen). Coomassie Blue-stained spots were isolated from the SDS gel, and the excised gel pieces were destained for 20 min at 30°C with 500 μl of 100 mM Tris-Cl, pH 8.5, in 50% acetonitrile under permanent shaking. Digestion buffer D (100 mM Tris-Cl, pH 8.1, 10% (v/v) acetonitrile) was added, and after 30 min at 30°C, the supernatant was discarded, and the gel pieces were dried under vacuum to 20% of the initial gel volume in a speedvac concentrator (Savant). Tryptic in-gel digestion was started after addition of 12.5 ng/μl (375 ng) trypsin (sequence grade, Promega) in 100 mM Tris-Cl, pH 8.1, 1 mM CaCl₂, 1 mM acetonitrile, and 6 mM acetic acid. The gel pieces were incubated overnight at 37°C. The digestion was stopped by addition of 100 μl of 75% (v/v) acetonitrile and 2% (v/v) trifluoroacetic acid, and the tryptic peptides were eluted and then analyzed by MALDI-TOF-MS using an ALADIM II mass spectrometer (constructed by the Department of Analytic Chemistry, University of Giessen) equipped with an N₂-UV laser (337 nm, 3-ns pulse length) and a two-stage reflector. External mass calibration was performed close to each sample spot using calibration mixtures. The search criteria for the received molecular masses were set to a mass accuracy of ±0.15 Da and preferably none (maximum of one) mis-caleded peptides per protein. Proteins were considered as identified when more than 30% of the amino acid sequence was covered by the identified peptides and four or more peptides matched the search criteria.

RESULTS

Purification of BsGlnK and BsGlnK-ST and Determination of the Molecular Weight—The B. subtilis glnK (nrgB) gene was overproduced in E. coli BL21(DE), and the resulting BsGlnK was purified to apparent electrophoretic homogeneity (see Supplemental Material). The purified protein was used for determination of native molecular weight as well as for the production of antibodies. The size of the native BsGlnK was analyzed by fast protein liquid chromatography gel filtration experiments on a Superdex 200 column (see Supplemental Material), revealing an apparent size of about 34 kDa. With a subunit size of 12.68 kDa, calculated from the glnK coding sequence, this is consistent with a trimeric structure of BsGlnK.

Binding of [³²P]ATP or [¹⁴C]ADP to BsGlnK—Based on the highly conserved amino acid sequence and subunit composition, binding of ATP and 2-oxoglutarate was suggested as a common property of proteins of the PII family (5, 7, 8). Initial attempts to detect ATP binding of BsGlnK by using the buffer conditions (including MgCl₂) and the ultrafiltration procedure that was used to measure ATP binding of E. coli and Synechococcus elongatus PII proteins (7, 8), failed to reveal binding. Therefore, ATP binding at higher ligand concentrations and with different buffer conditions was tried using UV photolabeling experiments with [³²P]ATP or [¹⁴C]ADP, a method that was successfully employed previously (20). As shown in Fig. 1, an appreciable amount of ATP binding to BsGlnK could be detected in the absence of divalent cations, and binding was slightly enhanced in the presence of 2-oxoglutarate. In the presence of 2 mM MgCl₂, the amount of [³²P]ATP that bound to BsGlnK was strongly reduced. However, 2-oxoglutarate partially rescued ATP binding to BsGlnK. By contrast, in the pres-
ence of 2 mM MnCl₂, ATP binding was not affected by 2-oxoglutarate. Binding analysis using [¹⁴C]ADP revealed no detectable photoaffinity labeling (data not shown).

To gain further insights into the ligand binding properties of BsGlnK, ITC experiments were performed. As shown in Fig. 2, A and B, BsGlnK exhibits high affinity toward ATP in the absence of Mg²⁺ ions, irrespective of the absence or presence of 2-oxoglutarate. When the data are fitted according to a model assuming three independent binding sites, the $K_d$ value for ATP binding by BsGlnK is about 17.3 μM in the absence and 20.3 μM in the presence of 2-oxoglutarate, with a respective stoichiometry of 2.07 or 2.1 per PII trimer. According to a binding model assuming three sequential bindings, all three sites may be occupied, irrespective of the presence or absence of 2-oxoglutarate (data not shown). In contrast to ATP, 2-oxoglutarate is bound much less strongly (Fig. 2C) with an estimated dissociation constant in the millimolar range, and its binding is apparently not affected by ATP (not shown). As already observed by UV cross-linking, the presence of MgCl₂ strongly affected ATP binding. In the presence of 1 mM MgCl₂, addition of ATP did not yield calorimetric signals, neither in the absence (Fig. 2D) nor in the presence of 2-oxoglutarate (not shown). Experiments with submillimolar concentrations of MgCl₂ showed that already 0.1 mM MgCl₂ strongly reduced the signals detected by ITC. In the presence of 0.1 mM MgCl₂, 2-oxoglutarate favored ATP binding (compare Fig. 2E and F). Fitting these data according to a model assuming three sequential binding sites, one site would be occupied in the absence of 2-oxoglutarate, whereas three sites may be occupied in its presence. Calculating the thermodynamic constants for ATP binding in the absence of Mg²⁺.
ions results in a $\Delta H$ of $-11,070$ cal/mol, a $\Delta S$ of $-14.7$, and a $\Delta G$ of $-6.61$ kcal/mol, whereas in the presence of $0.1$ mM Mg$^{2+}$ ions, the apparent values indicate a $\Delta H$ of $-2284$ cal/mol, a $\Delta S$ of $+16.9$, and a $\Delta G$ of $-7.41$ kcal/mol. This suggests that Mg$^{2+}$ ions strongly affect the enthalpy ($\Delta H$) of the reaction, whereas the entropy ($\Delta S$) rises. Thus, ATP binding in the presence of Mg$^{2+}$ ions might cause conformational changes, which consume the free energy and increase the entropy. Together, these data show that the ligand binding properties of BsGlnK, albeit principally conserved, are quite distinct from that of other P$_{II}$ proteins characterized so far, most notably, the strongly reduced affinity toward ATP in the presence of Mg$^{2+}$ ions.

**Physiological Characterization of BsGlnK**—The amount of BsGlnK protein in *B. subtilis* cells was determined by immunoblot analysis using BsGlnK-specific antibodies. As expected from the TnrA dependence of amtBglnK expression (28, 33), no BsGlnK was detectable when the cells were grown with rich nitrogen sources such as arginine or ammonium. Using various poor nitrogen sources, growth in the presence of nitrate caused the highest accumulation of BsGlnK, whose abundance increased up to 0.1% of the total cellular protein (data not shown). Consequently, growth with nitrate as nitrogen source was used in this study to achieve maximum accumulation of BsGlnK.

To analyze, whether BsGlnK may be covalently modified in *B. subtilis*, nondenaturing PAGE analysis was used, a method that revealed covalent modification of P$_{II}$ proteins in other bacteria (7, 37). Although bands of higher electrophoretic mobility could be observed, treatment of extracts with alkaline phosphatase or phosphodiesterase did not change electrophoretic mobility of BsGlnK (not shown). Furthermore, nitrogen upshift of nitrate-grown cells did not change the electrophoretic mobility of BsGlnK, arguing against a rapid modification/de-modification system.

To determine a potential unknown modification of BsGlnK, a Strep-tagged variant of BsGlnK (BsGlnK-ST) was overproduced in *E. coli* and in the glnk-deficient *B. subtilis* strain GP253 grown in the presence of nitrate. After purification, the proteins were subjected to electrospray ionization (ESI) analysis (see Supplemental Material). Two masses were obtained, differing by 131 Da, which corresponds to the molecular weight of the N-terminal methionine residue. No further molecular species were obtained, indicating the absence of a stable covalent modification of BsGlnK.

**Cellular Localization of BsGlnK**—Previous analysis of the localization of BsGlnK revealed that most of BsGlnK protein was associated with the membrane fraction, apparently in association with the ammonium transporter AmtB, both under conditions of nitrogen limitation (growth with 0.2% (w/v) glutamate) and after ammonium shock (33). In this study, the partial membrane association of BsGlnK was initially confirmed in extracts from nitrate-grown cells. To analyze whether the ligands ATP and 2-oxoglutarate affect the binding of BsGlnK to the membrane, extracts of nitrate-grown cells were fractionated in the absence or presence of divalent cations with or without 2-oxoglutarate and in the presence of increasing amounts of ATP (added prior to ultracentrifugation). The distribution of BsGlnK between the soluble and membrane fraction was determined by immunoblot analysis and densitometric quantification of the resulting bands (Fig. 3). In the absence of effector molecules and divalent cations (Fig. 3A), only 15% of total BsGlnK was membrane-associated, whereas in the presence of MnCl$_2$ (Fig. 3B), 40% was recovered in the membrane fraction. In the presence of MgCl$_2$ (Fig. 3C), 50% of total BsGlnK was membrane-associated, in agreement with previous results (33). Increasing concentrations of ATP caused a release of membrane-associated BsGlnK to the soluble fraction; at a concentration of about 4 mM ATP, BsGlnK was almost completely soluble. The effect of ATP on the membrane association of

![FIGURE 3. ATP affects membrane association of BsGlnK. Extracts were prepared from cells grown in the presence of 20 mM nitrate and were fractionated in the absence of divalent cations (A), in the presence of 2 mM MnCl$_2$ (B), or in the presence of 2 mM MgCl$_2$ (C) with (triangles) or without (squares) 1 mM 2-oxoglutarate together with increasing concentrations of ATP, as indicated, and analyzed by immunoblotting. For experimental details, see "Experimental Procedures."](image-url)
Interaction of GlnK and TnrA in B. subtilis

BsGlnK was slightly enhanced in the presence of 2-oxoglutarate (Fig. 3, triangles), which is particularly evident in the presence of MgCl₂. To determine the specificity of the ATP effect, other nucleotides (ADP, GTP, CTP, and UTP) were tested under the above-mentioned conditions. However, these metabolites showed no effect on the cellular localization of BsGlnK nor did glutamine or glutamate, confirming the specificity of the ATP effect (data not shown). These data suggest that ATP concentrations in the millimolar range are sensed by BsGlnK and affect its cellular localization.

Immunoprecipitation of BsGlnK and Mass Spectrometric Analysis—In a further approach to reveal potential modifications of BsGlnK, immunoprecipitation experiments of soluble and membrane-bound BsGlnK were performed. Extracts of nitrate-grown cells were fractionated into a cytosolic and a membrane fraction (see "Experimental Procedures") and incubated with BsGlnK-specific antibodies in the presence of nonionic detergent. These antigen-antibody complexes were collected with protein A-agarose, and after rigorous washing in nonionic detergent-containing buffer and elution of antibody-bound protein, the samples were separated by SDS-PAGE. Resulting protein bands were excised and subjected to tryptic in-gel digestion. The peptide fragments obtained by this procedure were then analyzed by MALDI-TOF-MS. No tryptic fragment of BsGlnK could be fitted to a potentially modified BsGlnK fragment (not shown). Most unexpectedly, however, the MALDI-TOF-MS analysis of the immunoprecipitate of membrane-bound BsGlnK showed additional peptides with m/z values of 803,42; 855,46; 916,55; 985,45; 1201,62 and 1356,68. Within an error range of 0.6 Da, these masses could be fitted to tryptic peptides of the transcription factor TnrA (25), yielding a sequence coverage of 46% (TnrA peptides corresponding to fragments from amino acid 37–43; 51–58; 59–65; 67–77, 83–93 and 88–94). By contrast, no additional fragments could be fitted to any B. subtilis protein in the immunoprecipitate of soluble BsGlnK.

To analyze whether TnrA was specifically co-immunoprecipitated with GlnK, TnrA-specific antibodies were generated. To examine the specificity of a potential interaction between TnrA and GlnK, immunoprecipitation experiments with membrane-bound and soluble BsGlnK were performed as described above. The immunoprecipitate and the supernatant of immunoprecipitation were analyzed with BsGlnK- and TnrA-specific antibodies. As shown in Fig. 4A, TnrA was almost completely co-immunoprecipitated with membrane-bound BsGlnK. Soluble BsGlnK instead did not co-immunoprecipitate TnrA (data not shown). Immunoprecipitation experiments were also performed with TnrA-specific antibodies, which were indeed able to co-immunoprecipitate nearly 90% of membrane-bound GlnK (Fig. 4B) together with TnrA. Confirming the results of the previous MALDI-TOF-MS and immunoprecipitation analysis, the reciprocal co-immunoprecipitations of TnrA and GlnK strongly indicates that membrane-bound BsGlnK and TnrA form a stable complex.

Co-localization of TnrA with BsGlnK—The immunoprecipitation experiments suggested that TnrA may be membrane-localized through interaction with BsGlnK. To test this supposition, the cellular localization of TnrA was investigated in the presence or absence of BsGlnK. Extracts of B. subtilis cells grown to exponential phase with nitrate as sole nitrogen source were fractionated by ultracentrifugation as described above. Aliquots of the fractions were analyzed by SDS-PAGE followed by immunoblot analysis using specific antibodies against BsGlnK and TnrA. The quality of the fractionation was verified by Western blot analysis using antibodies against the soluble enzyme GS (see "Experimental Procedures"). GS was detected in the cytosolic but not in the membrane fractions confirming that the membrane preparations were essentially free of cytoplasmic proteins (Fig. 5). In extracts from wild-type cells (with 1 mM MgCl₂ present), BsGlnK was distributed equally between the soluble and the membrane fraction after ultracentrifugation as shown in previous experiments (compare Fig. 3C). By contrast, TnrA could only be detected in the membrane fraction (Fig. 5A). In extracts from the glnK mutant GP253, the immunoblot against BsGlnK showed no signals, as expected (data not shown). However, TnrA was exclusively located in the cytosolic fraction (Fig. 5B), revealing that BsGlnK is indeed required for the membrane association of TnrA.

Because AmtB was shown to be required for membrane localization of BsGlnK (33), TnrA localization was also investigated in AmtB-deficient cells. Separation of extracts into soluble and membrane fractions revealed that in this strain TnrA is soluble, as well as BsGlnK (Fig. 6), indicating that AmtB-bound BsGlnK is necessary to localize TnrA to the membrane. As shown above, the effector molecules ATP and 2-oxoglutarate affect membrane localization of BsGlnK in vitro (Fig. 3). If the membrane association of TnrA depends on membraneAmtB-associated BsGlnK, it is expected that these effector molecules...
not only solubilize BsGlnK but also TnrA. To verify this, cell extracts containing 1 mM MgCl₂ were fractionated by ultracentrifugation as described above in the presence or absence of 4 mM ATP and 1 mM 2-oxoglutarate. The fractions were subjected to SDS-PAGE followed by immunoblotting using BsGlnK and TnrA-specific antibodies. As a control to detect a possible contamination of the particulate fraction with soluble proteins, a blot was performed with antibodies against the soluble enzyme GS. A, fractions prepared from the wild-type strain. B, fractions obtained from the BsGlnK-deficient mutant. 1. UC and 2. UC, first and second ultracentrifugation.

FIGURE 6. AmtB dependence of TnrA membrane association. Cell-free extracts from B. subtilis wild-type (wt) and AmtB-deficient mutant (ΔAmtB) grown under nitrogen-poor conditions were fractionated into soluble and membrane fractions, as described previously (33). The supernatant of the first centrifugation (S) and the washed membrane fraction (P) were analyzed by Western blotting for the presence of BsGlnK and TnrA.

FIGURE 7. ATP/2-oxoglutarate affect BsGlnK-dependent TnrA localization. Wild-type cells were grown under nitrogen limitation in SMM with 20 mM NaNO₃ as the sole nitrogen source. Whole-cell extracts (ce) were divided into an upper (S₁) and lower (S₂) soluble fraction and a particulate (P₁) fraction. P₁ was washed and fractionated again as before (upper and lower soluble fractions S₃ and S₄, and particulate fraction P₂). The fractions were subjected to SDS-PAGE followed by immunoblotting using BsGlnK- or GS-specific antibodies. The GS blot indicates the degree of contamination of membrane fractions with cytosolic proteins. 1. UC and 2. UC, first and second ultracentrifugation.

not only solubilize BsGlnK but also TnrA. To verify this, cell extracts containing 1 mM MgCl₂ were fractionated by ultracentrifugation as described above in the presence or absence of 4 mM ATP and 1 mM 2-oxoglutarate. The fractions were again subjected to immunoblot analysis; GS-specific antibodies were used to evaluate the fractionation. In the control (absence of ATP and 2-oxoglutarate), BsGlnK was distributed between the particulate and cytosolic fraction, whereas TnrA was just located in the particulate fraction, as expected (Fig. 7A). In the presence of ATP and 2-oxoglutarate instead (Fig. 7B), BsGlnK was soluble as well as TnrA, with no detectable association with the membrane fraction, further confirming the dependence on membrane-bound BsGlnK for membrane association of TnrA.

**DISCUSSION**

This study aimed to characterize the B. subtilis GlnK homologue and to reveal possible regulatory targets. We identified unusual ATP-binding properties of BsGlnK and revealed a novel potential regulatory target of BsGlnK, the nitrogen starvation-specific transcription factor TnrA. Interaction between BsGlnK and TnrA occurs in a membrane-associated complex with AmtB in an ATP- and, to a lesser extent, 2-oxoglutarate-dependent manner.

Compared with the P₁₁ consensus sequence, the BsGlnK-sequence is poorly conserved (38). Potential sites of modification in the T-loop sequence are not conserved. Indeed, mass spectrometric analysis failed to reveal covalently modified forms. The bands of higher electrophoretic mobility that were observed in nondenaturing gels may be because of C-terminal processing. Because we used C-terminal fused Strep-tagged BsGlnK expressed in B. subtilis for ESI analysis, this modification could not be detected. Thus, it appears that BsGlnK is indeed not covalently modified, as previously suggested (33). A further striking feature of the BsGlnK sequences is the poor conservation of its N-terminal part of the ATP binding cleft, as well as the lack of the almost universally conserved residue arginine 101 making contact with the γ-phosphate of ATP (6). Consistent with these sequence specificities, striking differences in ATP binding could be detected. Although ITC measurements...
clearly demonstrate that BsGlnK has the ability to bind ATP, Mg²⁺ ions affect ATP binding in a so far unreported manner. Although in previously analyzed P₆₈ proteins high affinity ATP binding could be detected in the presence of millimolar Mg²⁺ concentrations (7–9), this is not the case in BsGlnK. As deduced from ITC binding assays, Mg²⁺ changes the binding properties in such a way that the enthalpy of the reaction diminishes strongly, thereby preventing detectable ITC signals. The only means to detect ATP binding at millimolar Mg²⁺ concentrations was by UV cross-linking. By this qualitative method, it appears that 2-oxoglutarate stimulates ATP binding, an observation that qualitatively agrees with ITC measurements in the presence of 0.1 mM MgCl₂. Furthermore, BsGlnK appears to only weakly bind 2-oxoglutarate, and in contrast to other P₆₈ proteins (9, 20), no ADP binding could be observed. The reduced affinity toward Mg²⁺-ATP may be of physiological significance. The ATP dependence of the solubility of BsGlnK and of its interaction with TnrA/AmtB showed responses in the millimolar range of ATP, concentrations that were reported to be physiologically relevant in B. subtilis (39). An example for the regulatory role of the cellular ATP level in B. subtilis is the ATP-dependent activation of the nutritional stress-specific σB transcription factor (40).

This study showed that binding of effector molecules apparently affects the interaction of BsGlnK with its membrane receptor AmtB (33). Furthermore, the membrane-associated BsGlnK forms a stable complex with TnrA, the major transcription factor of genes responding to nitrogen limitation. Such an interaction is supported by three independent facts. (i) For reciprocal immunoprecipitation, anti-GlnK antibodies co-immunoprecipitate TnrA, and vice versa anti-TnrA antibodies co-immunoprecipitate BsGlnK. (ii) For BsGlnK/AmtB-dependent localization of TnrA, in wild-type cells TnrA is membrane-associated together with BsGlnK, whereas in BsGlnK- and AmtB-deficient mutants, TnrA is soluble. (iii) For co-solubilization of BsGlnK and TnrA, in the presence of 4 mM ATP/1 mM 2-oxoglutarate, both BsGlnK and TnrA are removed from the membrane fraction.

Apparently, TnrA only interacts with the AmtB-bound, membrane-associated BsGlnK protein. This suggests that the BsGlnK-AmtB complex is the target of TnrA association. The data presented here do not clearly distinguish whether GlnK and TnrA interact directly or assemble indirectly. Nevertheless, the co-immunoprecipitation assays suggest a direct interaction. Recently, P₆₈ sequestration to AmtB in Azospirillum brasilense was shown to cause membrane binding of DraG and DraT, enzymes that modulate the activity of nitrogenase reductase by covalent modification (22). Binding of P₆₈-like signaling proteins to AmtB could thus be a common mechanism to sequester regulatory proteins to the membrane.

What could be the physiological significance of BsGlnK/AmtB-mediated TnrA membrane association? When the ATP levels in the cell are sufficiently high, membrane association of BsGlnK is low, and TnrA should be mostly soluble. In the soluble state, TnrA is able to form a complex with feedback-inhibited GS (26, 31), which controls its transcriptional activity. In contrast, when the levels of ATP drop, BsGlnK binds to the membrane in an AmtB-dependent manner and sequesters TnrA. This potential regulatory scenario derived from the in vitro characteristics of the proteins needs to be further investigated by in vivo cellular localization and gene expression studies. Previous studies showed that the glnK mutant of B. subtilis was not impaired in TnrA-dependent gene expression under standard growth conditions; however, under acid stress conditions in the presence of low ammonium concentrations, the expression of the TnrA-dependent amtB-glnK operon was drastically decreased compared with the wild type (33). This implies that BsGlnK helps to maintain the activity of TnrA, a conclusion that seems to be in conflict with the BsGlnK-dependent sequestration of TnrA. According to the orthodox interpretation, transcription factors may be inactivated by membrane sequestration (41). However, there have been reports that membrane anchoring of transcription factors did not affect their activity (42) or that transcription factors are activated in their membrane-associated state (43). If this also applies for TnrA, bound to the BsGlnK-AmtB complex, this could be a means to prevent inhibitory interactions between TnrA and GS or could protect TnrA from degradation. Further investigations will address this issue.

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Interaction of GlnK and TnrA in B. subtilis

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