A novel regulatory mechanism for control of the ubiquitous 2-oxoglutarate dehydrogenase complex (ODH), a key enzyme of the tricarboxylic acid cycle, was discovered in the actinomycete Corynebacterium glutamicum, a close relative of important human pathogens like Corynebacterium diphtheriae and Mycobacterium tuberculosis. Based on the finding that a C. glutamicum mutant lacking serine/threonine protein kinase G (PknG) was impaired in glutamate utilization, proteome comparisons led to the identification of OdhI as a putative substrate of PknG. OdhI is a 15-kDa protein with a forkhead-associated domain and a homolog of mycobacterial GarA. By using purified proteins, PknG was shown to phosphorylate OdhI at threonine 14. The glutamine utilization defect of the ΔpknG mutant could be abolished by the additional deletion of odhI, whereas transformation of a ΔodhI mutant with a plasmid encoding OdhI-T14A caused a defect in glutamine utilization. Affinity purification of OdhI-T14A led to the specific copurification of OdhA, the E1 subunit of ODH. Because ODH is essential for glutamine utilization, we assumed that unphosphorylated OdhI might be caused by a disturbed tricarboxylic acid cycle.

Increasing numbers of eukaryotic-like serine/threonine protein kinases found in bacteria implicates that they play important roles in cell signaling, but their targets and specific functions are largely unknown (1). The genome of the important human pathogen Mycobacterium tuberculosis encodes 11 members of this protein family (2). Among these, protein kinase G (PknG) gained particular interest because it was reported to inhibit phagosome-lysosome fusion, thus allowing for intracellular survival of mycobacteria. Deletion of the pknG gene in Mycobacterium bovis BCG resulted in lysosomal localization and mycobacterial cell death in infected macrophages. PknG was detected in the cytosol of infected macrophages and was therefore suggested to interfere with host cell signaling pathways (3). A pknG deletion mutant of M. tuberculosis displayed decreased viability upon infection of immunocompetent mice but also reduced growth in vitro (4), implying that PknG function is not restricted to the pathogenic life style. This is supported by the fact that genes encoding PknG homologs are not only present in pathogenic mycobacteria but also in all other members of the suborder Corynebacterineae with known genome sequence, i.e. species of the genera Corynebacterium, Mycobacterium, Nocardia, and Rhodococcus, as well as in Streptomyces species. To determine the function of PknG, we chose Corynebacterium glutamicum, a nonpathogenic species used for biotechnological amino acid production (5), which already proved useful for understanding the function of homologous genes in M. tuberculosis (6).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—The strains and plasmids used in this study are listed in Table 1. C. glutamicum strains were cultivated aerobically in shake flasks at 150 rpm and 30 °C in brain heart infusion medium (Difco) or CGXII minimal medium (7) with 200 mM glucose. For growth experiments on glutamine as carbon and nitrogen source, C. glutamicum was pre-cultured overnight in brain heart infusion medium with 100 mM glucose. Cells were harvested, washed with 0.9% NaCl, and used to inoculate the main cultures in glucose-free (modified CGXII lacking ammonium sulfate and urea) and containing 100 mM glutamate supplemented with 5 mM glucose. Samples for determination of intracellular metabolites and for proteome analysis were taken after 12 h of cultivation when the ΔpknG strain just became stationary after glucose depletion. For the determination of growth rates, cells grown in brain heart infusion medium with glucose were cultivated in glutamate medium with 20 mM glucose. The cells from this second preculture were used to inoculate glutamate medium without glucose. For all cloning purposes, Escherichia coli DH5α was used and routinely grown in Luria-Bertani medium at 37 °C. When appropriate, kanamycin was added at concentrations of 25 mg/liter (C. glutamicum) or 50 mg/liter (E. coli).

Construction of Plasmids and Mutants—The oligonucleotides used as PCR primer in this study are listed in Table S1. Plasmids were constructed by standard molecular genetic methods and confirmed by DNA sequence analysis. Defined C. glutamicum deletion mutants were constructed by crossover PCR and double homologous recombination using the suicide vector pk19mobsacB (8). All deletions were verified by Southern blot analysis. Site-directed mutagenesis of odhI was carried out by PCR using the mutagenic primer pairs T14A-for/-rev and T15A-for/-rev. For construction of C. glutamicum strains synthesizing Streptagged OdhA or Strep-tagged AceE, 3′-terminal fragments of the respective genes were amplified with the primer pairs odhA-for/odhA-rev and aceE-for/aceE-rev. The reverse primers introduced a Strep-
C. glutamicum strains used in this study

| Strains | Description | Ref. |
|---------|-------------|------|
| ΔpknG   | Wild type derivative with in-frame deletion of pknG (cg3046) | 39 |
| ΔglnH   | Wild type derivative with in-frame deletion of glnH (cg3045) | This work |
| ΔglnX   | Wild type derivative with in-frame deletion of glnX (cg3044) | This work |
| ΔodhI   | Wild type derivative with in-frame deletion of odhI (cg1630) | This work |
| ΔpknG2ΔodhI | ΔpknG derivative with additional in-frame deletion of odhI | This work |
| WT-odhA<sub>St</sub> | Wild type derivative with plasmid pK18mob-odhA<sub>St</sub> integrated into the chromosomal odhA gene (cg1280) adding a Strep-tag-II coding sequence before the odhA stop codon | This work |
| ΔpknG2-odhA<sub>St</sub> | As above but ΔpknG derivative | This work |
| ΔpknG2–aceE<sub>St</sub> | Wild type derivative with plasmid pK18mob–aceE<sub>St</sub> integrated into the chromosomal aceE gene (cg2466) adding a Strep-tag-II coding sequence before the aceE stop codon | This work |

Plasmids

| Plasmids | Description | Ref. |
|----------|-------------|------|
| pEKEx2  | Kan<sup>R</sup>, C. glutamicum expression vector for IPTG-inducible gene expression | 40 |
| pEKEx2-ΔpknG<sub>St</sub> | pEKEx2 derivative containing the C. glutamicum pknG gene with the native ribosome-binding site and a Strep-tag-II coding sequence before the pknG stop codon | This work |
| pAN3K   | Kan<sup>R</sup>, derivative of the E. coli expression vector pASK-IAB3C (IBA, Göttingen, Germany) for anhydrotracyclene-inducible production of C-terminally Strep-tagged proteins, contains the Kan<sup>R</sup> gene and the C. glutamicum replicon from pJC1 allowing plasmid replication and gene expression in C. glutamicum | This work |
| pAN3K-odhI and derivatives | pAN3K derivative containing the C. glutamicum wild-type odhI gene (obtained by PCR with primers OdhI-for-1/rev-1) or mutated odhI genes with Thr-14 or -15 codons exchanged to alanine codons | This work |
| pJC1    | Kan<sup>R</sup>, E. coli-C. glutamicum shuttle vector | 41 |
| pJC1-odhI and derivatives (T14A, T15A) | pJC1 derivatives containing the C. glutamicum wild-type odhI gene (obtained by PCR with primers OdhI-for-2/rev-2) with its native promoter or mutated odhI genes with Thr-14 or -15 codons exchanged to alanine codons | This work |
| pK18mob | Kan<sup>R</sup>, E. coli vector unable to replicate in C. glutamicum | 42 |
| pK18m-odhA<sub>St</sub> | pK18mob derivative containing a 625-bp PCR product covering the 3′-terminal end of the odhA gene and a Strep-tag-II coding sequence before the odhA stop codon | This work |
| pK18m-aceE<sub>St</sub> | pK18mob derivative containing a 578-bp PCR product covering the 3′-terminal end of the aceE gene and a Strep-tag-II coding sequence before the aceE stop codon | This work |
| pK19mobsacB | Kan<sup>R</sup>, E. coli vector for generating C. glutamicum deletion mutants | 42 |
| pK19m-ΔpknG | pK19mobsacB derivative containing a crossover PCR product (primer ΔpknG-1–4) that covers the flanking regions of the pknG gene | This work |
| pK19m-ΔglnH | pK19mobsacB derivative containing a crossover PCR product (primer ΔglnH-1–4) that covers the flanking regions of the glnH gene | This work |
| pK19m-ΔglnX | pK19mobsacB derivative containing a crossover PCR product (primer ΔglnX-1–4) that covers the flanking regions of the glnX gene | This work |
| pK19m-ΔodhI | pK19mobsacB derivative containing a crossover PCR product (primer ΔodhI-1–4) that covers the flanking regions of the odhI gene | This work |

Preparation of Cell-free Extracts and Protein Purification by Affinity Chromatography—All steps were performed at 4 °C. Cells, which were resuspended in buffer A (100 mM Tris/HCl, pH 8.0, 150 mM NaCl) containing Complete EDTA-free protease inhibitor (Roche Diagnostics) and disrupted by sonication or French press treatment. Cell debris was removed by low speed centrifugation (18,000 × g for 10 min). Prior to purification, cell extracts from C. glutamicum were incubated with avidin (50 μg/ml) for 30 min to reduce copurification of the biotinylated proteins pyruvate carboxylase and acyl-CoA carboxylase. Strep-tagged PknG, OdhI, and mutated derivatives were purified from C. glutamicum chromosome by a single homologous recombination event. Recombinant strains were selected on agar plates containing kanamycin.

Determination of Internal Glutamine/Glutamate Concentrations—Culture samples containing 0.5–1 mg of biomass (dry weight) were rapidly filtered through glass fiber disks (Millipore), and the filter-bound cells were washed with 0.9% NaCl at room temperature (9). Internal metabolites were extracted by incubating harvested cells in 1.3 ml of 50 mM ornithine for 15 min at 95 °C. Amino acids in the extracts were quantified by high pressure liquid chromatography after precolumn derivatization with o-phthalaldehyde. Internal concentrations were calculated using a correlation of dry weight (mg/ml) = 0.25 × A<sub>600</sub> and a cytoplasmic volume of 1.5 μl/mg dry weight (10). The correlation between dry weight and OD<sub>600</sub> was determined for three independent cultures of wild type and ΔpknG mutant and was found to be identical.

Two-dimensional Gel Electrophoresis and Protein Identification—Cytoplasmic proteins were isolated and separated by two-dimensional gel electro-phoresis as described (11). Protein identification from Coomassie-stained one- or two-dimensional gels and analysis of the OdhI phosphorylation site was performed by peptide mass fingerprinting of tryptic digests and post-source decay analysis of the phosphorylated peptide using a Voyager DE-STR mass spectrometer (Applied Biosystems, Weiterstadt, Germany) as described (11).
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For 2-oxoglutarate dehydrogenase (ODH) and pyruvate dehydrogenase (PDH) activity assays and for purification of active ODH-PDH complexes, cells were disrupted in buffer B (50 mM TES/NaOH, pH 7.7, 10 mM MgCl₂) with 30% (v/v) glycerol. Cell extracts for enzyme assays were gel-filtered in the same buffer on PD10 columns (Amersham Biosciences) to remove interfering intracellular metabolites. ODH-PDH complexes were purified on Strept-Tactin-coated magnetic beads or Strept-Tactin-Sepharose (for subsequent enzyme assays) essentially as described above, but cell extracts were diluted with buffer B to 15% (v/v) glycerol, and buffer A was replaced by buffer B containing 10% (v/v) glycerol.

Enzyme Assays—Autokinase activity of PknG and PknG-dependent phosphorylation of OdhI was assayed in kinase buffer (25 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 2 mM MnCl₂, 1 mM dithiothreitol) as described (12). Oxoglutarate dehydrogenase and pyruvate dehydrogenase activity were measured at 30 °C in a photometric assay by following the initial increase in absorbance of NADH at 340 nm (13). Assays containing buffer B with 3 mM l-cysteine, 0.9 mM thiamine diphosphate, 2 mM NAD⁺, 50 μM chloropromazine (to prevent reoxidation of NADH (14)), 25–100 μl of cell extract, or purified ODH-PDH complexes and up to 30.4 nM OdhI were preincubated for 10 min at 30 °C before the reaction was started by addition of 1.5 mM 2-oxoglutarate or pyruvate and 0.2 mM coenzyme A. An activity of 1 unit refers to 1 μmol of NADH formed per min.

Miscellaneous—Protein concentrations were determined with the Bradford protein assay using bovine serum albumin as standard. Glutamine uptake rates of cells grown in CGXII/glucose to the mid-log phase were determined using 1-[14C]glutamine and a filtration assay as described (15). To study the role of GlnX and GlnH for glutamine utilization, internal concentrations of glutamate and glutamine were measured at 30 °C in a photometric assay by following the initial increase in absorbance of NADH at 340 nm (13). Assays containing buffer B with 3 mM l-cysteine, 0.9 mM thiamine diphosphate, 2 mM NAD⁺, 50 μM chloropromazine (to prevent reoxidation of NADH (14)), 25–100 μl of cell extract, or purified ODH-PDH complexes and up to 30.4 nM OdhI were preincubated for 10 min at 30 °C before the reaction was started by addition of 1.5 mM 2-oxoglutarate or pyruvate and 0.2 mM coenzyme A. An activity of 1 unit refers to 1 μmol of NADH formed per min.

RESULTS

PknG Is Required for Glutamine Utilization in C. glutamicum—The C. glutamicum pknG gene (cg3046) is located in a putative operon (Fig. 1A) with two genes, cg3044 (glnX) and cg3045 (glnH), that encode a hypothetical membrane protein with four potential transmembrane helices and a putative “periplasmic” glutamine-binding lipoprotein, respectively (16). This gene arrangement is conserved in bacteria containing pknG homologs except for Streptomyces coelicolor and Streptomyces avermitilis where an additional open reading frame is present between glnH and pknG. Because GlnH homologs of other bacteria, e.g. E. coli or Bacillus subtilis, are part of ABC transporters for high affinity glutamine uptake, we tested the effect of an in-frame pknG deletion in C. glutamicum on glutamine utilization. As shown in Fig. 1B, C. glutamicum wild type grew on minimal medium agar plates with glutamine as sole carbon and nitrogen source, whereas growth of the ΔglnX, ΔglnH, or ΔglnX, ΔglnH mutant was severely inhibited. This phenotype could be complemented by plasmid-borne pknG (data not shown). In liquid glutamate medium, C. glutamicum wild type showed a growth rate of 0.17–0.20 h⁻¹ and formed 5 g of cell dry weight liter⁻¹ within 20 h. The ΔpknG strain showed no growth within this period. The mutant started to grow only after a prolonged incubation. This growth was probably because of suppressor mutations, because the lag phase of different cultures varied considerably, and cells from these cultures could subsequently grow immediately on glutamine agar plates. No significant growth differences were observed in rich medium or glucose minimal medium (data not shown). To study the role of GlnX and GlnH for glutamine utilization, in-frame deletion mutants of C. glutamicum lacking either glnX or glnH were constructed (Fig. 1A). On glutamine agar plates, both mutants showed a growth defect (Fig. 1B). In liquid glutamate medium, the ΔglnX mutant showed a similar phenotype as the ΔpknG mutant. There was no growth within 50 h, but upon prolonged incubation suppressor mutants started to grow. By contrast, the ΔglnH mutant was able to grow in liquid glutamate medium, with growth rates varying from 0.14 to 0.16 h⁻¹. From these results, a function of PknG, GlnX, and GlnH in glutamine uptake or metabolism could be inferred.

C. glutamicum possesses a secondary Na⁺-dependent glutamine uptake system that has not yet been genetically identified (15). Transport assays were performed to study whether GlnH and GlnX are part of a glutamine transporter that is regulated by PknG. However, the C. glutamicum mutant strains lacking pknG, glnX, or glnH showed at least 80% of the glutamine transport activity of the wild type (data not shown), indicating that the defect of the mutants in glutamine utilization is not because of an impaired glutamine uptake. A similar result was reported for a M. bovis BCG ΔpknG mutant (17). Measurement of the internal amino acid concentrations revealed that the glutamate level was 2-fold higher in the ΔpknG mutant compared with the wild type and the complemented mutant, whereas the glutamine level was only slightly increased (Fig. 1C). Therefore, a defect in glutamate catabolism was likely to be responsible for the defect of the ΔpknG mutant in glutamine utilization.

Identification of an in Vivo Substrate of PknG—To find the molecular basis for inhibition of glutamate catabolism by PknG, we searched for in vivo phosphorylation substrates of PknG by proteome analysis of C. glutamicum wild type, ΔpknG mutant, and complemented mutant grown on 100 mM glutamate and 5 mM glucose as carbon sources. Comparison of Coomassie-stained two-dimensional gels revealed a series of three spots in the acidic, low molecular mass range that clearly differed between the wild type and complemented ΔpknG strain on the one hand and the ΔpknG mutant on the other hand (Fig. 1D). All three spots were identified by mass spectrometry as the protein encoded by gene cg1630, which was designated OdhI. OdhI (143 amino acid resi-
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FIGURE 2. Phosphorylation of OdhI by PknG and role of OdhI for glutamine utilization. A, autoradiogram showing in vitro phosphorylation of OdhI by PknG at threonine 14. A T14A exchange in OdhI abolishes phosphorylation, whereas a T15A exchange does not. 6.1 pmol of OdhI or the indicated variants were incubated with 5.6 pmol of PknG and 37 kBq [γ-32P]ATP for 30 min at 37 °C, denatured, and completely loaded on a 15% SDS gel. 8, alignment of the amino-terminal regions of OdhI homologs from different Actinomycetales. The filled circle denotes the residue in C. glutamicum OdhI that is phosphorylated by PknG and the open circle the residue of the M. tuberculosis OdhI homolog (designated GarA), which is phosphorylated in vitro by PknB (20). C. Corynebacterium efficiens; Cj, Corynebacterium jeikeium; Mp, Mycobacterium avium subsp. paratuberculosis; Mb, M. bovis; Mt, M. tuberculosis; Ms, M. smegmatis; Nf, Rhodococcus fascians; Sa, S. avermitilis; Sc, S. coelicolor. C, growth with glutamine as sole carbon source of the following C. glutamicum strains: 1, wild type; 2, ΔpknG; 3, ΔpknGΔodhI; 5, ΔodhI/pJC1-odhI-T14A; 6, ΔodhI/pJC1-odhI-T15A.

dues, 15,402 Da) is a homolog (69% sequence identity) of Mycobacterium smegmatis GarA (18) and contains a forkhead-associated (FHA) domain, which binds phosphothreonine epitopes on proteins and mediates phosphorylation-dependent protein-protein interactions (19). The pl values of 4.64, 4.56, and 4.47 observed for the three OdhI spots were close to the theoretical values calculated for unphosphorylated, monophosphorylated, and doubly phosphorylated OdhI protein, respectively. The amount of the different OdhI isoforms was relatively quantified by densitometric analysis of the two-dimensional gels. In the wild type 61.5 ± 6.4% of total OdhI was present in the monophosphorylated and 5.5 ± 3.9% in the doubly phosphorylated form (all data are mean values ± S.D. of three gels from independent cultures). The complemented ΔpknG mutant showed no significant differences with corresponding values of 53.1 ± 14.3 and 2.0 ± 1.8%, respectively. In contrast, the ΔpknG mutant contained only 5.9 ± 0.1% monophosphorylated OdhI, and the doubly phosphorylated form was undetectable. These data suggest that OdhI is phosphorylated by PknG but also by at least one additional protein kinase. Besides PknG, C. glutamicum possesses homologs of the M. tuberculosis serine/threonine protein kinases PknA, PknB, and PknL (2), encoded by the genes cg0059, cg0057, and cg2388, respectively. As M. tuberculosis PknB was recently shown to phosphorylate mycobacterial GarA in vitro (20), PknB is a favorite candidate for PknG-independent OdhI phosphorylation.

To confirm that OdhI is a substrate of PknG, both proteins were synthesized as Strep-tagged derivatives and purified to apparent homogeneity. As shown by in vitro kinase assays, PknG catalyzed autophosphorylation and transphosphorylation of OdhI, whereas OdhI alone did not incorporate 32P (Fig. 4A). By comparing the tryptic peptide mass fingerprints of phosphorylated and unphosphorylated OdhI, the amino-terminal peptide covering amino acid residues 2–19 was found to be the only one phosphorylated by PknG (Fig. 3, A and B). Post-source decay analysis of this peptide identified threonine 14 as the phosphorylated residue (Fig. 3C). This result was verified by in vitro kinase assays, which showed that a T14A exchange in OdhI abolished PknG-dependent phosphorylation (Fig. 2A). In contrast, a T15A exchange only reduced the phosphorylation efficiency, indicating that Thr-15 might be involved in the binding of OdhI to PknG. OdhI/GarA homologous proteins with a strictly conserved ETTS motif in the amino-terminal region occur in all species that contain PknG homologs (Fig. 2B). The first threonine residue of this motif is the one phosphorylated by PknG in C. glutamicum, and the second threonine residue corresponds to Thr-22 of GarA from M. tuberculosis that was shown to be phosphorylated by PknB (20).

Functional Characterization of the PknG Substrate OdhI—To address the in vivo function of OdhI, in particular its involvement in glutamine utilization, its structural gene was deleted in C. glutamicum wild type as well as in the ΔpknG mutant. Additionally, the ΔodhI mutant was transformed with plasmids pCl-odhI, pCl-odhI-T14A, or pCl-odhI-T15A directing the synthesis of Strep-tagged OdhI, OdhI-T14A, or OdhI-T15A, respectively. The ΔodhI mutant was able to grow on glutamine, and the defect of the ΔpknG mutant in glutamine utilization was abolished by additional deletion of odhI (Fig. 3C), showing that OdhI is an essential component of the PknG signaling pathway and pointing to an inhibitory function of unphosphorylated OdhI. Consistently, the ΔodhI strain synthesizing the OdhI-T14A protein could not grow on glutamine agar plates, whereas the one with the OdhI-T15A protein could (Fig. 3C). In summary, the presence of OdhI, which cannot be phosphorylated on Thr-14 by PknG, inhibited growth on glutamine and consequently glutamate degradation.

In contrast to proteins containing the FHA domain as part of a larger protein whose activity is modulated by phosphorylation (21, 22), the FHA domain of the OdhI protein is flanked only by an amino-terminal extension of about 50 amino acid residues. This suggested that OdhI exerts its inhibitory function in glutamate catabolism by interaction with other cellular proteins. To identify OdhI interaction partners, plasmid-encoded Strep-tagged OdhI-T14A was purified from C. glutamicum strain ΔodhI. As shown in Fig. 4A, large amounts of a protein with an apparent mass of 140 kDa were copurified, which was identified by mass spectrometry as OdhA (cg1280), the E10 subunit of the 2-oxo-glutarate dehydrogenase complex (23). In contrast, only small amounts of OdhA were copurified with unmutated OdhI from strain ΔodhI/pJC1-odhI, which was present predominantly in the phosphorylated form, suggesting that OdhA interacts preferably with unphosphorylated OdhI (Fig. 4A, lane 2). This was further strengthened by copurification of unphosphorylated OdhI with Strep-tagged OdhA (Fig. 4A, lane 3). The phenotype of the odhI mutants along with the observation that

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FIGURE 3. Mapping of the PknG-dependent phosphorylation site of OdhI. Peptide mass fingerprints of tryptic digests of unphosphorylated (A) and in vitro phosphorylated (B) Strep-tagged OdhI. Peaks are labeled with their monoisotopic masses and the assigned amino acid residues are shown in parentheses. The covered sequence (87% of 153 amino acids) included all serine and threonine residues. The putative phosphopeptide is marked with an asterisk. C, post-source decay analysis of the OdhI phosphopeptide (average mass 2059.0). For noise filtering and smoothing, the Data Explorer software (Applied Biosystems) was used. Fragment ions attributable to the y-series within a mass accuracy of 0.7 Da were labeled. β-Elimination of phosphoric acid (mass shift −98) is indicated.
ODH activity is crucial for glutamate catabolism (24) led us to suppose that unphosphorylated OdhI can function as an inhibitor of ODH.  

**Purification of a Mixed ODH-PDH Complex**—Generally, ODH is a large multienzyme complex consisting of oxoglutarate dehydrogenase (E1o), dihydrolipoamide succinyltransferase (E2o), and dihydrolipoamide dehydrogenase (E3) subunits. The proteins encoded by *sucB* (cg2421) and *lpd* (cg0790) were annotated as putative E2 and E3 subunits of ODH in *C. glutamicum* (16), and Lpd has been biochemically characterized as dihydrolipoamide dehydrogenase (25). Remarkably, corynebacteria and mycobacteria display two unusual features with respect to ODH. The E1o subunit carries an amino-terminal extension bearing sequence similarity to the catalytic domain of E2o subunits of ODH and the related PDH complex. Therefore, an altered protocol lacking endogenous PknG and OdhI upon addition of purified, unphosphorylated OdhI. As shown in Fig. 5A, this resulted in a concentration-dependent hyperbolic inhibition of ODH activity. Half-maximal inhibition was observed at 4.3 nM OdhI. For a more detailed analysis of the inhibition pattern, ODH activity was measured at oxoglutarate concentrations between 0.08 and 1.5 mM and different fixed OdhI concentrations. Primary plots, e.g., according to Lineweaver and Burk, were nonlinear, thereby indicating a slightly positive cooperative binding of oxoglutarate (data not shown), as reported for ODH from other organisms (29). The best linear fits in primary Eadie-Scatchard plots (Fig. 5B, v/[[S] versus v) were obtained by using a Hill coefficient, n_H, of 1.24. In these plots, the activities at different OdhI concentrations gave a series of nearly parallel lines with slopes of ≈1/K_{app} and intersection points with the abscissa at V_{max(app)}. For noninhibited ODH, a V_{max} value of 94 milliunits/mg and an [S]_{0.5} value of 0.22 mM (K' = [S]_{0.5}) were determined. Double-reciprocal replots of V_{max} versus K_{app} (DV_{m}) and K' versus K' (DK') versus the inhibitor concentration yielded parabolic curves (Fig. 5B, inset). From the intersection points of these replots with the ordinate, values for α and β of 1.63 and β of 0.024 were calculated (nomenclature according to Ref. 30). The latter value has to be treated with caution because the very low ODH activity at high OdhI concentrations is difficult to measure. From these results it can be concluded that OdhI acts as a partial mixed type, essentially noncompetitive inhibitor with respect to oxoglutarate. Application of a linear equation for hyperbolic tight-binding inhibition (31) yielded a K_i, value of 2.4 nM.  

By using affinity-purified ODH, it was possible to study the influence of PknG-dependent phosphorylation of OdhI on ODH activity without possible interferences from other protein kinases, phosphatases, and ATPases present in cell extracts. As shown above with cell extract, addition of unphosphorylated OdhI inhibited ODH activity. Upon phosphorylation of OdhI by PknG, no inhibition but even a slight stimulation of...
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FIGURE 5. Regulation of ODH activity by the phosphorylation status of OdhI. A, concentration-dependent inhibition of ODH activity in cell extracts of C. glutamicum ΔpknGΔodhI by addition of purified, unphosphorylated OdhI. The line represents the fit of the measured data to the general velocity equation for hyperbolic mixed type inhibition (30). B, Eadie-Scatchard plot (using a Hill coefficient n = 1.24) of ODH activity in cell extracts at varying oxoglutarate concentrations (0.08, 0.25, 0.5, and 1.5 mM) and different fixed OdhI concentrations: 0 nM (filled circles), 0.9 nM (open circles), 1.8 nM (filled triangles), 3.6 nM (open triangles), 6.1 nM (filled squares), 12.1 nM (open squares), and 30.4 nM (filled diamonds). The solid lines represent linear fits of the data; the dashed lines were calculated as connection to the common intersection point. The inset shows a double-reciprocal replot of Vmax minus Vmax(app) (ΔVmax; filled circles) and Kmax minus K’ (ΔK; open circles) versus the OdhI concentration as calculated from the primary plot. C, inhibition of ODH activity by OdhI is abolished by PknG-dependent phosphorylation of OdhI. 2 μg of ODH complex purified from C. glutamicum ΔpknG-odhIΔh9262 was incubated for 1 h before activity assays (in triplicate) either without additives (bar 1), or with 0.2 μg of OdhI and 2 μg ATP (bar 2), or with 0.2 μg of OdhI, 2 μg ATP and 2.2 μg of PknG (bar 3). The phosphorylation status of OdhI was verified by SDS-PAGE and mass spectrometric analysis: bar 1, native unphosphorylated OdhI; bar 2, native and Streptagged unphosphorylated OdhI; bar 3, native and Streptagged phosphorylated OdhI.

ODH activity was observed (Fig. 5C), providing further evidence that only the unphosphorylated form of OdhI acts inhibitory. The stimulation can be explained by the assumption that partial inhibition of ODH caused by small amounts of copurified OdhI was abolished after phosphorylation by PknG. The phosphorylation status of OdhI in these experiments was confirmed by SDS-PAGE (Fig. 5C, upper part) and mass spectrometric analysis. In contrast to ODH, no indications for an influence of OdhI or PknG on PDH activity were found (data not shown).

DISCUSSION

The finding that PknG regulates ODH activity via the phosphorylation status of OdhI represents the first example for participation of serine/threonine protein kinases in the control of this key enzyme of the tricarboxylic acid cycle. In contrast to the well known regulation of mitochondrial PDH activity via phosphorylation of E1p by pyruvate dehydrogenase kinase (32), the mechanism shown here for ODH does not involve direct phosphorylation of ODH subunits by PknG. Rather, it requires the small soluble protein OdhI to mediate signal transduction from PknG, which is at least partially membrane-associated (4, 12), and from membrane integral serine/threonine protein kinases like PknB to cytosolic ODH. Control of the OdhI phosphorylation status by several protein kinases allows integration of different environmental signals to optimally adjust carbon flux distribution at the 2-oxoglutarate node to the prevailing conditions. This mechanism is of particular importance for the biotechnological production of about 1.5 million tons/year of L-glutamate with coryneform bacteria, where attenuation of ODH activity is required for growth of C. glutamicum (17) as well as of M. tuberculosis (4), because of the regulatory function of OdhI and its assumed ability to respond to different stimuli, this protein is the prime candidate for linking the various treatments with the observed metabolic response and therefore a promising target for improving amino acid production.

Considering that the proteins PknG, OdhI (GarA, respectively), and OdhA are highly conserved in corynebacteria and mycobacteria, the signaling cascade involving these proteins might operate also in mycobacteria, an assumption supported by the increased intracellular glutamate/glutamine level measured in an M. tuberculosis ΔpknG strain (4). PknG is dispensable for growth of M. bovis (17) as well as of C. glutamicum in nutrient-rich media. In M. tuberculosis, the growth defect of the ΔpknG mutant was most pronounced in nutrient-depleted media, and an involvement of PknG in sensing nutritional stress was proposed (4). This agrees with our preliminary observation of increased levels of phosphorylated OdhI in carbon-starved C. glutamicum (data not shown). Although the metabolism of mycobacteria growing inside macrophages is poorly understood, it has been shown that fatty acids serve as carbon source via the glyoxylate pathway (36), and the tricarboxylic acid cycle is thought to be required to drive ATP synthesis (37). The importance of an intact tricarboxylic acid cycle is further supported by the finding that OdhA is required for growth of M. tuberculosis (38). Thus, besides the postulated interference of secreted PknG with host cell signaling pathways (3), delayed virulence and decreased ability to block phagosomelysosome fusion of mycobacteria lacking PknG might also be explained by a disturbed energy metabolism because of inhibition of ODH.

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2 A. Niebisch, A. Kabus, C. Schultz, B. Well, and M. Bott, unpublished results.
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