Regulation of nitrogenase by 2-oxoglutarate-reversible, direct binding of a PII-like nitrogen sensor protein to dinitrogenase

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Posttranslational regulation of nitrogenase, or switch-off, in the methanogenic archaeon *Methanococcus maripaludis* requires both *nifI*$_1$ and *nifI*$_2$, which encode members of the PII family of nitrogen-regulatory proteins. Previous work demonstrated that nitrogenase activity in cell extracts was inhibited in the presence of NifI$_1$ and NifI$_2$, and that 2-oxoglutarate (2OG), a potential signal of nitrogen limitation, relieved this inhibition. To further explore the role of the NifI proteins in switch-off, we found proteins that interact with NifI$_1$ and NifI$_2$ and determined whether 2OG affected these interactions. Anaerobic purification of His-tagged NifI$_2$ resulted in copurification of NifI$_1$ and the dinitrogenase subunits NifD and NifK, and 2OG or a deletion mutation affecting the T-loop of NifI$_2$ prevented copurification of dinitrogenase but did not affect copurification of NifI$_1$. Similar results were obtained with His-tagged NifI$_1$. Gel-filtration chromatography demonstrated an interaction between purified NifI$_{1,2}$ and dinitrogenase that was inhibited by 2OG. The NifI proteins themselves formed a complex of ≈85 kDa, which appeared to further oligomerize in the presence of 2OG. NifI$_{1,2}$ inhibited activity of purified nitrogenase when present in a 1:1 molar ratio to dinitrogenase, and 2OG fully relieved this inhibition. These results suggest a model for switch-off of nitrogenase activity, where direct interaction of a NifI$_{1,2}$ complex with dinitrogenase causes inhibition, which is relieved by 2OG. The presence of *nifI*$_1$ and *nifI*$_2$ in the *nif* operons of all nitrogen-fixing Archaea and some anaerobic Bacteria suggests that this mode of nitrogenase regulation may operate in a wide variety of diazotrophs.

ammonia switch-off | Archaea | *Methanococcus maripaludis* | NifI | posttranslational regulation

Biological nitrogen fixation is a key step in the global nitrogen cycle and is carried out by a variety of Bacteria and methanogenic Archaea (1). This process is catalyzed by the nitrogenase complex, which consists of dinitrogenase (an $\alpha_2\beta_2$ heterotetramer of the NifD and NifK proteins), the site of substrate reduction, and its specific electron-donor dinitrogenase reductase (a homodimer of the NifH protein) (2). Nitrogen fixation is energetically demanding and highly regulated, often at multiple levels. Posttranslational regulation of nitrogenase activity in response to conditions such as addition of ammonium is termed switch-off and is present in some free-living diazotrophs (1). The best characterized mechanism of switch-off involves the reversible ADP-ribosylation of an arginine residue in NifH by the dinitrogenase reductase ADP ribosyltransferase/dinitrogenase reductase-activating glycohydrolase (DraT/DraG) system (3). This mode of regulation occurs in various Proteobacteria, however other diazotrophs apparently do not effect switch-off by this process (4–7).

PII proteins are widespread and ancient nitrogen sensors that function in nearly all aspects of nitrogen regulation (reviewed in refs. 8 and 9), including ADP-ribosylation of NifH (10–12). The best studied examples, GlnB and GlnK of *Escherichia coli* and other Proteobacteria, are homotrimers (13). Studies have shown that *E. coli* GlnB binds ATP and 2-oxoglutarate (2OG), and is covalently modified in response to low glutamine levels (14, 15). Depending on its state of ligand binding and modification, GlnB participates in transcriptional and posttranslational regulation of nitrogen metabolism through its interaction with a variety of other proteins (16). The T-loop domain, also the site of covalent modification, has been shown to be important in these interactions (17). The roles of PII proteins in regulation of nitrogen metabolism have also been studied in cyanobacteria (18) and Gram-positive bacteria (19–21), as well as in Archaea (22) and plastids (23). Although glutamine seems to be the primary nitrogen signal (reflecting excess) sensed by PII proteins in enteric bacteria (24), 2OG may be the main signal (reflecting nitrogen deficiency) in some cyanobacteria (25).

NifI$_1$ and NifI$_2$ form two distinct subfamilies of PII proteins, whereas all other known PII proteins, including GlnB and GlnK, form a third subfamily (26). The genes encoding these proteins are present in the *nif* operons of all nitrogen-fixing Archaea as well as some anaerobic nitrogen-fixing Bacteria, including *Chlorobium tepidum*, *Dehalococcoides ethanogenes* (27), *Heliobacterium chlorum*, and some members of the *Clostridia* and δ-Proteobacteria (26). In contrast to other members of the PII family, the functions of *nifI*$_1$ and *nifI*$_2$ have been studied only in the model methanogenic archaeon *Methanococcus maripaludis*, where both genes were essential for nitrogenase switch-off (28). Previous work with crude extracts of *M. maripaludis* demonstrated that addition of 2OG relieved a *nifI*$_1$- and *nifI*$_2$-dependent inhibition of nitrogenase activity (29). In this study, we extend these findings by identifying proteins that interact with NifI$_1$ and NifI$_2$ and determining the effect of 2OG on these interactions.

Results

Copurification of Proteins with His-Tagged NifI$_1$ and NifI$_2$. Because other PII proteins regulate nitrogen metabolism by protein–protein interactions, often mediated by the T-loop domain, we attempted to find proteins that might specifically copurify with NifI$_1$ and NifI$_2$ and to determine whether any copurification was affected by 2OG or mutations in the T-loop. His-tagged NifI$_1$ and His-tagged NifI$_2$, with and without deletion mutations affecting the T-loops, were constructed and expressed on low-copy plasmids in *M. maripaludis* strains containing null mutations in *nifI*$_1$ and *nifI*$_2$, respectively (see *Materials and Methods*). Expression of C-terminal His-tagged NifI$_1$ and N-terminal His-tagged NifI$_2$ restored switch-off in Δ*nifI*$_1$ and Δ*nifI*$_2$ background strains, respectively. A Δ43–49 mutation in the T-loop of His-tagged NifI$_1$ was deficient in switch-off and a Δ48–52 mutation in the T-loop of His-tagged NifI$_2$ was only slightly capable of effecting switch-off (data not shown).

Immobilized metal-ion-affinity chromatography was used to

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Abbreviation: 2OG, 2-oxoglutarate.

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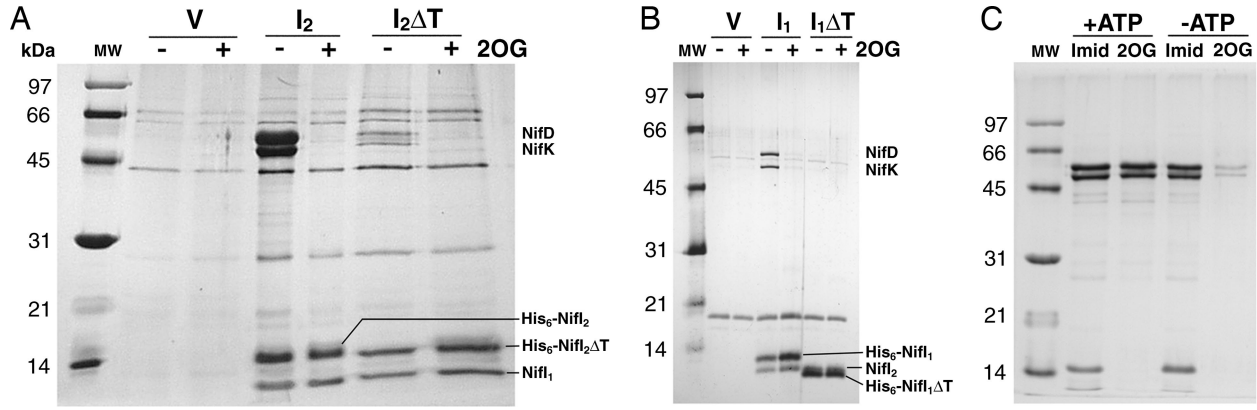

Fig. 1. Copurification of proteins with His-tagged $NifI_1$ and $NifI_2$ and interaction of $NifI_{1,2}$ with dinitrogenase. Extracts were bound to Ni-NTA agarose, and elution fractions were analyzed by gel electrophoresis. (*A*) $NifI_2$ interactions: V, strain Mm1012 [Δ*nifI*$_2$ (null) background containing pLW40neo (vector control)]; I_2, strain Mm711 (Δ*nifI*$_2$ background containing pLW40neo *nifI*$_2$ expressing His-tagged $NifI_2$); I_2ΔT, strain Mm1017 (Δ*nifI*$_2$ background containing pLW40neo *nifI*$_2$ΔT expressing His-tagged $NifI_2$ with a deletion in the T-loop). Extracts were bound to Ni-NTA agarose in the absence (−) or presence (+) of 10 mM 2OG and eluted with 100 mM imidazole. Proteins corresponding to the indicated bands were identified by 2D gel electrophoresis and MALDI-TOF MS. Masses of the molecular weight markers (MW) are shown on the left. (*B*) $NifI_1$ interactions: V, strain Mm1050 [Δ*nifI*$_1$ (null) background containing pLCW40neo (vector control)]; I_1, strain Mm1051 (Δ*nifI*$_1$ background containing pLCW40 *nifI*$_1$ expressing his-tagged $NifI_1$); I_1ΔT, strain Mm1067 (Δ*nifI*$_1$ background containing pLCW40neo *nifI*$_1$ΔT expressing His-tagged $NifI_1$ with a deletion in the T-loop). Binding and elution are as in *A*. The band corresponding to $NifI_2$ is partially obscured by His-tagged $NifI_1$ΔT but was separated and identified on 2D gels. (*C*) Effect of ATP on $NifI_2$ interactions using extract of strain Mm711. Binding, washing, and elution were done in the presence (+) or absence (−) of 5 mM ATP. Elution was done with either 100 mM imidazole (Imid) or 10 mM 2OG. $NifI_1$ is present at the dye front of the gel.

purify the His-tagged constructs under conditions similar to those used for measuring nitrogenase activity *in vitro* (anaerobic, presence of dithionite, ATP and Mg^{+2}; see *Materials and Methods*). When an ATP-regenerating system (creatine phosphate and creatine phosphokinase) and acetylene were included in the binding step, nitrogenase activity was observed and was enhanced ≈3-fold with 10 mM 2OG (data not shown), similar to previous results in crude protein extracts (29). After washing, proteins bound to the Ni-NTA agarose were eluted with imidazole, and copurifying proteins were identified by MS. Results for His-tagged $NifI_2$ are shown in Fig. 1*A*. $NifI_1$ copurified with both the full-length and Δ48–52 His-tagged $NifI_2$, regardless of 2OG. The subunits of dinitrogenase, NifD, and NifK also copurified with His-tagged $NifI_2$ but only in the absence of 10 mM 2OG. Copurification of dinitrogenase was significantly decreased with the Δ48–52 $NifI_2$ but, similar to wild-type $NifI_2$, could still be eliminated in the presence of 2OG. No other proteins were observed to consistently copurify with the His-tagged constructs. Similar and complementary results were obtained with the His-tagged $NifI_1$ (Fig. 1*B*): $NifI_2$ copurified with $NifI_1$, and copurification of dinitrogenase was observed only in the absence of 2OG. The Δ43–48 mutation in $NifI_1$ prevented any detectable copurification of dinitrogenase. These data suggest that $NifI_1$ and $NifI_2$ act as a heteromeric complex ($NifI_{1,2}$) that binds to dinitrogenase, that 2OG inhibits this binding, and that the T-loops of both $NifI_1$ and $NifI_2$ are important for binding.

Binding of ATP to PII proteins has been shown to increase their affinity for 2OG (14). To determine whether ATP might affect the formation or stability of the $NifI_{1,2}$–NifDK complex, purification of the His-tagged $NifI_2$ was repeated with (as before) or without 5 mM ATP in the binding, wash, and elution buffers (Fig. 1*C*). By comparing the imidazole elutions, lack of ATP did not significantly affect the amount of dinitrogenase that copurified with the His-tagged $NifI_2$, suggesting that the complex is stable in the absence of ATP. Elution with 10 mM 2OG instead of imidazole in the presence of ATP resulted in the elution of dinitrogenase because of its separation from $NifI_{1,2}$, showing that the $NifI_{1,2}$–NifDK complex was disrupted by 2OG. In the absence of ATP, however, much less dinitrogenase was eluted with 2OG, suggesting that ATP is required for 2OG to efficiently disrupt the $NifI_{1,2}$–NifDK complex.

Other factors that affected the copurification were explored. A marked decrease in copurification of dinitrogenase with His-tagged $NifI_2$ was observed when dithionite was not included in the wash and elution steps, and no copurification was detected when the sodium chloride concentration was increased from 0.1 to 1 M. Glycerol (10%) did not alter the copurification of dinitrogenase, and none of the above conditions had an effect on the copurification of $NifI_1$ with His-tagged $NifI_2$. Attempts at purification of His-tagged $NifI_2$ under aerobic conditions resulted in copurification of $NifI_1$, but no copurification of dinitrogenase or other proteins was detected with or without 2OG (data not shown). These results suggest that the interaction between the $NifI_{1,2}$ and dinitrogenase may be ionic in character and sensitive to redox conditions. When expressed in a Δ*nifI*$_1$Δ*nifI*$_2$ background, His-tagged $NifI_2$ was not eluted from the Ni-NTA and could not be detected in cell extracts by Western blotting with an antibody for the His-tag (data not shown), suggesting that it is not stable in the absence of $NifI_1$.

Purification of $NifI_{1,2}$ and Nitrogenase. To investigate the interactions of the NifI proteins and nitrogenase by using purified components as well as to reconstitute switch-off *in vitro*, the $NifI_{1,2}$ complex and the nitrogenase components dinitrogenase and dinitrogenase reductase were purified. The $NifI_{1,2}$ complex and the dinitrogenase were purified separately by immobilized metal-ion-affinity chromatography (IMAC) from extracts of the strain expressing the His-tagged $NifI_2$. $NifI_{1,2}$ was purified by including 2OG in all purification steps, thereby preventing copurification of dinitrogenase. Dinitrogenase was purified by eluting with 2OG instead of imidazole. His-tagged dinitrogenase reductase was also purified by IMAC from a strain expressing His-tagged NifH from a plasmid in a Δ*nifH* (Nif$^-$) background. Expression of this His-tagged NifH conferred a Nif$^+$ phenotype to the background strain, indicating that it could form a functional nitrogenase. Components were purified to at least 95% purity, as assessed by Coomassie-stained gels. Sometimes, a second round of IMAC purification was used for the $NifI_{1,2}$ complex to obtain the desired level of purity. Typical activity of

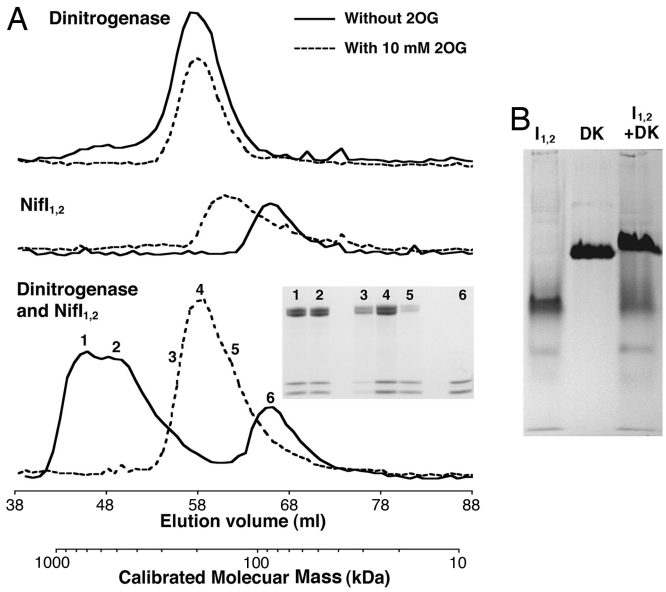

Fig. 2. Interaction of purified $NifI_{1,2}$ and dinitrogenase. (*A*) Gel filtration of dinitrogenase alone, $NifI_{1,2}$ alone, or a mix of the two was performed anaerobically with ATP and $MgCl_2$, with or without 10 mM 2OG. Protein concentration in fractions, measured as A_{600} nm, is shown versus the elution volume. The secondary *x* axis shows the molecular mass corresponding to the fraction volume calibrated by known protein standards. Protein in the fractions indicated by numbers 1–6 were concentrated and run on SDS/PAGE, and the resulting Coomassie-stained gel is shown (*Inset*). Amounts of protein loaded on the column were dinitrogenase, 1 mg and 2 mg with and without 2OG, respectively; $NifI_{1,2}$, 0.75 mg and 0.5 mg with and without 2OG, respectively; and dinitrogenase:$NifI_{1,2}$ mix, 2:1 mg and 3:1.5 mg with and without 2OG, respectively. (*B*) Purified $NifI_{1,2}$ ($I_{1,2}$), dinitrogenase (DK), or a 1:2 mix ($NifI_{1,2}$/NifDK, by mass) run on an 8% native PAGE gel and stained with Coomassie.

purified nitrogenase was 100–300 nmol of C_2H_2 reduced per min^{-1} per mg $protein^{-1}$, an approximate 400-fold increase in specific activity from crude extracts (29). When assayed individually, purified components had no activity. As observed for purified nitrogenase from *Methanosarcina barkeri* (5), activity of purified nitrogenase components decreased after prolonged storage, together or separately, on ice; however, about a third of the activity was present after storage in a 1:1 mass ratio at −20°C in 50% glycerol or frozen at −80°C for 2 weeks. All of the experiments shown here were performed by using freshly purified dinitrogenase, dinitrogenase reductase, and NifI proteins.

Interaction of Purified $NifI_{1,2}$ and Dinitrogenase. Gel filtration chromatography was used to confirm the interaction of $NifI_{1,2}$ with dinitrogenase and to determine the effect of 2OG on this complex as well as to estimate the molecular masses of $NifI_{1,2}$ and the complex (Fig. 2*A*). Because of the effects of ATP and anaerobiosis observed in the copurification experiments, gel filtration was performed under anaerobic conditions in the presence of ATP. In the absence of 2OG, dinitrogenase eluted mainly in a single peak corresponding to a molecular mass of ≈200 kDa, close to the predicted 208 kDa for an $\alpha_2\beta_2$ heterotetramer of NifD (53.6 kDa) and NifK (50.5 kDa). A small amount of protein eluted in higher molecular mass fractions, possibly because of complex formation with small amounts of $NifI_{1,2}$ present as impurities in the dinitrogenase preparation (see below). 2OG did not affect the elution profile of the dinitrogenase peak; however, no protein was observed in the higher-molecular-mass fractions. In the absence of 2OG, purified $NifI_{1,2}$ eluted in a single peak corresponding to a molecular mass of ≈85 kDa. This peak eluted in earlier fractions when 2OG was present, at a molecular mass of ≈145 kDa, although the peak had a broad tail that overlapped with the $NifI_{1,2}$ run without 2OG. These results are not consistent with $NifI_{1,2}$ being a trimer ($NifI_1$, 11.5 kDa; His-tagged $NifI_2$, 14.8 kDa) as with other PII proteins but, instead, suggest that $NifI_{1,2}$ is a larger complex, possibly a hexamer, that further oligomerizes in the presence of 2OG.

When a 2:1 (by mass) mixture of dinitrogenase and $NifI_{1,2}$ was loaded on the column in the absence of 2OG, a peak corresponding to dinitrogenase alone was not observed; however, most of the protein eluted as a broad peak corresponding to a higher molecular mass (500–700 kDa). SDS/PAGE revealed that both dinitrogenase and $NifI_{1,2}$ were present in this higher-molecular-mass peak (Fig. 2*A Inset*, lanes 1 and 2). An amount of $NifI_{1,2}$ corresponding to approximately half of the $NifI_{1,2}$ loaded eluted in the same fractions as $NifI_{1,2}$ run alone (Fig. 2*A Inset*, lane 6). When the mixture was run in the presence of 2OG, the higher-molecular-mass peak was not observed, and protein eluted in the same fractions as dinitrogenase and $NifI_{1,2}$ run alone with 2OG. This peak was similar in shape to that obtained by summing the peaks of the dinitrogenase and $NifI_{1,2}$ run alone in the presence of 2OG, and the dinitrogenase and $NifI_{1,2}$ were unevenly distributed within this peak, with more dinitrogenase in the earlier fractions and more $NifI_{1,2}$ in the later fractions (Fig. 2*A Inset*, lanes 3–5). This peak, therefore, likely represents the $NifI_{1,2}$ and dinitrogenase running through the column independently rather than as a complex. These results are consistent with $NifI_{1,2}$ and dinitrogenase forming a complex that is disrupted by 2OG.

Anaerobic native-gel electrophoresis was also used to confirm the interaction between $NifI_{1,2}$ and dinitrogenase (Fig. 2*B*). When run alone, dinitrogenase and $NifI_{1,2}$ each formed a single major band. Combined with the results from the gel filtration above, this result suggests that $NifI_{1,2}$ exists primarily as a single, defined complex. Interaction of $NifI_{1,2}$ with dinitrogenase was observed as a retardation in the mobility of the band corresponding to dinitrogenase when $NifI_{1,2}$ was present. The 2OG did not prevent the gel shift when included in the loaded samples (data not shown); however, neither 2OG nor ATP were included in the gel or running buffer.

Effects of $NifI_{1,2}$ and 2OG on Nitrogenase Activity. To try to reconstitute switch-off *in vitro*, the effects of 2OG and purified $NifI_{1,2}$ on the activity of purified nitrogenase were determined. In all of these assays, high levels of ATP were maintained (see *Materials and Methods*). Fig. 3*A* shows a sample of data from one of these experiments. Nitrogenase activity was linear from 15 min to at least 45 min after the start of the reaction, and activity was calculated from this portion. The addition of $NifI_{1,2}$ resulted in a decrease in activity of >50-fold. The presence of 2OG (10 mM) in reactions containing $NifI_{1,2}$ resulted in the restoration of activity to the level of nitrogenase alone. For unknown reasons, 2OG had a negative effect on activity of nitrogenase alone, reducing it by about half. These results show that switch-off can be reconstituted *in vitro*, with a $NifI_{1,2}$-dependent inhibition of nitrogenase activity that is fully relieved by 2OG.

To determine the amount of $NifI_{1,2}$ needed to fully inhibit nitrogenase and to infer the stoichiometry of the complex, the effect of increasing $NifI_{1,2}$ concentrations on nitrogenase activity was determined (Fig. 3*B*). Because the exact oligomeric structure of $NifI_{1,2}$ is not known, the amount of $NifI_{1,2}$ added is expressed as a fraction of the mass of dinitrogenase present in the reaction. Although the effect of small amounts of $NifI_{1,2}$ was variable, nitrogenase activity decreased consistently with addition of $NifI_{1,2}$ above a 0.1 mass ratio. Activity was decreased by half between 0.2 and 0.25 mass ratio, and loss of >90% of the activity was achieved at a mass ratio of 0.4. Combined with the molecular masses calculated from the gel filtration (85 kDa for

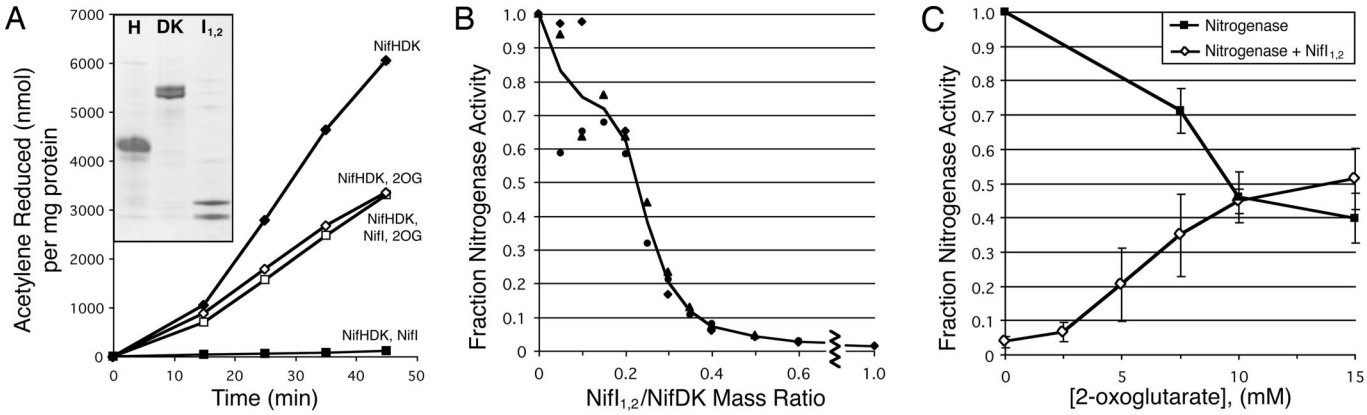

Fig. 3. Effects of $NifI_{1,2}$ and 2OG on nitrogenase activity. (*A*) Acetylene (C_2H_2) reduced per milligram of nitrogenase (75 μg each of NifH and NifDK) as a function of time, with or without 10 mM 2OG or 60 μg of $NifI_{1,2}$. Data are from a representative experiment. (*Inset*) A Coomassie-stained SDS/PAGE gel of the purified NifH, NifDK, and $NifI_{1,2}$. (*B*) The effect on nitrogenase activity of increasing amount of $NifI_{1,2}$ as a fraction of the nitrogenase activity in the absence of $NifI_{1,2}$. The concentration of $NifI_{1,2}$ is expressed as a ratio to the total amount of dinitrogenase (NifDK, 75 μg) in the reaction. Results from three independent experiments (circles, diamonds, and triangles) and the average (solid line) are shown. (*C*) Nitrogenase activity without (filled squares) or with (open diamonds) 60–75 μg of $NifI_{1,2}$ as a function of 2OG concentration, expressed as the fraction of activity without $NifI_{1,2}$ or 2OG. Error bars show the SEM of at least three independent experiments.

$NifI_{1,2}$ and 200 kDa for dinitrogenase), these results suggest that $\approx$1 mol of $NifI_{1,2}$ is sufficient to almost completely inhibit the activity of 1 mol of dinitrogenase.

Once an inhibitory level of $NifI_{1,2}$ was established, the effect of increasing concentrations of 2OG on nitrogenase activity was determined in the presence and absence of $NifI_{1,2}$ (Fig. 3*C*). Addition of 2OG from 2.5 to 10 mM resulted in a steady increase in nitrogenase activity in the presence of $NifI_{1,2}$, reaching 100% of the activity of nitrogenase alone at 10 mM. The 2OG had a negative effect on nitrogenase activity alone, decreasing the activity by $\approx$50% at 10 mM. In all but one of the experiments, 2OG concentrations >10 mM did not increase the activity of nitrogenase with $NifI_{1,2}$ above the activity of nitrogenase alone, suggesting that the positive effect of 2OG on activity is solely the relief of inhibition by $NifI_{1,2}$. Addition of other potential signaling molecules, glutamine, glutamate, or pyruvate at 7.5 mM, did not increase activity in the presence of $NifI_{1,2}$. When equal amounts (7.5 mM) of glutamine and 2OG were present, the activity was the same as with 2OG alone. Thus, 2OG appears to be the main nitrogen signal sensed by $NifI_{1,2}$.

Discussion

Results presented here support a model for regulation of nitrogenase activity in *M. maripaludis*: $NifI_{1,2}$, a heteromeric nitrogen sensor composed of subunits related to PII proteins, inhibits nitrogenase activity by direct binding to dinitrogenase. This binding is prevented, and inhibition thus relieved, by high levels of 2OG, which act as a signal of nitrogen limitation. The model is supported by copurification experiments and gel filtration with purified components that demonstrate the protein–protein interactions and *in vitro* nitrogenase assays with purified components. The mechanism is relatively simple and contrasts with the more complex regulation found in some Proteobacteria involving ADP-ribosylation of nitrogenase reductase by DraT and DraG (3), binding and membrane sequestration of DraG to the ammonia transporter AmtB by PII proteins, and covalent modification of the PII proteins by the glutamine sensor GlnD (12). The wide phylogenetic distribution of *nifI*$_1$ and *nifI*$_2$ genes, and their almost exclusive linkage to *nifHDK* genes, suggests that this mode of regulation may be conserved in a variety of diazotrophic Archaea and Bacteria (30).

Two features of our results are anomalous but do not, in our opinion, diminish the evidence for our model of regulation. First, 2OG, in addition to reversing binding of $NifI_{1,2}$ to dinitrogenase and preventing inhibition of nitrogenase activity, lowered nitrogenase activity when $NifI_{1,2}$ was not present. This effect could be due to the action of 2OG as a weak chelator of either magnesium or iron, lowering the MgATP concentration or damaging the nitrogenase metal cofactors by removal of iron. Second, the amount of 2OG required for relief of $NifI_{1,2}$ inhibition of nitrogenase, 2.5 mM to 10 mM, was $\approx$10-fold higher than the concentration range we estimated to be present in cells (29). A similar concentration was required to restore activity in crude extracts from wild-type *M. maripaludis*, suggesting that the high levels of 2OG needed were not simply an alteration of its binding to $NifI_{1,2}$ because of the His-tag fusions. It is possible that some factor might increase the affinity of $NifI_{1,2}$ for 2OG *in vivo*; however, this factor is apparently either not present or not active in the crude cell extracts.

The mechanism for regulation of nitrogenase activity proposed here has significant similarities with the proteobacterial mechanism. PII-related proteins are involved, and the T-loops are important for the interaction with the target protein. 2OG appears to act as an indicator of nitrogen deficiency. Also, ATP is required for the effect of 2OG (14). However, the two mechanisms have many contrasting features. The two PII-related proteins, $NifI_1$ and $NifI_2$, represent two subfamilies of PII that are distinct from all those that have been previously studied (26). Although heterotrimers of GlnB and GlnK have been observed (31), these proteins function primarily as homotrimers; in contrast, $NifI_{1,2}$ appears to function as a heteromer. It is interesting that the T-loops of both $NifI_1$ and $NifI_2$ seem to be required for the interaction with dinitrogenase but are not required for the interaction of $NifI_1$ with $NifI_2$. The $NifI_{1,2}$ heteromer has a molecular mass consistent with a hexamer and, in the presence of 2OG, appears to oligomerize to a higher molecular mass. The 2OG seems to indicate only the nitrogen state of the cell, not glutamine as well (32). We have found no evidence of covalent modification of $NifI_1$ or $NifI_2$ that would mediate an additional signal, as is the case with other PII-like proteins. The reconstitution of switch-off *in vitro* suggests either that modification is not necessary or that the purified $NifI_1$ and/or $NifI_2$ are already in their appropriately modified form. Finally, the immediate target of regulation is dinitrogenase rather than dinitrogenase reductase, and inhibition occurs by

direct binding of $NifI_{1,2}$ rather than a cascade of events leading to covalent modification.

It is interesting to speculate on how binding of $NifI_{1,2}$ to dinitrogenase inhibits nitrogenase activity. $NifI_{1,2}$ could occlude access of dinitrogenase reductase to dinitrogenase. Alternatively, binding of $NifI_{1,2}$ could interfere with substrate binding or result in a conformational change that somehow prevents electron transfer within dinitrogenase. In any case, the mechanism of inhibition may shed more light on the function of nitrogenase in general. Further study of the interaction between $NifI_{1,2}$ and dinitrogenase, as well as between $NifI_1$ and $NifI_2$ themselves, and how this interaction inhibits nitrogenase activity should yield intriguing results.

Materials and Methods

Cell Growth, Harvesting, and Extract Preparation. Standard procedures for culturing methanogens were used (33). Strains were grown in N-free medium in either 150-ml volumes using 1-liter bottles (for copurification experiments), as described previously, (29) or 10-liter volumes (for protein purification) by using a Microferm fermenter (New Brunswick Scientific). Puromycin (2.5 μg/ml) and neomycin (1 mg/ml) were used for selection when necessary. Medium composition for fermenter growth was altered, by using 2 g/liter sodium bicarbonate and 0.5 g/liter cysteine. The cysteine allows for better growth in the fermenter but cannot be used as a sole nitrogen source when added after autoclaving (A. K. Haydock and J.A.L., unpublished results). Fermenter growth was at 37°C with an agitation rate of 200 rpm, and gassing rates were 500 ml/min H_2, 200 ml/min CO_2, 200 ml/min N_2, and 20 ml/min 1% H_2S (balance N_2). Culture was harvested anaerobically from the fermenter at an OD_{660} of $\approx$1.0 via an 18-gauge syringe needle inserted into a 4-liter vacuum flask that was previously incubated in an anaerobic chamber and sealed with butyl rubber stoppers. Cells were pelleted anaerobically as described (29). Fermenter-grown cells were resuspended in a buffer containing 50 mM Hepes, pH 7.5, 50 mM NaCl, and 4 mM sodium dithionite and stored up to 8 months at −80°C in 5-ml serum vials (Wheaton Science Products, Millville, NJ) sealed with butyl rubber stoppers. Crude protein extracts were prepared by sonication as described (29). Protein quantitation was performed with Coomassie Plus Protein Assay reagent (Pierce) using BSA as a standard.

Strain Construction. Strains, plasmids, and oligonucleotide sequences used in this study appear in Table 1, which is published as supporting information on the PNAS web site. All strains are derivatives of *M. maripaludis* strain S2 (34). Two plasmids were constructed for expression of proteins with His_6 tags at their N or C termini in *M. maripaludis* by insertion of a polylinker directly downstream of the *Methanococcus voltae* histone promoter present in the vector pWLG40NZ-R (35). Polylinkers were constructed by annealing the oligonucleotides WHN coding and WHN noncoding (for N terminus His-tagging) or WHC coding and WHC noncoding (for C terminus His-tagging) and ligated into pWLG40NZ-R digested with NsiI and BglII, creating pLW40neo and pLCW40neo, respectively. His-tagged $NifI_2$ was constructed by PCR amplifying $nifI_2$ with primers containing ApaI (at the 5′ end of $nifI_2$) and AscI (3′ end) restriction sites and ligating into ApaI–AscI-digested pLW40neo. A similar process was used for $nifI_1$ and *nifH* using pLCW40neo and digestion with NsiI instead of ApaI. The resulting constructs coded for the amino acid sequence MHHHHHHIEGRGP preceding the N-terminal methionine for $NifI_2$ or GRAIEGRHHHHHH(stop) at the C terminus for $NifI_1$ and NifH. Deletions in the T-loop domains were constructed by amplifying the 5′ and 3′ regions of the gene flanking the desired deletion with primers that contained a BamHI site at their 5′ ends (resulting in the insertion of codons for glycine and serine). PCR products were digested with BamHI, AscI, and either ApaI (for $NifI_2$) or NsiI (for $NifI_1$) and ligated with pLW40neo digested with ApaI and AscI (for $NifI_2$) or pLCW40neo digested with AscI and NsiI (for $NifI_1$). The resulting mutations were the replacement of amino acids 48–52 with a serine (the glycine codon in the BamHI site replaced a glycine codon in the wild-type $nifI_2$) in $NifI_2$ and the replacement of amino acids 43–49 with a glycine and serine in $NifI_1$. A *M. maripaludis* strain (Mm1036) with an in-frame deletion in *nifH* (Δ4–268) was constructed from strain Mm900 for expression of the His-tagged NifH. Regions flanking *nifH* were PCR amplified and cloned into ApaI-XbaI digested pCRPrtNeo, and marker replacement was performed as described (36). All constructs were confirmed by sequencing. *M. maripaludis* strains Mm55 (Δ$nifI_2$), Mm56 (Δ$nifI_1$), and Mm1036 were transformed (37) with the following plasmids: Mm55 with pLW40neo, pLW40neo $nifI_2$, and pLW40neo $nifI_2$ΔT (creating strains Mm1012, Mm711, and Mm1017, respectively); Mm56 with pLCW40neo, pLCW40neo $nifI_1$, and pLCW40neo $nifI_1$ΔT (creating strains Mm1050, Mm1051, and Mm1067, respectively); and Mm1036 with pLCW40neo *nifH* (creating strain Mm1046).

Copurification Experiments. Nickel-ion-affinity chromatography was used to identify proteins that copurify with the His-tagged $NifI_1$ and $NifI_2$ proteins. Binding reactions were prepared in 18-ml modified Balch tubes (shortened to 10-cm length at the Physics Department glass shop, University of Washington) in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of H_2/N_2 (5:95). Protein extracts were bound to 0.25 ml of Ni-NTA agarose (Novagen) in binding buffer (25 mM Hepes, pH 7.5, 10 mM sodium dithionite, 100 mM NaCl, 10 mM imidazole, 5 mM ATP, 12.5 mM $MgCl_2$, and 3.4 mg/ml phosphocreatine) with or without 10 mM 2OG. The total volume was 3 ml, with a protein concentration of 2–3 mg/ml. Tubes were stoppered, removed from the anaerobic chamber, and incubated with shaking at 30°C for 30–45 min. If nitrogenase activity was to be measured during the binding, 0.1 mg/ml creatine phosphokinase and 0.5 mg/ml BSA were included in the mixture, the headspace was flushed for 2 min with 100% argon gas, and 0.2 ml of 100% acetylene gas was added before incubation. Tubes were returned to the anaerobic chamber, and the binding mixture was applied to 10-ml PolyPrep Columns (Bio-Rad) drained by gravity flow. The Ni-NTA agarose was washed twice with 5 ml of binding buffer, and bound proteins were eluted with 1.5 ml of binding buffer containing 100 mM imidazole (for His-tagged $NifI_2$) or 500 mM imidazole (for His-tagged $NifI_1$). Elution fractions were run on 12% SDS/PAGE (Fig. 1 *A* and *C*) or 10% Tris-tricine gels (Fig. 1*B*), and proteins were visualized with Silver Stain Plus kit (Bio-Rad).

Protein Identification by 2D Gel Electrophoresis and MS. Elution fractions (1 ml) were precipitated with 0.3 volumes of acetone, and 2D gel electrophoresis was performed as described (38), with the following modifications: pH 3–10 NL (nonlinear) immobilized pH gradient (IPG) strips (Amersham Pharmacia Biosciences) were used, 1% pH 3–10 NL buffer was included in the rehydration buffer, and 12% acrylamide gels were used for the SDS/PAGE. Gels were silver stained (39), and spots to be identified were excised by using a clean scalpel. As a negative control, a portion of the gel with no visible protein was also taken. In-gel digestion with sequencing-grade modified trypsin (Promega) was performed essentially as described (39), but without the treatment with iodoacetamide. Resulting peptide fragments were desalted by using C_{18} Zip-Tips (Millipore) according to the manufacturer's instructions. MALDI-TOF MS was performed at the Medicinal Chemistry Mass Spectrometry Center, University of Washington, using α-cyano-4-hydroxy-cinnamic acid as a matrix. Peaks that were not in the mass spectrum of the negative control were used to identify the corresponding protein with PEPTIDESEARCH version 3.0.5b0 (40) using the *M. maripaludis* strain S2 genome sequence (41) as an index file.

Purification of NifI and Nitrogenase Components. Proteins were purified by nickel affinity essentially as described above for the copurification experiments, with some modifications. The volumes (binding, 12 ml; washes, 25 ml; elution, 3 ml) and the amount of Ni-NTA (1 ml) were increased, 20-cm length Econo-columns (Bio-Rad) were used, and protein concentrations were 10–15 mg/ml. Dinitrogenase (NifDK) and the NifI proteins (His-tagged $NifI_2$ and $NifI_1$) were purified from strain Mm711. For purification of NifI proteins, 20 mM 2OG was included in all of the steps to prevent dinitrogenase copurification, and elution was performed with imidazole as above. For dinitrogenase purification, elution was performed with 10 mM 2OG. His-tagged dinitrogenase reductase (NifH) was purified by using extracts from strain Mm1046. ATP, phosphocreatine, and $MgCl_2$ were omitted, and elution was done with 100 mM imidazole. Elution fractions were concentrated to $\approx$100 μl by using Vivaspin 0.5-ml centrifugal concentrators with 10-kDa (for NifI proteins) or 30-kDa (for NifH and NifDK) molecular mass cut-off PES membranes, by using a Spectrafuge (Labnet International, Edison, NJ) microcentrifuge in the anaerobic chamber. Concentrated proteins were then desalted three times with 10 volumes of resuspension buffer (25 mM Hepes, pH 7.5, 25 mM NaCl, and 2–5 mM sodium dithionite) by using the centrifugal concentrators and stored on ice until use.

Gel Filtration. Gel filtration was performed at room temperature in an anaerobic chamber using a Hi-Prep 16/60 Sephacryl S-300 column (Amersham Pharmacia Biosciences) with a flow rate of 0.5 ml/min. The buffer contained 50 mM Hepes, pH 7.5, 100 mM sodium chloride, 2.5 mM ATP, 6.25 mM $MgCl_2$, and 2.5 mM sodium dithionite. For some runs, 10 mM 2OG was included. Before loading, proteins were incubated at room temperature for 10 min in resuspension buffer containing 5 mM ATP and 12.5 mM $MgCl_2$, with or without 20 mM 2OG. Molecular mass calibration was done by using Low and High Molecular Weight Calibration kits (Amersham Pharmacia Biosciences). Protein in fractions (0.75 ml) eluted from the column was quantitated with Coomassie Plus Protein Assay reagent (Pierce). To identify the proteins present in certain peaks, corresponding fractions were concentrated by using 0.5-ml centrifugal concentrators with 3-kDa molecular mass cut-off membranes (Vivaspin), and SDS/PAGE was performed by using Precise 4–20% gradient gels (Pierce).

Native Gel Electrophoresis. Native gel electrophoresis was performed anaerobically by using a modified version of the Laemmli method without SDS (42). Resolving gels (8% acrylamide) were cast aerobically and brought into an anaerobic chamber, where the stacking gels were cast and the gels were run. Sodium dithionite was used in the cast gels (0.5 mM) and the running buffer (2 mM), which was bubbled with 100% N_2 gas to remove oxygen.

Nitrogenase Activity Assays. *In vitro* nitrogenase activity assays, using an ATP regenerating system (phosphocreatine and creatine phosphokinase) and sodium dithionite as a reductant, were performed as described (29), with modifications. Reactions (0.5 ml) were prepared in 5-ml serum vials with 20 mM dithionite, 1 ml of 100% acetylene was added to the headspace, and 0.3-ml gas samples were taken by syringe at various times after the beginning of the 30°C incubation. Reactions typically contained 75 μg each of NifH and NifDK, and activities were calculated based on 150 μg of total nitrogenase.

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