JOURNAL OF BACTERIOLOGY, Oct. 1991, p. 6159–6161
0021-9193/91/196159-03$02.00/0

Vol. 173, No. 19

Glycine 100 in the Dinitrogenase Reductase of *Rhodospirillum rubrum* Is Required for Nitrogen Fixation but Not for ADP-Ribosylation

LORI J. LEHMAN† AND GARY P. ROBERTS*

Department of Bacteriology and Center for the Study of Nitrogen Fixation, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 15 April 1991/Accepted 22 July 1991

Dinitrogenase reductase (Rr2) is required for reduction of the molybdenum dinitrogenase in the nitrogen fixation reaction and is the target of posttranslational regulation in *Rhodospirillum rubrum*. This posttranslational regulation involves the ADP-ribosylation of Rr2. To study the structural requirements for these two functions of Rr2, i.e., activity and regulation, two site-directed mutations in *nifH*, the gene encoding Rr2, were constructed and analyzed. The mutations both affected a region of the protein known to be highly conserved in evolution and to be relevant to both of the above properties of the protein. These mutants were both Nif⁻, but one of the altered Rr2s was a substrate for ADP-ribosylation. This demonstrates that the ability of Rr2 to participate in nitrogen fixation can be separated from its ability to act as a substrate for ADP-ribosylation.

Nitrogenase is a protein complex that contains two components, dinitrogenase (Rr1) and dinitrogenase reductase (Rr2). Rr2 reduces Rr1, which then reduces dinitrogen to ammonia. In *Rhodospirillum rubrum*, Rr2 is posttranslationally regulated by two enzymes, dinitrogenase reductase ADP-ribosyl transferase (DRAT) (10) and dinitrogenase reductase-activating glycohydrolase (DRAG) (20), which respond to levels of light and ammonia sensed by the cell (5). When the cell is exposed to darkness or ammonia, DRAT ADP-ribosylates one of the two identical subunits of Rr2 and inactivates the enzyme (12). This modification by DRAT is a highly specific reaction, as nondenatured Rr2 is its only substrate in the cell (10, 11). When the ammonia is exhausted or when light is again present, DRAG removes the ADP-ribose group and the enzyme is activated (5). The site of ADP-ribosylation on Rr2 is Arg-101 (16), which is part of a region conserved in all dinitrogenase reductases sequenced to date (17). This region also contains a cysteine, Cys-98, involved in 4Fe-4S cluster binding (4).

Klebsiella pneumoniae lacks this posttranslational modification system, and a mutant strain, UN1041 (13), has been shown to contain histidine in place of the conserved arginine residue (2). The altered protein was purified and found to be unable to pass electrons to dinitrogenase. It is also not a substrate for modification by DRAT in vitro (9), though the wild-type protein from this organism is a substrate for DRAT in vitro (10). This residue thus appears to be important for both nitrogen fixation activity and in vitro ADP-ribosylation.

The *R. rubrum nifH* gene, which encodes Rr2, has previously been cloned, sequenced, and functionally characterized (6). In the present study, two strains with point mutations in the previously mentioned conserved region of *nifH* were constructed by using site-directed mutagenesis. The site of ADP-ribosylation, Arg-101, and the adjacent Gly-100 were changed. Characterization of these mutants demonstrated that while mutations at Arg-101 and Gly-100 eliminated nitrogen fixation in *R. rubrum*, only the alteration at Arg-101 prevented ADP-ribosylation. This is the first demonstration that an alteration in this region can separate the ability of Rr2 to participate in nitrogen fixation from its ability to act as a substrate for ADP-ribosylation.

* Corresponding author.
† Present address: Department of Genetics, North Carolina State University, Raleigh, NC 27695.

MATERIALS AND METHODS

Strains and growth. Strains used in this study are listed in Table 1. The following growth media for *R. rubrum* are described by Lehman and Roberts (7): MN (minimal), MN⁻ (N free), and MG (a low-N medium for nitrogenase derepression that is MGC (minimal medium with glutamate as the N source) with 10.5 g of MOPS [morpholinepropanesulfonic acid] per liter). Antibiotic levels for *R. rubrum* were as follows: kanamycin, 10 μg/ml; streptomycin, 100 μg/ml; spectinomycin, 15 μg/ml; and tetracycline, 1 μg/ml. Cultures were grown and derepressed for nitrogenase as previously described (6, 7). Derepression of nitrogen fixation was assayed in vivo by whole-cell acetylene reduction (5). ADP-ribosylation of derepressed cultures was initiated by placing cultures in the dark for 1 h.

Mutagenesis. Unless otherwise mentioned, DNA manipulations were performed as described by Maniatis et al. (14). Single-stranded DNA was generated from the phagemid pLL120 by using the helper phage R408 (19). Mutagenic oligonucleotides of 15 (Ala-100) and 21 bp (Val-101) were synthesized by Operon (San Pablo, Calif.) and by the University of Wisconsin Biotechnology Center (Madison, Wis.). The single-stranded DNA was mutagenized by the method of Vandeyar et al. (23) with several alterations. The concentration of the mutagenic oligonucleotide was raised fourfold, and the annealing mix was heated to boiling for 5 min and then allowed to cool to room temperature. T7 polymerase (U.S. Biochemical, Cleveland, Ohio) was substituted for Klenow large fragment, and 2 mM dithiothreitol was added to the polymerization-ligation step. The mutagenized DNA was transformed into fresh competent cells (3) of strain ER1562. The resulting clones were screened by restriction digestion and confirmed by sequencing with the Sequenase Kit (U.S. Biochemical) and [^{35}S]dATP (Amersham, Arlington Heights, Ill.).

The mutagenized region was excised with *Eco*RI and cloned into the *Eco*RI site of pUX3, which is unable to replicate in *R.*

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant traits	Source or reference
R. rubrum		
UR2	Sm^r *nif*$^+$	6
UR249	Sm^r *nifH1071*	This work
UR250	Sm^r *nifH1072*	This work
E. coli		
DH5α	*recA*	Gibco-BRL
ER1562	*mcrAB*	New England Biolabs
S17-1	*pro tra*$^+$ Km^r	22
Plasmids		
pBSKS(−)	Ap^r f1 origin	Stratagene
pSUP202	Ap^r Cm^r *mob*$^+$	22
pUC-4K	Km^r	24
pUX3	pSUP202 with Km^r	This work
pLL120	pBSKS(−) with 2.1-kb *Eco*RI fragment carrying *nifH*	This work
pLL129	Cm^r Km^r *nifH1071 mob*$^+$	This work
pLL130	Cm^r Km^r *nifH1072 mob*$^+$	This work

TABLE 2. Characterization of mutants

Property	UR2	UR249	UR250
Alteration	None	Gly-100 to Ala-100	Arg-101 to Val-101
Sequence (bp 301–306)[a]	GGCCGT	GCCCGT	GGGGTG
Growth on nitrogen-free plates	+	−	−
Whole-cell acetylene reduction (% of wild type)[b]	100	<1	<1
Rr2 accumulation	+	+	+[c]
Rr2 modification in vivo[d]	+	+	−
Spontaneous reversion rate	NA[e]	$<10^{-9}$	$<10^{-9}$
NTG-stimulated reversion rate	NA	10^{-8}	$<10^{-9}$

[a] Numbered from start codon (see reference 6; altered bases are underlined).
[b] 8-h derepression.
[c] Charge-changed product.
[d] 48-h derepression.
[e] NA, not applicable.

rubrum. pUX3 was constructed by inserting the Km cassette from pUC-4K into the *Pst*I site of pSUP202. The resulting plasmid was conjugated from S17-1, which contains the *tra* functions, into UR2, selecting for Km^r on MN plates to demand plasmid integration. A colony was then grown without kanamycin for 17 to 25 generations and screened for loss of Km^r caused by recombination between the two copies of the *nifH* region flanking the inserted vector. The mutant was confirmed by sequencing polymerase chain reaction-amplified (21) chromosomal DNA or by cloning polymerase chain reaction product into pBSKS(−) followed by sequencing.

Characterization of mutants. The two-dimensional gel electrophoresis protocol used was that of Roberts et al. (18); samples were prepared as described previously (1). Proteins were visualized by silver staining (15). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis was performed as described by D. Lies et al. (8).

RESULTS

Construction of mutants. Two mutations were constructed in the *nifH* gene carried on pLL120. The site of ADP-ribosylation, Arg-101, was changed to Val-101 in a 4-bp alteration that also eliminated a *Nae*I site, allowing isolates to be screened by restriction digestion. The adjoining, highly conserved Gly-100 was converted to Ala-100 by a 1-bp change that eliminated the same *Nae*I site.

To allow reintroduction into *R. rubrum*, each mutagenized fragment was cloned into pUX3, creating pLL129 (Gly-100 to Ala-100) and pLL130 (Arg-101 to Val-101). After reintroduction of the mutations, Km^s strains were obtained at a frequency of 2×10^{-4} to 5×10^{-4}. Twenty-five percent of these Km^s isolates from each reintroduction were found to contain the mutation. The resulting strains were UR249 (Gly-100 to Ala-100) and UR250 (Arg-101 to Val-101). The Rr2 from UR249 will be referred to as Rr2GA and that of UR250 will be referred to as Rr2RV.

Characterization of mutants. The resulting strains were derepressed and shown to have no significant acetylene reduction activity (Table 2). They were also unable to grow on N-free plates. No reversion to Nif$^+$ was seen with either strain on N-free plates (Table 2). Following NTG treatment, the Ala-100 mutation, a 1-bp change, reverted to Nif$^+$ at a frequency of 10^{-8}. Two of these revertants were sequenced and proved to be genotypically wild type. The 4-bp change of UR250, however, yielded no revertants, even with mutagenesis.

Following derepression of the mutants, two-dimensional gel analysis demonstrated that both strains synthesized and accumulated Rr2 (Fig. 1). Rr2RV appeared as a charge-altered protein, as would be expected in light of the loss of the arginine residue. Additionally, Rr2RV displayed an apparently higher molecular weight, an anomaly also seen with the analogous protein from *K. pneumoniae* UN1041 (9). Rr2GA behaved like Rr2 on gels. When derepressed strains were exposed to darkness for 1 h, no modification of Rr2RV was detected on silver-stained two-dimensional gels, but modification of Rr2GA was seen. This modification was reversible upon reexposure to light, as in UR2 (Fig. 1). The identical electrophoretic pattern of behavior in the wild type (UR2) has been shown to be due to ADP-ribosylation (5), indicating that Rr2GA can be ADP-ribosylated but that Rr2RV cannot be.

DISCUSSION

Neither the radical change of Arg-101 to Val-101 nor the conservative change of Gly-100 to Ala-100 yielded an Rr2 able to support growth on N_2 on N-free plates or to reduce acetylene in vivo. The region of Rr2 containing these two residues is highly conserved in all dinitrogenase reductases that have been sequenced, and the Gly-100 and Arg-101 residues are completely conserved (17). This conservation is true regardless of whether the organism has a regulatory ADP-ribosylation system and argues that these residues are important to the enzyme for structural and/or functional reasons. The lack of activity due to the mutations described in this report agrees with this hypothesis. The lack of activity of the Val-101 mutation is not surprising, since an Arg-100 to His-100 mutation in *K. pneumoniae* UN1041 had the same effect (9). However, it is interesting that the relatively minor alteration from Gly-100 to Ala-100 does not just reduce

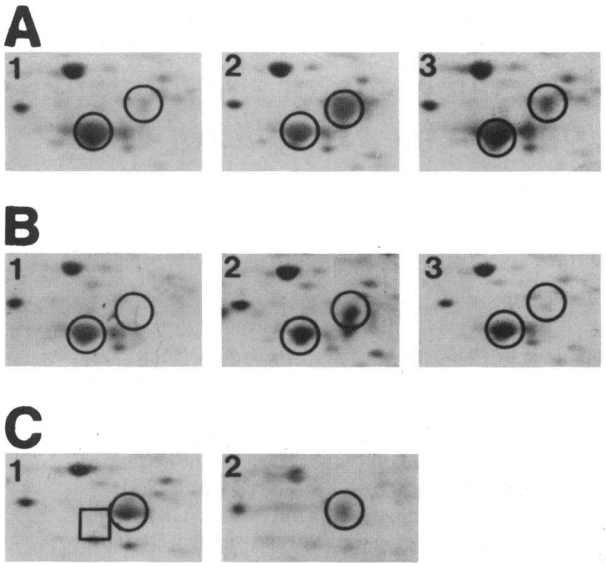

FIG. 1. Two-dimensional gel analysis of Rr2 accumulation and modification status in the wild type and in mutants. In panels A and B, the lower circles show the positions of unmodified proteins and the upper circles show the positions of the modified protein. In panel C, only the unmodified protein is circled. (A) 1, Rr2; 2, Rr2 after 1 h of darkness; 3, Rr2 after reexposure to light for 1 h. (B) 1, Rr2GA; 2, Rr2GA after 1 h of darkness; 3, Rr2GA after reexposure to light for 1 h. (C) 1, Rr2RV (the square indicates the position that Rr2 would occupy); 2, Rr2RV after 1 h of darkness.

activity but completely abolishes it. Sequence analysis of two of the NTG-generated Nif^+ revertants of this strain showed that both had regained the wild-type sequence in this region. A glycine in this position not only appears to be the best amino acid but may be the only amino acid that confers sufficient activity for detectable growth.

In view of the requirement of Gly-100 for activity, it is interesting that it is not required for ADP-ribosylation. DRAT does not ADP-ribosylate any other proteins in vivo in *R. rubrum* and is specific for undenatured dinitrogenase reductase (11). It will not modify arginine, *N*-dansyl arginine, or the Rr2-derived hexapeptide Gly-Arg-Gly-Val-Ile-Thr containing Arg-101 (10). In light of this extreme substrate specificity, it is surprising that a residue immediately adjacent to the site of ADP-ribosylation can be altered without loss of that specificity. Either Rr1 and DRAT do not interact with identical domains on Rr2, or there are distinct conformational requirements for the two reactions.

ACKNOWLEDGMENTS

We thank Wayne Fitzmaurice, Douglas Lies, and Paul Ludden for helpful discussions.

This work was supported by the College of Agricultural and Life Sciences, by the University of Wisconsin-Madison Graduate School, and by grant 89-37262-4740 from the U.S. Department of Agriculture.

REFERENCES

1. **Bonham, D., L. J. Lehman, G. P. Roberts, and P. W. Ludden.** 1989. Regulation of carbon monoxide dehydrogenase and hydrogenase in *Rhodospirillum rubrum*: the effects of CO and oxygen on synthesis and activity. J. Bacteriol. **171**:3102–3107.
2. **Chang, C. L., L. C. Davis, M. Rider, and D. J. Takemoto.** 1988. Characterization of *nifH* mutations of *Klebsiella pneumoniae*. J. Bacteriol. **170**:4015–4022.
3. **Hanahan, D.** 1983. Studies on the transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
4. **Hausinger, R. P., and J. Howard.** 1983. Thiol reactivity of the nitrogenase Fe-protein from *Azotobacter vinelandii*. J. Biol. Chem. **258**:13486–13492.
5. **Kanemoto, R. H., and P. W. Ludden.** 1984. Effect of ammonia, darkness, and phenazine methosulfate on whole-cell nitrogenase activity and Fe protein modification in *Rhodospirillum rubrum*. J. Bacteriol. **158**:713–720.
6. **Lehman, L. J., W. P. Fitzmaurice, and G. P. Roberts.** 1990. The cloning and functional characterization of the *nifH* gene of *Rhodospirillum rubrum*. Gene **95**:143–147.
7. **Lehman, L. J., and G. P. Roberts.** 1991. Identification of an alternative nitrogenase system in *Rhodospirillum rubrum*. J. Bacteriol. **173**:5705–5711.
8. **Lies, D., W. P. Fitzmaurice, L. J. Lehman, and G. P. Roberts.** Unpublished data.
9. **Lowery, R. G., C. L. Chang, L. C. Davis, M. C. McKenna, P. J. Stevens, and P. W. Ludden.** 1989. Substitution of histidine for arginine-101 of dinitrogenase reductase disrupts electron transfer to dinitrogenase. Biochemistry **28**:1206–1212.
10. **Lowery, R. G., and P. W. Ludden.** 1988. Purification and properties of dinitrogenase reductase ADP-ribosyltransferase from the photosynthetic bacterium *Rhodospirillum rubrum*. J. Biol. Chem. **263**:16714–16719.
11. **Lowery, R. G., L. L. Saari, and P. W. Ludden.** 1986. Reversible regulation of the nitrogenase iron protein from *Rhodospirillum rubrum* by ADP-ribosylation in vitro. J. Bacteriol. **166**:513–518.
12. **Ludden, P. W., G. E. Preston, and T. E. Dowling.** 1982. Comparison of active and inactive forms of iron protein from *Rhodospirillum rubrum*. Biochem. J. **203**:663–668.
13. **MacNeil, T., D. MacNeil, G. P. Roberts, M. A. Supiano, and W. J. Brill.** 1978. Fine structure genetic mapping and complementation analysis of *nif* (nitrogen fixation) genes in *Klebsiella pneumoniae*. J. Bacteriol. **136**:253–266.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Morissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
16. **Pope, M. R., S. A. Murrell, and P. W. Ludden.** 1985. Covalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginine. Proc. Natl. Acad. Sci. USA **82**:3173–3177.
17. **Pretorius, I.-M., D. E. Rawlings, E. G. O'Neill, W. A. Jones, R. Kirby, and D. R. Woods.** 1987. Nucleotide sequence of the gene encoding the nitrogenase of *Thiobacillus ferrooxidans*. J. Bacteriol. **169**:367–370.
18. **Roberts, G. P., T. MacNeil, D. MacNeil, and W. J. Brill.** 1978. Regulation and characterization of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. J. Bacteriol. **136**:267–279.
19. **Russell, M., S. Kidd, and M. R. Kelley.** 1986. An improved filamentous helper phage for generating single-stranded DNA. Gene **45**:333–338.
20. **Saari, L. L., E. W. Triplett, and P. W. Ludden.** 1984. Purification and properties of the activating enzyme for iron protein of nitrogenase from the photosynthetic bacterium *Rhodospirillum rubrum*. J. Biol. Chem. **259**:15502–15508.
21. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **230**:1076–1078.
22. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
23. **Vandeyar, M. A., M. P. Weiner, C. J. Hulton, and C. A. Batt.** 1988. A simple and rapid method for the selection of oligodeoxynucleotide-directed mutants. Gene **65**:129–133.
24. **Vierra, J., and J. Messing.** 1982. The pUC plasmids, an M13-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.