Nif\textsuperscript{−} Phenotype of Azotobacter vinelandii UW97

CHARACTERIZATION AND MUTATIONAL ANALYSIS*

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We have identified the molecular basis for the nitrogenase negative phenotype exhibited by Azotobacter vinelandii UW97. This strain was initially isolated following nitrosoguanidine mutagenesis. Recently, it was shown that this strain lacks the Fe protein activity, which results in the synthesis of a FeMo cofactor-deficient apoprotein. Activation of this apoprotein requires the addition of both MgATP and wild-type Fe protein to the crude extracts made from A. vinelandii UW97 (Allen, R. M., Homer, M. J., Chatterjee, R., Ludden, P. W., Roberts, G. P., and Shah, V. K. (1993) J. Biol. Chem. 268 23670–23674). Earlier, we proposed the sequence of events in the MoFe protein assembly based on the biochemical and spectroscopic analysis of the purified apoprotein from A. vinelandii DJ 54 (Gavini, N., Ma, L., Watt, G., and Burgess, B. K. (1994) Biochemistry 33, 11842–11849). Taken together, these results imply that the assembly process of apoprotein is arrested at the same step in both of these strains. Since A. vinelandii DJ 54 is a nifH strain, this strain is not useful in identifying the features of the Fe protein involved in the MoFe protein assembly. Here, we report a systematic analysis of an A. vinelandii UW97 mutant and show that, unlike A. vinelandii DJ 54, the nifH gene of A. vinelandii UW97 has no deletion in either coding sequence or the surrounding sequences. The specific mutation responsible for the Nif\textsuperscript{−} phenotype of A. vinelandii UW97 is the substitution of a non-conserved serine at position 44 of the Fe protein by a phenylalanine as shown by DNA sequencing. Furthermore, oligonucleotide site-directed mutagenesis was employed to confirm that the Nif\textsuperscript{−} phenotype in A. vinelandii UW97 is exclusively due to the substitution of the Fe protein residue serine 44 by phenylalanine. By contrast, replacing Ser-44 with alanine did not affect the Nif phenotype of A. vinelandii. Therefore, it seems that the Nif\textsuperscript{−} phenotype of A. vinelandii UW97 is caused by a general structural disturbance of the Fe protein due to the presence of the bulky phenylalanine at position 44.

Nitrogenase is one of the most intriguing, complex metalloenzymes and is the protein that catalyzes the MgATP-dependent reduction of N\textsubscript{2} to ammonia (1–4). The properties of the nitrogenase proteins, isolated from diverse bacteria, are very similar (5–7). This enzyme is unusual because it is composed of two separately purified proteins, both of which are extremely oxygen sensitive. The smaller of the two proteins, designated the Fe protein, has a molecular mass of about 60,000 daltons and is a dimer of identical subunits encoded by the nifH gene (1, 8). The larger of the two proteins, designated the MoFe protein, has a molecular mass of 230,000 daltons (6, 7). The MoFe protein is a tetramer in its biologically active form. It is composed of two identical halves, each containing an α-subunit and a β-subunit encoded by the nifD and nifK genes, respectively. Each of these identical halves contains one FeMo cofactor and one P-cluster center. These two halves are believed to be unable to communicate with each other. A major breakthrough in understanding the structural properties of nitrogenase came with the publication of the crystallographic structures of both component proteins of nitrogenase and their metal centers (9–16). Cloning and sequencing most of the genes involved in the synthesis and assembly of nitrogenase, in turn, have provided us with an elegant picture of the genetic organization of this complex metalloenzyme (1, 17, 18). Thus, now the stage is set for concentrating on studies to elucidate how the component proteins of this multi-centered metalloenzyme are regulated by their structural constraints during their interplay in the biological N\textsubscript{2} fixation reaction.

For N\textsubscript{2} reduction to occur, electrons need to be transferred one at a time from the Fe protein to the MoFe protein. The sequence of events involved in this reaction is generally referred to as the "Fe protein cycle" and is described elsewhere (19). Basically, the Fe protein is reduced by either flavodoxin or ferredoxin (in vivo) or SO\textsubscript{2} (in vitro from dithionite). The reduced Fe protein, in turn, binds two MgATPs, undergoes a conformational change, and then binds to the MoFe protein (20, 21). Mutants that bind MgATP normally but cannot undergo the MgATP-induced conformational change have been identified (22–27); therefore, it is presumed that these two functions are independent of each other. Once the Fe protein-MgATP complex binds to the MoFe protein, both the MgATPs are hydrolyzed and one electron is transferred from the Fe protein to the MoFe protein (28). The Fe protein and the MoFe protein need to interact for MgATP hydrolysis to occur. Cross-linking experiments and mutagenesis data indicate that the MoFe protein probably induces a conformational change in the Fe protein, which is necessary to bring the internal groups close together to catalyze the hydrolysis (29, 30).

The Fe protein of nitrogenase has been reported to have four distinct functions. As mentioned above, the Fe protein is the specific physiological electron donor to the MoFe protein. A second function of the Fe protein involves the initial biosynthesis of FeMo cofactor (31–37). Although the pathway for FeMo cofactor synthesis has not yet been established, it has been known for some time that the FeMo cofactor is synthesized separately from the MoFe protein polypeptides. In fact, its synthesis requires the combined action of the nifQ, -B, -N, -E, and -V genes (38, 39). It was documented that the mutants of Klebsiella pneumoniae and Azotobacter vinelandii that did not
synthesize the nifH polyptide also did not synthesize FeMo cofactor (31, 33). Recently, this was further confirmed by purifying the FeMo cofactor-deficient MoFe protein from one of these mutants and characterizing it by biochemical and spectroscopic analysis (36). Based on these and other experiments, it is now accepted that the nifH gene product is required for an early step in FeMo cofactor biosynthesis. However, it is not known what features of the Fe protein are required for this action. The third Fe protein function involves the insertion of pre-formed FeMo cofactor into an inactive FeMo cofactor-deficient MoFe protein (23, 36, 37). This step in the assembly of an active MoFe holoprotein requires not only the nifH polyptide but also MgATP. Once again, it is not known what features of the Fe protein are required for FeMo cofactor insertion. Finally, the Fe protein has been implicated in the regulation of the transcription of its own operon and in the expression of related nitrogenase systems (40).

Earlier, we showed that the ΔnifH strain of A. vinelandii, designated DJ 54, accumulates a FeMo cofactor-deficient MoFe protein that is distinct from the FeMo cofactor-deficient MoFe proteins synthesized by nifB, nifN, or nifE strains (35). Recently, we have purified apodinitrogenase from A. vinelandii DJ 54 and shown that it can be activated by the addition of wild-type Fe protein and MgATP when the additional required components are supplied by cell-free extracts from a ΔnifD strain of A. vinelandii (36). This observation highlights the crucial role of the Fe protein in FeMo cofactor synthesis/insertion. However, in the DJ 54 strain the entire nifH gene is deleted. Therefore, no information regarding the domains on the Fe protein responsible for its involvement in FeMo cofactor synthesis or insertion can be obtained from this strain. To understand what features of the Fe protein are responsible for its ability to function in FeMo cofactor synthesis or insertion, we needed to obtain Nif− mutants with point mutations in the nifH gene. Here, we report on the analysis and identification of a point mutation in nifH that impaired FeMo cofactor biosynthesis/insertion.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids used in this study are listed in Table I. A. vinelandii strains were grown at 30 °C in modified Burk nitrogen-free (BN−) medium (41). The BN− medium contained 20 g of sucrose, 0.64 g of KH2PO4·H2O, 0.16 g of KH2PO4, 0.142 g of Na2SO4, 0.203 g of MgCl2·6H2O, 0.074 g of CaCl2·2H2O, and 0.034 g of FeC8H6O4 per liter. When it was necessary to include fixed nitrogen in the medium, ammonium acetate (NH4)2O·H2O was added to a final concentration of 400 μg per ml. Escherichia coli strains were grown at 37 °C in Luria broth, ZTY, or minimal medium supplemented with glucose (0.2%) and 10 μg/ml thiamine (42). The antibiotic ampicillin was used to a final concentration of 50 μg/ml wherever the selection was made.

| Strains or plasmids | Relevant characteristics or genotype | Source |
|---------------------|-------------------------------------|--------|
| E. coli TG1         | K12, (lac-pro), supE, thi, hsdS5'/ F' traD36 proA' B−, lacIΔ71, lacZΔM15 endA1, recA1, hsrR171, supE44, λ−, thi−1, gyrA, relA1, deaR1, F' lacZΔM15(laszZAargaF) | Amersham |
| E. coli 1NVuF        |                                    | Invitrogen |
| A. vinelandii OP     | Wild type                          | D. R. Dean |
| A. vinelandii UW     | Wild type                          | M. Homer and G. P. Roberts |
| A. vinelandii UW97   | nif−; described in this paper      | Ref. 38  |
| A. vinelandii D154   | nif−; defined deletion in the nifH gene | Ref. 52  |
| pCR™II              | 3.9-Kilobase plasmid with colE1 origin, the lacZ for blue/white selection, ampC, kanamycin resistance genes, and a versatile polylinker. It is designed for cloning PCR products directly from a PCR reaction | Invitrogen |
| M13 mp18 and mp19    | 9-Bislinkage vectors constructed for cloning, sequencing of DNA fragments and containing the lacZ for blue/white selection | B.B. Roberts |
| pDB6                | 6-Kilobase Smal fragment spanning A. vinelandii nifHDKY genes cloned in the Smal site of pUC8; Amp′, with colE1 origin of replication | Ref. 6  |
| pBG120              | Derived from pDB6; contained intact nifH and 5′ region of the nifD | This study |

**TABLE I**

**Bacterial strains and plasmids**

General Molecular Techniques—DNA subcloning, plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations, and E. coli transformations were carried out as described in the laboratory manuals (42) or as suggested in the manufacturer's instructions. A. vinelandii was transformed with plasmid or recombinant M13 DNA by the method of Page and Von Tiggesem (43).

Isolation of Chromosomal DNA—Chromosomal DNA was prepared from A. vinelandii strains by a modification of the method described by Dillida and Woo (44). Cells were harvested from 100 ml of an overnight culture and resuspended in 10 ml of STE buffer (10 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM Na2EDTA). This cell suspension, proteinase K and SDS were added to give final concentrations of 100 μg/ml and 0.5% (w/v), respectively. The mixture was incubated at 55 °C for 10–16 h. The lysate was gently extracted with an equal volume of phenol/chloroform/isoamyl alcohol (24:1), and after repeating this step two or more times, the aqueous layer was extracted once with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous layer was collected and adjusted to 0.2 μl of NaOAc, pH 5.5; then, two volumes of 100% ethanol were gently layered on top, and the DNA was spooled from the aqueous-ethanol interface using a sterile glass rod. The spooled DNA was washed with 70% (v/v) ethanol, and the excess ethanol was evaporated off. The DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM Na2EDTA) at 4 °C overnight. This DNA was treated with DNase-free RNase (100 μg/ml) for 1 h at 37 °C; then, the extraction and spooling of the DNA was repeated as described above. Finally, the DNA was recovered by dissolving in TE buffer.

Southern Blotting and Hybridizations—Southern blotting and hybridizations were carried out as described (55). Briefly, the chromosomal DNA was cleaved with appropriate restriction endonucleases and separated on agarose gels in 0.5 × TBE buffer (42) (1× buffer). To determine the transfer of large DNA fragments, the agarose gels were submerged in 250 mM HCl for 10 min to accomplish limited depurination of the DNA. After the gels were subjected to denaturation and neutralization steps, the DNA was transferred to a nylon membrane (MagnaGraph Nucleic Acid Transfer Membrane, Micron Separations, Inc.) with capillary action in 10 × SSC for 16–24 h. The transferred DNA was fixed to the nylon membrane by baking in a vacuum oven for 1 h at 80 °C. The DNA hybridizations were performed with digoxigenin-labeled DNA probes (Boehringer Mannheim). The DNA was labeled with digoxigenin-11-UTP using the random-primed method (Boehringer Mannheim). Detection of digoxigenin-labeled DNA probe was accomplished by using "Lumi-Phos 530" and repeated exposures to the Kodak-XAR-5 films.

Polymerase Chain Reaction Amplifications, Cloning, and Nucleotide Sequencing—The PCR was performed by using the reagents from GeneAmp kit (Perkin-Elmer Corp.). 100 ng of the chromosomal DNA linearized with PstI and about 50 nmol of the primers corresponding to sense and antisense DNA strands were added immediately after and before the open reading frame for the Fe protein region were used in the reaction. PCR amplification was continued for 20 cycles under stringent conditions. The DNA fragment obtained by this method was cloned into the pCR™ vector (purchased from Invitrogen), and selection of recombinants was made as described in the instruction manual of the supplier. The resulting fragments corresponding to nifH were subcloned by ligating this region of DNA into the M13 cloning vector mp18 or mp19 (45).

1 The abbreviation used is: PCR, polymerase chain reaction.
followed by sequence determination using the method of Sanger et al. (46).

Site-directed Mutagenesis—The oligonucleotide-directed mutagenesis was carried out by using Sculptor™ In vitro Mutagenesis System Version 2 (Amersham Corp.) and is based on the phosphorothioate technique of Eckstein’s group (47). To do this, chemically synthesized oligonucleotide primers corresponding to the region of interest and containing the complementary sequence with the appropriate base change were hybridized to the single-stranded recombinant phage DNA and extended by using T₇ DNA polymerase and T₄ DNA ligase to generate mutant heteroduplex. During this synthesis, dCTP was replaced by dCTP·PS so that the newly synthesized strand was phosphorothioato DNA, which cannot be cleaved by the restriction enzyme NciI. The heteroduplex DNA was treated with NciI to generate nicks in the non-mutant strand. This nicked non-mutant strand was removed by treating the sample with exonuclease III, leaving a fragment of approximately 800 bases that acts as a primer for repolymerization. A double-stranded mutant homoduplex was generated using DNA polymerase I and T₄ DNA ligase. E. coli TG1 was transfected with this mutant recombinant phage DNA to prepare phage stocks for plagues for mutant analysis. The mutations were confirmed by dideoxy-nucleotide sequencing (46).

Construction of A. vinelandii nifH Mutant Strains—To construct various A. vinelandii strains that contain altered forms of nifH, a gene replacement technique previously described in detail was used (48, 49). A. vinelandii strains have a very efficient recombination system that allows homologous recombination between the newly delivered sequence and the host chromosome. This was done by transforming A. vinelandii with plasmids that cannot replicate in A. vinelandii cells and would be lost during cell division.

Growth Characteristics of A. vinelandii nifH Mutant Strains—To compare the growth characteristics of A. vinelandii nifH mutant strains to that of the wild type strain, overnight cultures were prepared by allowing them to grow in BN⁺ medium at 30°C for about 15 h. These fully grown cultures were diluted (1:250, overnight culture:fresh medium) into fresh BN⁺ medium and continued the growth at 30°C. The cell densities were recorded at various time intervals.

RESULTS AND DISCUSSION

Earlier, it was shown that the apodinitrogenase from different genetic backgrounds are biochemically distinct (35). For example, to activate apodinitrogenase from A. vinelandii DJ 54 with FeMo cofactor, we needed to add the Fe protein, isolated FeMo cofactor, MgATP, and an unknown additional component(s) that is present in cell-free extracts from a ΔnifD strain of A. vinelandii (36). However, the FeMo cofactor-deficient MoFe proteins from A. vinelandii strains with mutations in the nifB or nifEN genes can be activated in vitro by simple addition of isolated FeMo cofactor in N-methylformamide (35, 50). Recently, it was reported that the activation of apodinitrogenase from A. vinelandii UW97 with FeMo cofactor required very similar conditions to those that are used to activate apodinitrogenase from A. vinelandii DJ 54 (37).

The strain A. vinelandii DJ 54 has a well characterized deletion in the nifH gene (33); the strain A. vinelandii UW97 was originally isolated following nitrosoguanidine mutagenesis. While both of these strains accumulate the FeMo cofactor-deficient apodinitrogenase protein, the dinitrogenase reductase (the Fe protein) is accumulated only in the A. vinelandii UW97 cells. However, the apodinitrogenase of UW97 also needs a pre-treatment with wild-type Fe protein and MgATP for its activation by FeMo cofactor (37). Since an inactive Fe protein is synthesized in UW97, we argued that localizing the mutation will give us some clue about the features required for its participation in FeMo cofactor biosynthesis and insertion.

To check if the Nif⁻ phenotype of A. vinelandii UW97 was due to a mutation in the nifH, we replaced the DNA region spanning the nifH sequence of A. vinelandii UW97 chromosome with the nifH region of the A. vinelandii wild type. This was done by constructing a nifH-containing plasmid that cannot replicate in A. vinelandii for A. vinelandii transformation. To do this, we have taken a previously well characterized plasmid pDB6 (51) and cleaved it with the restriction endonuclease HindIII. The resulting products were subjected to self- ligation by using T₄ DNA ligase. The plasmids in which the HindIII DNA fragment corresponding to nifDKY was absent was identified by isolating DNA and analyzing it on agarose gels after restriction enzyme digestions. The resulting plasmid was designated pBG120. Then, A. vinelandii UW97 was transformed with the plasmid pBG120, which carries an intact wild-type nifH gene. Since pBG120 cannot replicate in A. vinelandii, it is lost during cell division and becomes instrumental in delivering the DNA region spanning the wild-type nifH sequence to the cell. A. vinelandii strains have a very efficient recombination system that allows homologous recombination between the newly delivered sequence and the host chromosome. If a mutation in the nifH sequence is responsible for the Nif⁻ phenotype, the transformants that have incorporated wild-type nifH sequences in their chromosome will become Nif⁺. As shown in Fig. 1, transformants of UW97 with pBG120 could grow on BN⁻ (nitrogen-free) plates. This experiment showed that the mutation causing the Nif⁻ phenotype of A. vinelandii UW97 is located in the nifH sequence and not in another gene that affects the Fe protein (e.g. nifM) (52).

To test whether this nifH mutation is due to a detectable deletion in that DNA corresponding to nifH, we have subjected the chromosomal DNA of A. vinelandii UW97 to Southern blot analysis and hybridization with DNA sequences corresponding to the wild-type nifH gene. The chromosomal DNA from A. vinelandii UW97 (mutant) and A. vinelandii UW (wild type) was isolated as described under “Experimental Procedures.” The DNA from each of these strains was subjected to restriction enzyme digestion with EcoR I, HindIII, or PstI, and Southern blots were prepared and hybridized with non-radioactive DNA probes spanning the coding sequence of nifH. This comparative analysis did not detect any visible deletions in the nifH gene of A. vinelandii UW97 chromosome (Fig. 2), and we infer that the Nif⁻ phenotype in A. vinelandii UW97 is not due to any detectable deletion in either the coding sequence for the Fe protein of A. vinelandii UW97 or its surrounding sequences.

If the Fe protein is synthesized in UW97, then the mutation responsible for making it inactive must be located in the open reading frame. To test this possibility, we decided to analyze the nucleotide sequence that encodes the A. vinelandii UW97 Fe protein. Since the nucleotide sequence of the nifH gene is known, we have made use of this information to design the oligonucleotide primers corresponding to the sequences upstream and downstream of the open reading frame to amplify
the nifH coding sequence from the chromosome of A. vinelandii UW97 by PCR amplification technique (53). The DNA fragment obtained by this method was cloned into the pCRTM II vector (purchased from Invitrogen). This 1.12-kilobase pair fragment was then cloned into M13 mp18 and subjected to nucleotide sequence analysis by dideoxy-nucleotide sequencing method (46). This analysis showed that the nucleotide sequence of the nifH gene of A. vinelandii UW97 differs from that of the wild type by a single base change that replaced the serine codon UCC at position 44 of the Fe protein by the codon UUC that encodes phenylalanine.

Since the Nif⁻ A. vinelandii strain UW97 was obtained by chemical mutagenesis, there was the possibility that there could be more than one mutation responsible for its Nif⁻ phenotype. Therefore, we decided to test whether the substitution of Ser-44 by phenylalanine alone is responsible for the Nif⁻ phenotype of A. vinelandii UW97. To accomplish this, we replaced the phenylalanine codon UUC of the A. vinelandii UW97 Fe protein with the serine codon UCC and tested its effect on the Nif phenotype of the Nif⁻ strains A. vinelandii UW97 and DJ54. This was done by subjecting the A. vinelandii UW97 Fe protein coding sequence cloned in the M13 mp18 to site-directed mutagenesis as described under “Experimental Procedures.” The Nif⁻ A. vinelandii strains UW97 and DJ54 were transformed with this in vitro mutagenesis construct. The homologous recombination between the nifH gene on the chromosome of these strains and the mutated A. vinelandii UW97 nifH gene (carrying the serine codon instead of the phenylalanine codon at the 44th position) on the M13 construct resulted in generating Nif⁺ transformants from both strains. This experiment confirmed that the Nif⁻ phenotype of A. vinelandii UW97 is due to the change of Ser-44 to phenylalanine. This serine is located in the second conserved domain spanning residues 37–45 (Fig. 3).

Crystallographic analysis indicates that in the conserved amino acid cluster encompassing the residues 37–45, Lys-41 is

Fig. 2. Southern blot analysis of the chromosomes of A. vinelandii strains UW97 and UW digested with HindIII (lanes 1 and 4), PstI (lanes 2 and 5), and EcoRI (lanes 3 and 6). The probe used was the DNA sequence corresponding to nifH gene and flanking regions. Autoradiograph shows that the chromosomes from both strains are indistinguishable with respect to nifH gene and flanking regions. kbp, kilobase pairs.

Fig. 3. Comparison of the amino acid residues of the second conserved domain in the Fe proteins that are deduced from 40 different nifH DNA sequences. The numbering corresponds to A. vinelandii NifHl. Upper case letters in the consensus sequence represent invariant residues in all 40 sequences, whereas lower case letters indicate the presence of one or more variants in that position.
involved in contacting the ribose group; whereas, the residues Asp-39 and Asp-43 could play a role in catalysis of ATP hydrolysis (9). Crystallographic data also suggest that the residues ranging from 39 to 80 could be involved in transmitting the MgATP-induced conformational change to the nucleotide binding site (9). Thus, the amino acids in the second conserved domain are implicated in taking part in the MgATP binding and the MgATP-induced conformational change. Studies on other Fe protein mutations at Lys-15 and Asp-125 point out that these mutations affected the MgATP-induced conformational change of the Fe protein. However, they did not interfere with its ability to take part in FeMo cofactor biosynthesis and insertion (22, 25). Our extensive analysis of another Nif- mutant, A. vinelandii UW91, has demonstrated that the Fe protein in this mutant has a substitution mutation in which Ala-157 has been replaced by a serine residue (23). This mutation does not seem to affect the ability of the Fe protein to participate in FeMo cofactor synthesis or insertion since the MoFe protein in this mutant is normal. The mutation also does not affect the ability of the Fe protein to bind MgATP. However, the Nif- phenotype is caused because the mutation prevents 1) the MgATP-induced conformational change that occurs in the wild-type Fe protein, 2) MgATP hydrolysis, and 3) productive electron transfer to the MoFe protein. These observations indicate that the ability of the Fe protein to take part in FeMo cofactor biosynthesis/insertion is not directly related to its involvement in MgATP binding and hydrolysis. Interestingly, our data on the A. vinelandii UW97 show that the mutation responsible for making the Fe protein non-functional in FeMo cofactor biosynthesis or insertion is located in a second conserved domain. Thus, it seems that the structural organization of the second conserved domain plays a crucial role in at least two seemingly independent functions of the Fe protein: the ability to take part in FeMo cofactor biosynthesis and the ability to induce a conformational change in response to MgATP binding. However, although serine 44 is in a highly conserved region of the Fe protein, the amino acid Ser-44 is not conserved. The structural model shows that the side chain of Ser-44 is pointing away from the proposed MgATP binding site and toward the core of the subunit. Moreover, within the 4-angstrom sphere of oxygen atom on the side chain of serine 44, there are more than five residues that can be found. Most importantly, the oxygen atom on the side chain of serine 44 is also within hydrogen bonding distance to the oxygen atom on the side chain of aspartic acid 125. An elegant work by Howard and colleagues (22) demonstrated the critical role played by the aspartic acid at 125 in bringing about a conformational change upon MgATP binding. It is possible that there is no space for a bulky phenylalanine side chain to be at position 44, hence the effect we see is probably due to a general conformational change of the mutant protein. We argued that a specific change at position 44 that could shed some light would be replacing serine with alanine at this position.

As described under “Experimental Procedures,” we have constructed an A. vinelandii strain in which the UCC codon specifying serine was replaced with GCC codon that was specifying alanine. The A. vinelandii strains were compared in their growth characteristics as described under “Experimental Procedures.” This analysis showed that the strain with alanine at the position 44 showed the characteristics similar to that of the wild type. In summary, the amino acid alanine could replace the serine at position 44 without affecting the functionality of the protein. By contrast, replacing this serine with phenylala-
nine affected the functionality of the protein and made the A. vinelandii UW97 strain Nif-. Thus, the phenotype of the corresponding mutant is more likely to result from a general structural disturbance rather than a specific amino acid interaction.

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