Evidence for the Direct Interaction of the nifW Gene Product with the MoFe Protein*

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The Azotobacter vinelandii nifW gene, under control of the nifH promoter, was subcloned into the broad host range multicopy plasmid pKT230 for overexpression in both wild-type and Δnif strains of A. vinelandii. Unlike the parent Δnif strain, which grows slowly relative to wild-type under N2-fixing conditions, both overproduction strains grow at the same rate, showing that the overexpressed nifW product is functional in vivo. The 40-fold overexpressed protein was purified, and sequence analysis confirmed its identity. During purification it was observed that NifW in crude extracts ran above the predicted molecular weight on denaturing gels and that as the purification proceeded lower molecular weight forms appeared. Mass spectrometry and studies with protease inhibitors revealed that this abnormal behavior was due to proteolysis. Native molecular weight determinations demonstrate that NifW is a homodimer, most likely a trimer. Native gel electrophoresis analysis shows that the behavior of wild-type and overexpressed NifW are identical and that when extracts are prepared anaerobically only the homomultimeric forms of NifW are observed. When extracts are exposed to oxygen, however, NifW becomes part of a very high molecular weight complex. Immunoprecipitation with NifW antibodies demonstrate that under those conditions NifW specifically associates with the MoFe protein. These data are consistent with a model whereby NifW is not involved in the initial assembly of an active MoFe protein but rather is part of a system design to protect the MoFe protein from O2 damage.

Molybdenum nitrogenase is composed of two separatively purified proteins. The iron protein (Fe protein) is a dimer of two identical subunits that has a single [4Fe-4S]12+ cluster and two binding sites for MgATP. The molybdenum-iron protein (MoFe protein) is an α2β2 tetramer that contains two [8Fe-8S] clusters (P-clusters) and two iron-molybdenum cofactor (FeMo cofactor) centers that each have the stoichiometry Mo:7Fe:9S: homocitrate (1–6). The in vivo assembly of a functional nitrogenase requires the participation of a large number of nitrogen fixation (nif)-specific gene products (7). In most cases, the specific functions of these additional proteins are not known. In general, two experimental approaches have been used to understand the role that an individual gene product plays in biological nitrogen fixation. In the first approach strains are constructed that have mutations or deletions in the gene of interest, and then the phenotype of the mutants with respect to growth and nitrogenase activity is studied. In a few cases involving mutations in the nifB (8, 9), nifH (10), nifW (11), and nifV (12) genes these studies have been extended, and the defective MoFe proteins have been purified and characterized. A second approach, which sometimes requires overexpression of the gene product of interest, has been to purify and characterize the gene product itself. This approach has recently been most successful for the characterization of the nifS (13) and nifU (14) gene products and has also been used to study the electron transport proteins encoded by the nifB (15) and nifR (16) genes.

NifW is one of many nif gene products whose specific function has yet to be determined. The nifW gene has so far been identified in eight diazotrophs (11). Early studies of strains that contained mutations or deletions in the nifW gene established that the gene product appeared to be somehow involved in the assembly of the MoFe protein (17–22) and suggested that it might have something to do with homocitrate, an essential component of the FeMo cofactor (11, 18). More recently, nitrogenase was purified from a ΔnifW strain of Azotobacter vinelandii (11). That study showed that the Fe protein was normal but that the MoFe protein was present in cell-free extracts in at least two forms, one of which was not properly folded while the other had altered substrate reactivity (11). Here we report the overexpression of the nifW gene product in its native background in A. vinelandii and the demonstration that its function involves direct binding to the MoFe protein.

EXPERIMENTAL PROCEDURES

Materials—The construction of the A. vinelandii Δnif strain, DJ 224 is described elsewhere (22). The restriction enzymes Bsu36I, BglII, and BamHI were from New England Biolabs. HindIII, T4 DNA ligase, and calf intestinal phosphatase were from Boehringer Mannheim. The GeneClean II kit was from Bio 101 Inc. Streptomycin, CsCl, and MOPS1 were from Sigma. The Wizard Miniprep kit was from Promega. The multiprimere DNA labeling kit, [γ-32P]ATP, and [α-32P]CTP were from Amersham Corp. All oligonucleotides were purchased from Integrated DNA Technologies, Inc. The Electro Cell Manipulator 600 for electroporation was from BTX Inc. Ammonium sulfate was from Fisher, DEAE-cellulose 52 was from Whatman, AcA 44 was from Bio Sep, Q-Sepharose, Superdex 75 HR 10/30, Superose 12 HR 10/30, and Protein A-Sepharose 6MB were from Pharmacia Biotech Inc. The silver staining kit, acrylamide, bisacrylamide, TEMED, and SDS were from Bio-Rad. Enhanced Chemiluminescence Western reagents were from Amersham. Horseradish peroxidase conjugated to goat anti-rabbit IgG was from Boehringer Mannheim.

Overexpression of the nifW Gene Product—Hyperproduction of the A. vinelandii nifW gene product in Escherichia coli was accomplished by

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1 The abbreviations used are: MOPS, 3(N-morpholino)propanesulfonic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonl fluoride; DEAE, diethylaminoethyl; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TEMED, N,N′,N′-tetramethylethylenediamine.
constructing a nifW gene cartridge in vitro using the polymerase chain reaction method and cloning this cartridge into the pT7-7 plasmid such that nifW gene expression is controlled by T7. The resulting hybrid plasmid was designated pDB611 (Fig. 1), and the recipient strain was E. coli (BL21(DE3)). When hyperproduced in E. coli NifW accumulated to approximately 35% of the total soluble protein in crude extracts and was readily purified by passing the supernatant from a 40% ammonium sulfate-saturated crude extract over a DEAE-Sepharose column. This purified NifW was used to produce antibodies that were raised commercially by Cocalico Biologicals, Inc. (Reamstown, PA). For overexpression the plasmid pDB611 was digested with BglII and BamHI and an 800-base pair A. vinelandii DNA fragment containing the nifW gene behind the nifH promoter was purified and concentrated. The plasmid pKT230 was digested with BamHI using a buffer supplied by New England Biolabs and was treated with calf intestinal phosphatase to prevent self-ligation. The plasmid pKT230 was designated pDB611 (Fig. 1), and the recipient strain was E. coli (BL21(DE3)). When hyperproduced in E. coli NifW accumulated to approximately 35% of the total soluble protein in crude extracts and was readily purified by passing the supernatant from a 40% ammonium sulfate-saturated crude extract over a DEAE-Sepharose column. This purified NifW was used to produce antibodies that were raised commercially by Cocalico Biologicals, Inc. (Reamstown, PA). For overexpression the plasmid pDB611 was digested with BglII and BamHI and an 800-base pair A. vinelandii DNA fragment containing the nifW gene behind the nifH promoter was purified and concentrated. The plasmid pKT230 was digested with BamHI using a buffer supplied by New England Biolabs and was treated with calf intestinal phosphatase to prevent self-ligation. The 800-base pair nifW fragment and the BamHI-digested/calf intestinal phosphatase-treated pKT230 were ligated and transferred into C600 E. coli cells by electroporation, and the transformants were screened for streptomycin (20 \(\mu\)g/ml) resistance and by hybridization (23) to nifW. Ninety percent of the streptomycin-resistant colonies were positive in the colony hybridization screening. Plasmids were recovered by minipreparation of positive colonies, and the presence and orientation of the 800-base pair insert was confirmed by digestion with a number of restriction enzymes. pKTW611 (Fig. 1) was selected for introduction into A. vinelandii using a transformation procedure for that organism (24).

**Fig. 1. Strategy for subcloning the nifW gene into pKT230.**

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**Purification and Characterization of NifW**—The protein was purified according to Scheme 1. Unless otherwise indicated all steps were carried out using 0.025 M Tris-HCl pH 7.4, all centrifugation used a GS-3 rotor, and all fractions were monitored by dot blot Western analysis using the convertible filtration manifold system from Life Technologies, Inc. The A4A column was run in two or three batches of 10–15 ml each, and the final Superose 12 FPLC column had to be run on a small scale with 200 µl for each sample. Following all the runs the NifW fractions were combined and concentrated using a Centricon 10 centrifugation system. The NifW protein samples were stored at ~20°C between manipulations and after purification was complete. For studies involving protease inhibitors frozen JG611 cells were resuspended in 2 volumes of sonication buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 50 \(\mu\)g/ml RNase), the resuspended cells were aliquoted into four separate tubes, and protease inhibitors were added as recommended by Calbiochem: AEBSF (0.2 mM), leupeptin (10 \(\mu\)M), pepstatin (1 \(\mu\)M), and EDTA (10 mM). Cells were then broken separately by sonication and centrifuged at 9000 rpm in an SS-34 rotor for 20 min. The supernatant was transferred separately to fresh tubes, and more protease inhibitors were added to make final individual concentrations of 0.4 mM AEBSF, 20 \(\mu\)M leupeptin, 2 \(\mu\)M pepstatin, and 20 mM EDTA. Denaturing gel electrophoresis was carried out as described elsewhere (26). Immunoblots used the enhanced chemiluminescence method of Amersham according to the manufacturer’s instructions. Anaerobic native gel electrophoresis was performed by a modification of published procedures (27, 28) whereby all buffers were degassed and the gel was prerun with 2 mM Na2S2O4 under argon for 15 min before loading the samples. The 12% acrylamide gels were run for 2.5 h at 100 V in a Vacuum Atmosphere’s anaerobic box. Protein sequencing was carried out at the UCI Biological Sciences core facility. Electrospray mass spectrometry was performed at the Beckman Research Institute of The City of Hope in Los Angeles. Samples were first separated by C18 HPLC column chromatography using a 2–90% gradient from buffer A (0.1% trifluoroacetic acid) to buffer B (0.07% trifluoroacetic acid, 90% acetonitrile). Individual peaks were then directly sent to the electrospray ionization chamber for mass spectrometry.

**Immunoprecipitation**—JG611 and DJ224 crude extracts were first incubated at 4°C separately with prebleeding serum (normal serum without NiF antibody) and Protein A-Sepharose 6MB (from Pharmacia) for 2 h to remove all the nonspecific interactions (preclearing step). After a 2-h incubation, the samples were spun, and the supernatant was transferred to a clean tube. These preclarified crude extracts were then incubated at 4°C with the NiF antibody for 1.5 h and with a 1:1 slurry of Protein A-Sepharose, TBS-T buffer 6MB for another 1.5 h. After a total 3-h incubation, the samples were centrifuged, and the resulting pellets were washed twice with TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100), once with TBS buffer (the same as TBS-T buffer except no Triton X-100), and once with 50 mM Tris-HCl, pH 6.8. The volume ratio of the crude extracts to NiF antibody to Protein A-Sepharose to wash buffer was 50:3:100:1000. The proteins...
clearly demonstrate that the
NifW gene product specifically associated with any other
but also because we wished to examine whether or not the
because proteins are most stable in their native background
A. vinelandii
was constructed and transformed into two different strains of
gene in the same orientation as the Kan promoter on pKT230
mid designated pKTW611, which had the
under "Experimental Procedures" and shown in Fig. 1, a plas-
that precipitated with Protein A-Sepharose 6MB were then eluted by
boiling for 5 min with SDS sample loading buffer, and subsequently,
that the protein in its native background in
A. vinelandii
cracked.

**RESULTS AND DISCUSSION**

Overexpression of the nifW Gene Product in Its Native Background—The nifW gene product is normally present at low
levels in wild-type A. vinelandii cells. We chose to overexpress
the protein in its native background in A. vinelandii not only
but also because we wished to examine whether or not the
NifW gene product specifically associated with any other nif
gene product(s). As shown in Fig. 1 our strategy was to intro-
duce multiple copies of the nifw gene into A. vinelandii using
the broad host range multicopy plasmid pKT230 that had pre-
viously been used to overexpress A. vinelandii ferredoxin I (25).
The starting material was a plasmid designated pDB611,
which is a pUC-derivative plasmid that has the nifw gene
subcloned behind the nifH promoter (Fig. 1). As described under "Experimental Procedures" and shown in Fig. 1, a plas-
mid designated pKTW611, which had the nifH promoter/nifw
gene in the same orientation as the Kan promoter on pKT230
was constructed and transformed into two different strains of
A. vinelandii, the wild-type strain and a ∆nifW strain design-
nated DJ224 (22). The strain derived from the wild-type host
is designated JG611, and the one derived from the ∆nifW host is
designated JG612.

Fig. 2 shows a SDS-PAGE gel electrophoresis separation of
cracked whole cells of three strains of A. vinelandii after blot-
ting and reacting with anti-NifW antibodies. The results
demonstrate that the nifw gene product has been suc-
cessfully overexpressed in both JG611 and JG612. Fig. 3 shows
that the nifw gene product is not expressed when the cells are
grown on ammonia and is therefore being regulated by the A.
vineandii nifH promoter and not by the Kan promoter on the
pKT230 vector. It should be noted that the growth rates of the
∆nifW host strain are much slower than that of the wild-type
host strain (22), whereas the growth rates of JG611 and JG612
under N₂-fixing conditions are very similar. Therefore, as ex-
pected, the overexpressed nifw gene product appears to be
functional in vivo.

Purification of the nifW Gene Product—Fig. 4 shows the
predicted sequence of the NifW protein with the highly
conserved regions underlined (11). Computer analysis of this
sequence indicates that NifW is expected to have a molecular
weight of 13,500, to be 70% helix oriented, and to be very acidic
with a pI of 4.32. The protein contains no cysteine residues,
and searches failed to reveal homology to any known metal or
organic cofactor binding site indicating that NifW is likely to be
an air-stable, colorless protein. With these features in mind the
protein was purified at 4 °C according to Scheme I. Fig. 5 shows
a Coomassie-stained SDS-polyacrylamide gel of the fractions
obtained during the purification of the NifW protein according to
Scheme I. b, Western analysis of the same samples. Lane 1, rainbow molecular
weight markers; lane 2, crude extracts (30 μg); lane 3, ammonium
sulfate cut (25 μg); lane 4, DEAE-cellulose (25 μg); lane 5, AcA44 (8 μg
for a, and 0.9 μg for b); lane 6, Q-Sepharose (5 μg for a, 0.9 μg for b); lane
7, Superose 12 FPLC (3 μg for a, 0.5 μg for b); lane 8, rainbow molecular
weight markers.
the deduced amino acid sequence of the NifW protein shown in Fig. 4 except that the first methionine was missing. This result confirmed that the desired protein had been purified and showed that there were no other contaminating proteins present.

Identification of the Nature of the NifW Modification—As shown in Fig. 5, the purification NifW was monitored using SDS-PAGE and Western analysis. Surprisingly, examination of cell-free extracts revealed that the NifW band ran at 17 kDa (Fig. 5) while the predicted molecular mass of the gene product was only 13.5 kDa (Fig. 4). Thus, either the protein was running abnormally on SDS-PAGE or it was covalently modified to a higher molecular weight form. As shown in Fig. 5, this situation was further complicated by the observation that a lower band that ran at 14.3 kDa started to appear as the purification proceeded. In fact, the upper band that was present at 17 kDa in the cell-free extracts disappeared completely after Q-Sepharose, and an intermediate band appeared that ran between the upper band and the lower band. As shown in Fig. 5 all bands reacted with the NifW antibody. Mass spectrometry was employed to determine if these gel patterns were due to a removal of a covalent modification or to proteolytic degradation.

A NifW sample that showed the same two-banded Western pattern as that shown in Fig. 5, lane 7, was first separated into two NifW fractions using a C18 HPLC column. Then each fraction was subjected to electrospray mass spectrometry. The masses of these two fractions were then calculated by deconvoluting the mass spectrometry profile (Fig. 6). The largest peaks present in the profile corresponded to masses of 8509 and 7731 daltons for the two HPLC fractions. These masses were well below the predicted molecular weight of the intact NifW, suggesting that the multiple bands observed on SDS-PAGE are due to proteolytic cleavage. As shown in Fig. 7 a variety of protease inhibitors were then examined to try to eliminate proteolysis during purification with EDTA showing the greatest degree of protection. These results lend further support to the conclusion that the abnormal SDS-PAGE behavior shown in Fig. 5 is due to proteolytic degradation and not to covalent modification. These data also strongly suggest that intact NifW runs abnormally at a higher molecular weight on SDS-PAGE, a phenomenon commonly observed for small acidic proteins (29).

Subunit Organization of NifW—Repeated attempts to purify NifW in the presence of protease inhibitors were unsuccessful in yielding homogenous NifW in the uncleaved state. Therefore experiments designed to determine the subunit organization were carried out both with the homogeneous cleaved protein shown in Fig. 5 and with the uncleaved protein at earlier stages of purification. When crude NifW was applied to a Superose 12 column it eluted in a volume similar to ovalbumin, which has a molecular mass of 42,750 daltons, indicating that uncleaved NifW was definitely a multimer, most probably a trimer. As shown in Fig. 8 the native molecular weight of the homogenous cleaved NifW was determined using Superdex 75 and Superose 12 FPLC columns. The estimated native molecular weights...
The presence of 0.4 mM AEBSF (a multimeric enzyme because in some organisms the likely a trimer. Together, these data suggest that NifW forms a multimer, most likely a trimer.

Determined for the homogenous cleaved protein were 23,600 and 27,500 for the two columns, respectively. Within experimental error this native molecular weight data for the homogenous cleaved protein also supports a trimeric model. Taken mentally error this native molecular weight data for the homogenous cleaved protein were 23,600 and 27,500 for the two columns, respectively. Within experimental error this native molecular weight data for the homogenous cleaved protein also supports a trimeric model.

The effect of various protease inhibitors on the degradation of the NifW protein. Shown is a SDS-PAGE separation after blotting and reacting with NifW-specific antibodies. Each lane contains 30 μg of JG611 crude extracts incubated at room temperature for 8 h in the presence of 0.4 mM AEBSF (A), 20 μM leupeptin (B), 2 μM pepstatin (C), 20 mM EDTA (D).

Fig. 7. The effect of various protease inhibitors on the degradation of the NifW protein. Shown is a SDS-PAGE separation after blotting and reacting with NifW-specific antibodies. Each lane contains 30 μg of JG611 crude extracts incubated at room temperature for 8 h in the presence of 0.4 mM AEBSF (A), 20 μM leupeptin (B), 2 μM pepstatin (C), 20 mM EDTA (D).

Fig. 8. Native molecular weight determination of the homogenous cleaved protein. a, molecular weight determination using a Superdex 75 FPLC gel filtration column. b, molecular weight determination using a Superose 12 FPLC gel filtration column. BSA, bovine serum albumin; OVA, ovalbumin; TI, Trypsin inhibitor; RNase A, ribonuclease A; Cyt C, cytochrome c.

Fig. 8. Native molecular weight determination of the homogenous cleaved protein. a, molecular weight determination using a Superdex 75 FPLC gel filtration column. b, molecular weight determination using a Superose 12 FPLC gel filtration column. BSA, bovine serum albumin; OVA, ovalbumin; TI, Trypsin inhibitor; RNase A, ribonuclease A; Cyt C, cytochrome c.

Western blots of native gel electrophoresis separations of cell-free extracts from wild-type (AvOP) and NifW overproduction strain JG611 after blotting and reacting with NifW-specific antibody. a, anaerobic native gel separation. Lane 1, 20 μg of anaerobically prepared JG611 extracts; lane 2, 40 μg of anaerobically prepared AvOP extracts. These samples did not contain the protease inhibitor EDTA. b, aerobic native gel separation. Lane 1, 40 μg of JG611 that was prepared aerobically in the presence of 10 mM EDTA; lane 2, 80 μg of AvOP that was prepared aerobically in the presence of 10 mM EDTA; lane 3, 40 μg of the JG611 sample shown in a after exposure to air; lane 4, 80 μg of the AvOP sample shown in a after exposure to air. These samples did not contain EDTA. All gels were 12% acrylamide. Species A, B, and C are discussed under “Results.”

Fig. 9. Western blots of native gel electrophoresis separations of cell-free extracts from wild-type (AvOP) and NifW overproduction strain JG611 after blotting and reacting with NifW-specific antibody. a, anaerobic native gel separation. Lane 1, 20 μg of anaerobically prepared JG611 extracts; lane 2, 40 μg of anaerobically prepared AvOP extracts. These samples did not contain the protease inhibitor EDTA. b, aerobic native gel separation. Lane 1, 40 μg of JG611 that was prepared aerobically in the presence of 10 mM EDTA; lane 2, 80 μg of AvOP that was prepared aerobically in the presence of 10 mM EDTA; lane 3, 40 μg of the JG611 sample shown in a after exposure to air; lane 4, 80 μg of the AvOP sample shown in a after exposure to air. These samples did not contain EDTA. All gels were 12% acrylamide. Species A, B, and C are discussed under “Results.”

nifZ genes are adjacent to each other and because the phenotypes of NifW− and NifZ− strains are similar (17, 22). On the other hand, the two genes are not adjacent in all organisms, and NifZ− strains of both K. pneumoniae and A. vinelandii have much lower MoFe protein activities than do NifW− strains (17, 22). More recently, Masepohl et al. (18) suggested that NifW might be involved in processing homocitrate and might therefore work together with the nifZ gene product that is known to encode homocitrate synthase (7). This suggestion was based on the observation that mutation of NifW alone gave no phenotype in Rhodobacter capsulatus, while mutation of nifW and nifV together yielded an organism that grew more slowly than a strain with a mutation in nifV alone (18).

In this study the possibilities that nifW might normally form a heteromultimer with another nif protein or that it might interact directly with a separate nif encoded protein were examined. At no time during our attempts to purify NifW did we observe any proteins that copurified with NifW. In addition, the homogenous NifW protein shown in Fig. 5 must be a homomultimer because no other contaminating proteins were detected by gel electrophoresis or by the highly sensitive method of protein sequencing. Therefore, it appears to be extremely unlikely that NifW normally forms a heteromultimer with some other nif gene product.

The first indication that NifW might associate more loosely with another protein came from examining the native gel electrophoresis results shown in Fig. 9. In these experiments proteins present in cell-free extracts from wild-type A. vinelandii and the NifW overproduction strain JG611 were separated by native gel electrophoresis and probed with NifW antibodies. This experiment was performed both anaerobically and aerobically and in the presence and absence of protease inhibitors. As shown in Fig. 9 three forms of NifW (A, B, and C) were present in both wild type and JG611, indicating that the behavior of NifW in JG611 is natural rather than an artifact of overexpression. The forms B and C are likely to be the intact and cleaved homomultimeric forms of NifW, respectively, because C appeared in increasing quantities when samples were stored over a period of time, it ran at the same position as the purified cleaved NifW homomultimer (data not shown), and its presence could be eliminated by adding EDTA (Fig. 9).

An additional form of NifW (form A) migrates too slowly to be NifW alone because it barely enters the separating gel on a 12%
acrylamide native gel. This form is only observed if the cells are ruptured aerobically or if the anaerobic sample is exposed to oxygen (Fig. 9). This observation is interesting because there is a large body of literature showing that under the same conditions nitrogenase forms a very high molecular weight O2-stable complex (30–35). To further investigate the possible association of NifW with other proteins to form a high molecular weight complex we used immunoprecipitation. This experiment was carried out aerobically using Protein A-Sepharose, which was expected to precipitate the NifW-MoFe antibody complex. As has been demonstrated in other systems, any protein present in cell-free extracts that interacts with NifW should also coprecipitate with the NifW-MoFe antibody complex under these conditions (36–37). For these experiments A. vinelandii ΔnifW strain DJ 224 cell-free extracts and mixtures that contained no cell-free extracts were both used as negative controls. NifW overexpression strain J G 611 cell-free extracts were used as the experimental samples because the level of NifW in the wild-type cell is very small and because the same high molecular weight complex was observed for both J G 611 and the wild type (Fig. 9).

Fig. 10 shows an SDS-12% polyacrylamide gel electrophoresis separation of the control and experimental samples after precipitation with the NifW antibody-Protein A-Sepharose complex. As shown in Fig. 10A, there are only two obvious bands that are exclusively present in the J G 611 sample that are not present in the negative controls. Of these two bands only the lower band reacts with the NifW antibody (Fig. 10B), demonstrating that the upper band was precipitated through NifW and not by the NifW antibody alone. As shown in Fig. 11, to further resolve the region of the gel that contains the upper band 8% polyacrylamide gels were run. Under those conditions the band separates into a clear doublet. As shown in Fig. 11A that doublet ran in the identical position to the MoFe protein α and β subunits, and as shown in Fig. 11B the protein cross-reacted with antibodies raised against the purified A. vinelandii MoFe protein. Taken together these data show that NifW binds specifically to the MoFe protein to form a high molecular weight complex. It should be noted that two other proteins that are known to associate with the MoFe protein to form an O2-stable high molecular weight complex under these conditions, the Fe protein subunit (M, ~30,000), and Fe-SII subunit (M, ~13,000), did not coprecipitate with NifW (Fig. 9). This result does not provide evidence against a multiprotein complex but rather suggests that NifW’s role involves specific association with the MoFe protein.

Working Hypothesis for the Role of NifW—Prior to this study NifW was believed to play a role in the initial assembly of an active nitrogenase (17, 19–22). A more specific function in homocitrate synthesis or processing had also been suggested (11, 18). The data presented here show that NifW specifically binds directly to the MoFe protein. This result is difficult to rationalize with a role for NifW in homocitrate processing because homocitrate is believed to be added at an early step in FeMo cofactor biosynthesis (2, 38) and because intact FeMo cofactor that contains homocitrate is assembled in the complete absence of the MoFe protein polypeptides (2, 7, 39). The data presented here further show that NifW is initially present as a homomultimer that associates with the MoFe protein to form a high molecular weight complex only when the cell-free extracts are exposed to oxygen (Figs. 8 and 10). Since this occurs in wild-type cell-free extracts (Fig. 8) the MoFe protein should be
already fully assembled at the point at which it associates with NifW.

The simplest explanation for these new data is that in A. vinelandii NifW is not involved in the initial assembly of an active MoFe protein. Rather, it is more likely to be part of an O2 protection system where its role would be to specifically protect the MoFe protein. This working hypothesis can be used to rationalize information in the literature concerning the phenotype of NifW− strains. For example, many NifW− strains that have been reported to date grow fairly well under N2-fixing conditions, indicating that they contain significant quantities of fully assembled MoFe protein (17–22). Also, NifW− strains from different organisms have very different properties as the native complex (34). The data presented here suggest that NifW may be an additional component of this complex or that it may specifically associate with the MoFe protein during the assembly of the final complex.

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