Isolation and Characterization of a Second Nitrogenase Fe-Protein from Azotobacter vinelandii*

Brian J. Hales, Dieter J. Langosch, and Ellen E. Case
From the Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803

Wild-type Azotobacter vinelandii strain UW was transformed with plasmid pDB12 to produce a species (LS10) unable to synthesize the structural proteins of component 1 and component 2 of native nitrogenase. A spontaneous mutant of this strain was isolated (LS15) which can grow by nitrogen fixation in the presence or absence of either Mo or W. It is proposed that LS15 fixes nitrogen solely by an alternative nitrogen-fixing system which previously has been hypothesized to exist in A. vinelandii. Under nitrogen-fixing conditions, LS15 synthesizes a protein similar to component 2 (Av2) of native nitrogenase in that it can complement native component 1 (Av1) for enzymatic activity. Isolation and characterization of this second component 2 shows it to be a 4Fe-4S protein of molecular mass about 62 kDa and is antigenically similar to Av2. This protein is also similar to Av2 in that in the reduced state it possesses a rhombic ESR spectrum in the g = 2 region, which changes to an axial spectrum upon addition of MgATP. It is suggested that this second Fe-protein is associated with the alternative nitrogen-fixing system in A. vinelandii.

Nitrogen fixation, the conversion of dinitrogen to ammonia, is an enzymatic process occurring in a wide range of species of bacteria. Despite the diversity of these bacteria, however, the nitrogen-fixing enzyme, nitrogenase, is highly conserved, always consists of two proteins (1). One of the proteins is an Fe-protein, called component 2, has a molecular weight of around 60,000, is composed of two identical subunits, and most likely contains a single 4Fe-4S center. The second protein, the MoFe-protein or component 1, has a molecular weight of about 240,000 in an αββ subunit pattern and contains 28-33 Fe and 2 Mo atoms in clusters of unknown structure. For enzymatic activity, electrons are funneled through the Fe-protein to the MoFe-protein where substrate reduction occurs. During this electron transfer, MgATP binds to the Fe-proteins and is hydrolyzed. Although not shown directly, it generally is felt that Mo exists at the catalytic site of this enzyme serving for both binding and reduction of substrate. Shah and Brill (2) first demonstrated that Mo could be extracted from the MoFe-protein as a Mo-Fe-S cluster called the FeMo-cofactor or FeMo-co. This cofactor has the ability of being able to reconstitute activity in apo (i.e. cofactor absent) component 1, further lending support to the notion of Mo being at the active site.

In 1980, Bishop et al. (3) showed that certain mutants of Azotobacter vinelandii unable to fix nitrogen (i.e. Nif') on medium containing 10 μM Mo were able to grow by nitrogen fixation (Nif' pseudorevertants) when Mo was absent. From these results they hypothesized the existence of a secondary (or alternative) nitrogen-fixing enzyme which is expressed under conditions of low Mo concentrations. ESR spectra of nitrogen-fixing whole cells of these mutants (4) failed to show the presence of component 1. Similarly, the two subunit polypeptides of the MoFe-protein could not be detected by SDS-PAGE of extracts from these cells. On the other hand, a polypeptide that migrated identical to native Fe-protein was observed as were the four new ammonia-repressible polypeptides.

Page and Collinson (5), also using Nif' mutants of A. vinelandii, found three polypeptides of molecular weights 57,000, 50,000, and 30,000 which were both ammonia and Mo repressible. While the identity of the first two polypeptides is unknown, the third could possibly be the same polypeptide observed by Bishop et al. (3) which migrates like native Fe-protein. Premakumar et al. (6) now state that this polypeptide does correspond to a second Fe-protein which is similar in electrophoretic mobility but different in isoelectric focusing from the native Fe-protein.

Although these results strongly suggest the presence of a second nitrogen-fixing enzyme, proving its existence has not been easy. In fact, there is much evidence in the literature which tends to disprove its existence. For example, Eady and Robson (7) demonstrated that the growth of A. vinelandii on limiting Mo is linear and stimulated by the addition of Mo, implying that Mo is needed for growth as would be expected if the Mo-containing enzyme were the only nitrogen-fixing enzyme present. Furthermore, Joerger et al. (8) and Terzaghi et al. (9) were unable to detect growth of either wild-type or Nif' mutants of A. vinelandii under Mo-deficient, N-free conditions.

Obviously, in order to prove the existence of a second nitrogen-fixing enzyme one must be able to demonstrate that growth and nitrogen fixation under Mo-deficient conditions is not due to small, albeit undetectable, amounts of native nitrogenase. This fact is also true when using Nif' mutants where the source and extent of the mutation is often unknown. To probe for the second enzyme, we used a deletion strain (LS15) void of the structural genes (nifH for the Fe-protein and nifDK for the MoFe-protein) of the native nitrogenase proteins. This paper describes the isolation and characterization of a protein from LS15 which is similar in both structure and function to native component 2 of A. vinelandii (Av2)
and is most probably associated with the alternative nitrogen-fixing enzyme in this species.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Wild-type and rifampicin-resistant strains of *A. vinelandii* (designated UW and UWW, respectively) were originally from the UW collection of W. Brill (University of Wisconsin). Plasmid pDB12 was a generous gift of Dr. Dennis Dean (Virginia Polytechnic Institute) and was constructed to contain a 3.6-kilobase Km\(^\text{R}\) deletion in a 12-kilobase XbaI nifHDK fragment of the Av genome (10).

Genomic DNA from strain UW\(^\text{W}\) was extracted and purified according to the method of Saito and Miura (11). Plasmid pDB12 was isolated from *Escherichia coli* as described by Bishop et al. (10).

UGW and DB2 DNA were cotransformed into *A. vinelandii* strain UW using the procedure of Page and von Tigerstrom (12). Transfornants were first isolated by their ability to grow in the presence of 10 mg/liter rifampicin. Since the deletion in pDB12 is in the genes encoding for the structural proteins of native nitrogenase (10), recombination of this plasmid with UW DNA renders the transformant Nif\(^-\) for native nitrogenase. The second selection used on these strains, therefore, was for their inability to grow by nitrogen fixation in the presence of 10 \(\mu\)M Mo. One of the strains isolated (LS10) was used in this study.

**Protein Isolation**—The enzyme was isolated and purified using a modification of the procedure of Burgess et al. (13). Cells (600 g) were harvested at mid-log phase (\(A_600 = 1.5\)), resuspended in 25 mM Tris, pH 7.4, containing 4 M glycerol, and lysed by osmotic rupture as described by Shak et al. (14). From this point on all solutions were anaerobic and contained 2 mM Na\(_2\)S\(_2\)O\(_4\). The extract was heat-treated at 56 °C for 5 min, centrifuged at 20,000 \(\times\) g for 60 min, concentrated to about 150 ml with a Millipore Minitan concentrator, and loaded on a Porapac N column.

**Activity Assay**—Acetylene reduction was used as a monitor of nitrogenase activity. *A. vinelandii* strain CA12 will fix nitrogen and reduce acetylene when grown on medium lacking Mo. The regulation that Mo places on the expression of the alternate enzyme presents a problem. It has been suggested that the alternate enzyme may be a Mo-dependent enzyme (11). As a result, we decided to attempt an isolation of a mutant of LS10 which contains the same deletion as LS10.

**RESULTS**

In order to probe for the possible existence of a second Fe-protein in *A. vinelandii*, we had to first remove the genes (nifHDK) which encode for the structural proteins of native nitrogenase. The resultant strain (LS10) which contains this deletion now lacks the ability to synthesize the conventional enzyme. This result is demonstrated by the inability of LS10 to grow by nitrogen fixation on N-free Burk's medium containing 10 \(\mu\)M Mo (Fig. 1). Furthermore, Southern blotting techniques (results not shown) confirm the absence of this fragment in the genomic DNA of LS10. Bishop et al. (10) recently have constructed a species (CA12) from *A. vinelandii* which contains the same deletion as LS10.

As already mentioned, Bishop et al. (3, 4) hypothesized the existence of an alternative nitrogen-fixing enzyme in *A. vinelandii* whose expression is regulated by the medium's Mo concentration. This group has demonstrated that their deletion strain CA12 will fix nitrogen and reduce acetylene when grown on medium lacking Mo. The regulation that Mo places on the expression of the alternate enzyme presents a problem to those who wish to isolate this enzyme. Typically large quantities of the bacterium have to be grown in order to isolate nitrogenase. Because of this, the expensive and time involved in obtaining large volumes of Mo-free medium could be very extensive. We, therefore, decided to attempt an isolation of a mutant of LS10 which would still express the native nitrogenase.
alternative enzyme even in the presence of Mo. Such a species could be grown without the need to extract Mo from the medium. This mutant was isolated by taking 1 ml of LS10 grown on Burk’s medium plus 5 mM NH₄CO₂CH₃, spreading it onto an agar plate containing Burk’s medium with 10 μM Mo but lacking the NH₄CO₂CH₃, and allowing the culture to incubate at 30 °C. After about 2 weeks several small colonies formed and were isolated. All of these colonies still possessed the same nifHDK deletion as LS10 yet were now able to grow by nitrogen fixation in the presence or absence of 10 μM Mo (Fig. 1) or even in the presence of 10 mM tungsten (present as sodium tungstate). One of these isolates, LS15, grew better than the rest and was used in this study.

The fact that LS15 can grow by nitrogen fixation even though it contains a deletion in the genes encoding for the structural proteins of native nitrogenase means that A. vineandii, as hypothesized by Bishop et al. (3), must possess an alternate means of fixing nitrogen. This alternative, however, is not necessarily a completely different nitrogenase enzyme. One way of probing for homology between different proteins is through antibody cross-reactivity. If A. vineandii has a second nitrogenase and if this enzyme has structural proteins similar to those of the native enzyme, it should be possible to detect these proteins with antibodies prepared against native nitrogenase. Rocket immunoelectrophoresis is one of the more sensitive techniques for quantitating concentrations of proteins, being able to detect less than 100 ng of protein. Fig. 2 shows the results of an experiment in which rocket immunoelectrophoresis was used to determine the amounts of cross-reactive material to anti-Avl and anti-Av2 in various protein preparations. This figure clearly shows that extracts from nitrogen-fixing LS15 contain no cross-reactive material to anti-Avl. On the other hand, there are approximately equal concentrations of protein which react with anti-Av2 in the crude extracts of both the wild-type and LS15 strains of A. vineandii. Therefore, nitrogen-fixing LS15 contains a protein which is antigenically similar to native Av2. In a separate experiment it was shown, as expected, that crude extract from LS10 derepressed on Burk’s N-free medium containing 10 μM Mo showed no cross-reactive material to either anti-Avl or anti-Av2. We have been able to quantify this technique and show that it has the ability to detect at least 0.1% Av1 and Av2 normally found in wild-type crude extract.

Despite the absence of cross-reactive protein in LS15 to anti-Av1, the alternative nitrogenase in this species was found to be very similar to the native enzyme. For example, crude extract of LS15, like that of the wild-type strain, has the ability to reduce acetylene in a MgATP- and reductant- (i.e. dithionite) dependent reaction. This ability is enhanced by the addition of either Av1 or Av2 implying that LS15 crude extract contains proteins which will enzymatically complement the two proteins of native nitrogenase. Furthermore, the ability of LS15 crude extract to reduce acetylene is not significantly affected by heating the extract to 56 °C for 5 min. The heat stability of the alternative nitrogenase in this strain is, therefore, similar to that found for native nitrogenase (13). It can further be stated that these results along with those from the rocket immunoelectrophoresis gel strongly imply that alternative nitrogenase contains a protein similar in both structure and function to native Av2. It is this protein, now referred to as Av2', which we have isolated and characterized.

During the initial phase of the isolation of Av2', crude extract was loaded onto a DEAE column and the components eluted with a linear NaCl gradient. In contrast to Av2, which elutes at a salt concentration of about 0.22 M, Av2' elutes at 0.17 M NaCl (Fig. 3). Because of this, the gradient used for the elution of the LS15 proteins was 0.10–0.35 M NaCl instead of the typical 0.1–0.5 M range.

Final purification on a Sephacryl S-200 gel filtration column showed Av2' to have a molecular mass similar to that of Av2 (around 63 kDa). After this stage of purification, the specific activity of Av2' was 1000–1400 nmol of C₂H₂ reduced min⁻¹ mg⁻¹ protein (four different isolations). In these activity determinations, Av1 was used as the complementing protein. SDS-PAGE of Av2' (Fig. 4) shows it contains a single polypeptide with an apparent molecular weight of about 31,000, running slightly lighter than Av2 (31,416) (20). In this figure, the gel in the region of this polypeptide has been enlarged to show this slight difference in apparent weight. It should be mentioned that Av2 typically migrates on our SDS gels with an apparent mass 2–3 kDa higher than that calculated from its amino acid composition (20). Therefore, we cannot rule out the possibility that the lighter apparent molecular weight of Av2' is not actually due to just a slight difference in electrophoretic migration rate.

Using the BPS procedure to quantitate the iron content of Av2' shows it to contain (Table I) 3.4 ± 0.4 Fe/protein. This is approximately the same value obtained by us for Av2 (3.6 ± 0.4) implying the Av2' like Av2 is a 4Fe-4S protein. When quantitating the Fe content of native component 2 this procedure utilizes the fact that MgATP induces a conformational change in this protein (18), exposing the Fe-S cluster thus making it available for chelation by BPS. This conformational change is apparently important for enzymatic activity. The fact that BPS chelation of the Fe in Av2' occurs only after the addition of MgATP to the medium implies that MgATP...
**Second Fe-Protein from A. vinelandii**

**FIG. 4.** Enlargement of the SDS gel of second component 2 (Av2; Lane A) and native component 2 (Av2, Lane B) showing the small difference in the apparent molecular weights of the two polypeptides. Both component 2 proteins migrate slower (i.e., heavier) than weight calculated from amino acid composition.

**TABLE I**

<table>
<thead>
<tr>
<th>Property</th>
<th>Av2</th>
<th>Av2'</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE elution salt concentration</td>
<td>0.22 M NaCl</td>
<td>0.17 M NaCl</td>
</tr>
<tr>
<td>Molecular weight*</td>
<td>63,000</td>
<td>62,000</td>
</tr>
<tr>
<td>Fe-protein</td>
<td>3.6 ± 0.4</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>g factors (+Na₂S₂O₄)</td>
<td>2.05</td>
<td>2.06</td>
</tr>
<tr>
<td>Spin concentration'</td>
<td>1.94</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>1.88</td>
<td>1.87</td>
</tr>
</tbody>
</table>

* Av2 molecular weight from amino acid composition (20); Av2' molecular weight from apparent molecular weight of polypeptide on SDS gel.

Fig. 5. ESR spectra of Av2 (-----) and Av2' (——) run at 11 K in 50 mM Tris, pH 8.0. Spectrometer settings: frequency, 8.99 GHz; modulation amplitude, 0.63 millitesla; microwave power, 1 milliwatt.

**FIG. 6.** ESR spectra of Av2' at 11 K in 50 mM Tris, pH 8.0, in the absence (a) and presence of MgATP (b). Spectrometer settings were the same as described in the legend to Fig. 5. Plot a has a x5 expansion of the low field (g = 4-6) region to show the presence of resonances indicative of an S = 3/2 spin center. For the spectrum in b, a 10-fold excess of MgATP was added to the concentration of Av2'.

Induces a conformational change in Av2' similar to that already observed in Av2.

In the dithionite reduced state, component 2 of native nitrogenase is paramagnetic exhibiting the ESR spectrum and g factors of a [4Fe-4S]⁺ cluster. The spectrum of Av2 is compared to that of Av2' in Fig. 5. As can be clearly seen in this figure, the spectra are similar but not identical with Av2' being slightly broader than Av2. We have found in past studies on Av2 that its spectrum in the g = 2 region changes, depending on the solvent composition. Therefore, this difference observed between the two proteins may not be significant. Fig. 6a shows the full spectrum of Av2' including the g = 5 region. For years one of the questions concerning native component 2 has been the consistently low spin quantitation of its paramagnetic signal. Recently, this question was answered (21-23) by showing that the Fe-S cluster of this protein can exist either as a S = 1/2 or S = 3/2 spin system with the relative amount of each state depending on the medium (21).

For example, in Tris buffer, pH 8.0, both forms are observable by ESR spectroscopy. While the S = 1/2 form yields the spectrum in the g = 2 region shown in Fig. 5, the S = 3/2 form produces a spectrum part of which is observable in the g = 5 region. Fig. 6A shows that Av2' possesses an inflection in the g = 5 region similar to that previously observed for Av2 (21) implying that the alternate component 2 also exists as a spin = 1/2, 3/2 mixture. Spin quantitation of the g = 2 region of the spectrum yields 0.18 ± 0.04 spin/molecule for Av2' which is comparable to the value of 0.22 ± 0.04 obtained for Av2.

Finally, as stated above, the BPS assay of the Fe content of Av2' suggests that MgATP induces a conformational change in this protein similar to that observed in the native
component 2. This conformational change is also detectable by ESR spectroscopy. When MgATP binds to component 2 the spectrum changes from rhombic to axial. Fig. 6b shows that a similar change occurs in the spectrum of Av2' when the protein is recorded in the presence of MgATP.

DISCUSSION

The possible existence of a second or alternative nitrogen-fixing enzyme in \textit{A. vinelandii} has been the subject of much debate in recent years. The one side argues that the ability of both wild-type and \textit{Nif}\textsuperscript{−} mutants of this bacterium to grow by nitrogen fixation in medium containing extremely low Mo concentrations strongly suggests the presence of a Mo-independent nitrogenase \cite{(3, 4)}. For the other side, the stimulatory effects of Mo on the rate of nitrogen fixation growth of this species implies that the native (Mo-containing) enzyme is the sole nitrogenase present \cite{(7)}. Many questions raised in this debate can now begin to be answered by LS15 which contains a deletion of the genes encoding for the structural proteins of native nitrogenase; this species, therefore, cannot fix nitrogen by the conventional enzyme. The fact that LS15 can still grow by nitrogen fixation shows that, in spite of the deletion, this bacterium still has the ability to produce a nitrogen-fixing enzyme. For the present, we will assume that this enzyme is the so-called alternative nitrogenase.

This paper focuses on the isolation and characterization of a component 2-like protein from the alternative system. As stated above, rocket immunoelectrophoresis studies of the parent deletion strain (LS10) derepressed on medium containing Mo failed to show the presence of cross-reactive material to anti-Av2. Since only the native and not the alternative nitrogenase is expressed in LS10 under these conditions, this result shows that Av2' is not synthesized under normal nitrogen-fixing conditions and, therefore, is not needed for nitrogen fixation by the native enzyme.

The physical properties of the alternative component 2 (Av2') are compared with those of Av2 from the parent strain (UW) in Table I. These two proteins differ in the apparent weight of their polypeptides and in the salt concentration needed to elute each of them off DEAE. The higher ionic strength needed to elute Av2 implies that it is a more acidic protein than Av2'. Other than these minor differences, including those noticed in the ESR spectra, it is remarkable how similar these two proteins are.

The existence of a component 2-like protein in the alternative nitrogen-fixing system of \textit{A. vinelandii} is not totally unexpected. As already mentioned, both Bishop et al. \cite{(3)} and Page and Collinson \cite{(5)} found a polypeptide from \textit{Nif}\textsuperscript{−} mutants of \textit{A. vinelandii} derepressed on medium free of Mo which migrated on SDS gels similar to that of native Av2. Riddle et al. \cite{(24)} obtained similar results from crude extracts of \textit{W}-resistant strains of the same bacterium grown on high concentrations of \textit{W}. More recently, Premakumar et al. \cite{(6)} found component 2 activity in crude extracts of \textit{Nif}\textsuperscript{−} strains which either lacked native Av2 or contained a defective Av2 when these strains were grown under conditions of Mo deficiency. This group also found that two-dimensional SDS-PAGE of these extracts showed a polypeptide which migrated with similar molecular weight to those of Av2 but with a slightly more basic pI. This latter result is consistent with our data of the elution profile of Av2' off DEAE (Fig. 3) implying it to be a more basic protein than Av2. Furthermore, it is interesting to note that in all of these studies no Av1 polypeptides could be detected on SDS gels. This is in agreement with our rocket immunoelectrophoresis analysis of LS15 extracts which show cross-reactive material to anti-Av2 but none to anti-Av1.

Genetic experiments also suggest the existence of a second \textit{nifH} gene. In experiments with the \textit{nif} DNA probe pSA30 (containing cloned \textit{nifHDK} from \textit{Klebsiella pneumoniae}), Medhora et al. \cite{(25)} found several hybridizing fragments with \textit{A. vinelandii} DNA which could not be explained in terms of a single restriction fragment and proposed the existence of more than one copy of these \textit{nif} genes. More recently, Bishop et al. \cite{(10)} constructed several strains containing deletions in the structural genes of native nitrogenase similar to LS10 described in this paper. Using a \textit{nifHDK} probe from \textit{A. vinelandii}, they found restriction fragments which showed sequence homology to \textit{nifH} but not to \textit{nifD} or \textit{nifK} suggesting the presence of a \textit{nifH}-like gene which may be involved in the alternative nitrogen-fixing system. Jacobson et al. \cite{(26)} have demonstrated the formation of two \textit{nifH} (but not \textit{nifD} or \textit{nifK}) homologous transcripts by \textit{A. vinelandii} when grown under nitrogen-fixing conditions with the Mo concentration of the medium less than 25 nM.

Multiple \textit{nifH} genes also have been found in the nitrogen-fixing bacteria \textit{Clostridium pasteurianum} \cite{(27)}, Azotobacter chroococcum \cite{(28)}, and \textit{Rhodopseudomonas capsulata} \cite{(29)}. In \textit{A. chroococcum}, Robson et al. \cite{(28)} have sequenced a second \textit{nifH} gene (called \textit{nifH*}) and found it to be similar but not identical (88% homology) to the gene for the native protein. Both genes encode for proteins with 289 amino acids, but the molecular weight of the \textit{nifH*} gene product is slightly lighter and slightly more basic than Av2. Using the \textit{nifHDK} probe pSA30, Chen et al. \cite{(27)} found three different \textit{nifH} genes in \textit{C. pasteurianum} (called \textit{nifH1}, \textit{nifH2}, and \textit{nifH3}). The gene product of \textit{nifH1} is that of the isolated native Fe-protein while \textit{nifH2} exists 412 base pairs upstream from \textit{nifH1} and has 92% homology to it. The gene product of \textit{nifH3} appeared to be very different from the first two. It seems that if, indeed, the second Fe-protein in \textit{A. vinelandii} is associated with the alternative nitrogen-fixing enzyme, then the presence of multiple \textit{nifH} copies in other nitrogen-fixing bacteria may suggest the possible existence of an alternative enzyme in these species. We are further studying the possible enzyme in \textit{A. vinelandii} by purifying and characterizing component 1.

REFERENCES

8. Joeger, D., Kopeynski, J. B., and Bishop, P. E. (1985) 14th \textit{Steenbock symposium on Nitrogen Fixation and CO\textsubscript{2} Metabolism} Abst. 61, University of Wisconsin, Madison
Second Fe-Protein from A. vinelandii