A Hybrid Azotobacter vinelandii-Clostridium pasteurianum Nitrogenase Iron Protein That Has in Vivo and in Vitro Catalytic Activity*

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Site-directed mutagenesis and gene replacement procedures were used to construct a mutant strain of Azotobacter vinelandii which expresses a hybrid nitrogenase Fe protein. This hybrid Fe protein has its carboxyterminal 18 residues replaced with the 5 analogous residues from the Clostridium pasteurianum Fe protein sequence. The hybrid Fe protein is 13 amino acids smaller than the wild-type A. vinelandii Fe protein and has a net loss of 4 negatively charged residues, resulting in a change in size and charge. The strain which produces the hybrid Fe protein remained capable of diazotrophic growth, albeit at a reduced rate. Also, the purified hybrid Fe protein exhibited a maximum activity about one-half that of native Fe protein. These results demonstrate that the tight, inactive complex which is formed when A. vinelandii MoFe protein and C. pasteurianum Fe protein are mixed in heterologous reconstitution experiments cannot be accounted for only by differences in the A. vinelandii and C. pasteurianum Fe protein primary sequences located at their respective carboxyl termini.

Nitrogenase catalyzes the ATP-dependent reduction of dinitrogen. Molybdenum-dependent nitrogenase is a complex enzyme system that consists of two components, the Fe protein (a homodimer, encoded by nifH) and the MoFe protein (an αβγ tetramer encoded by nifDK). The Fe protein acts as an ATP-binding, one-electron donor to the MoFe protein, which contains the substrate-binding site(s) (see Ref. 1 for a recent review). During catalysis, reduced Fe protein probably binds two MgATP molecules, associates with the MoFe protein, and donates a single electron to the MoFe protein in a reaction coupled with ATP hydrolysis and component protein dissociation. Because multiple electrons are required for N₂ reduction, multiple cycles of component protein association and dissociation are required for nitrogenase turnover. That nitrogenases from phylogenetically diverse diazotrophic organisms are similar in structure and mechanism was first indicated by experiments of Detroy et al. (2), who showed that complementary nitrogenase components isolated from different organisms can be mixed to form heterologous, catalytically competent enzymes. Structural similarities among nitrogenase components from different species was later confirmed by interspecies DNA-DNA hybridization studies (3, 4) and numerous DNA sequence analyses (for an example, see Ref. 5).

Although most heterologous Fe protein and MoFe protein complexes are catalytically active, a reconstituted mixture of Fe protein isolated from Clostridium pasteurianum (Cp2) and MoFe protein isolated from Azotobacter vinelandii (Av1) is ineffective in substrate reduction (6). This heterologous mixture results in the formation of a tight, catalytically inactive complex (7). Differences in the amino acid sequence of Cp2 when compared to Av2 have been used in attempts to explain this phenomenon and to assign a potential component protein interaction site located upon the Fe protein (8, 9). Av2 and Cp2 share high levels of identity throughout their primary sequences (10, 11). However, Av2 is elongated when compared to Cp2. This feature led two research groups to suggest that the carboxyl portion of the Fe protein could have an important role in nitrogenase component protein association and that sequence differences located at the carboxyl ends of the respective Fe proteins might account for the formation of the tight complex in heterologous Cp2-Av1 reconstitution experiments (8, 9). In the present study, we examined this hypothesis both in vivo and in vitro by constructing a hybrid Av2 protein which has its carboxy end replaced with the corresponding Cp2 sequence.

MATERIALS AND METHODS

The wild-type and mutant strains of A. vinelandii were grown at 30°C in modified Burk medium (12). When a fixed nitrogen source was required for growth, ammonium acetate was added to the growth medium to a final concentration of 30 mM. For antibiotic selection, kanamycin was added to a final concentration of 0.5 μg/ml. Growth rates were examined as follows. Cultures to be used as inoculum were grown to mid-log phase on ammonium acetate-supplemented Burk liquid medium. Cells were pelleted by centrifugation, washed once with Burk nitrogen-free medium, and inoculated into 30 ml of Burk nitrogen-free medium in a 300-ml baffled side-arm flask (Belco Glass, Vineland, NJ) to approximately 10 Klett units. Growth was followed using a Summerson-Klett meter equipped with a no. 66 filter. A. vinelandii crude extracts were prepared from cells grown to 100 Klett units in liquid minimal Burk medium as described previously (13). Proteins were quantitated by the Biuret method (14). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (15). Nitrogenase component proteins were isolated and characterized as previously described (16) except that Q-Sepharose (Pharmacia LKB Biotechnology Inc.) was used in place of DEAE-cellulose. Maximum Fe protein-specific activities were determined as previously described (17) by titrating a constant amount of Av2 with increasing amounts of purified wild-type Av1.

The Escherichia coli strain 71-18 (Δlac-proAB thi supE F'proAB) was used as the host for cloning experiments. The E. coli strain DSM 10 (Δlac-proAB thi supE F'proA) was used for cloning experiments. The E. coli strain DSM 10 (Δlac-proAB thi supE F'proA) was used for cloning experiments.

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lacIqZM15) served as host for recombinant M13 bacteriophage and plasmid vectors used in this study. Restriction enzymes, T4 DNA ligase and DNA polymerase I were purchased from Bethesda Research Laboratories or New England Biolabs (Beverly, MA) and used as recommended by the supplier. SDS PAGE molecular weight markers were purchased from Bio-Rad. Hybrid plasmids and hybrid M13 phage vectors used in this study are described in Table I and were constructed in the following ways. For site-directed mutagenesis, pDB20 was constructed by cloning a 2577-base pair PstI fragment from pDB6 (18) into the PstI site of M13mp18 (19). This PstI fragment contains the entire nifH gene and flanking regions. The relevant physical map of this region is shown in Fig. 1. The restriction site EcoRV was introduced into this phage construct by using site-directed mutagenesis as described by Zoller and Smith (20) (Fig. 2). The presence of the EcoRV site was confirmed by restriction enzyme digestion and DNA sequence analysis (21). The mutagenized phage was designated pDB122. The EcoRV site within pDB122 is located immediately after the termination codon for the nifH gene (Fig. 2). Plasmid pDB280 was prepared by cloning the 1106-base pair PstI fragment from pDB122 (Fig. 1) into the KpnI site of pUC19 (18). Plasmid pDB279 was constructed by removing the unique EcoRI site from pUC19 using restriction enzyme digestion, DNA polymerase I treatment, and DNA ligation. Plasmid pDB286 was constructed by cloning the 1106-base pair KpnI fragment from pDB279 into the KpnI site of pDB280. The region between the unique EcoRV site and EcoRV restriction site within pDB286 was replaced with the synthetic oligonucleotide cassette shown in Fig. 2.

### Table I

<table>
<thead>
<tr>
<th>Plasmid or phage</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDB6</td>
<td>Used as a source for the 2577-base pair PstI fragment containing nifH and flanking regions (see Fig. 1 and Ref. 18).</td>
</tr>
<tr>
<td>pDB20</td>
<td>Has the 2577-base pair PstI fragment containing nifH and flanking regions cloned into the PstI site of M13mp18 (Fig. 1).</td>
</tr>
<tr>
<td>pDB122</td>
<td>Derived from pDB20, contains an EcoRV site placed immediately past the nifH termination codon (Fig. 2).</td>
</tr>
<tr>
<td>pDB280</td>
<td>Prepared by cloning the 1106-base pair KpnI fragment from pDB122 into the KpnI site of pUC19 (Fig. 1).</td>
</tr>
<tr>
<td>pDB279</td>
<td>Derived from pUC19; the unique EcoRI site present in pUC19 is removed in pDB279.</td>
</tr>
<tr>
<td>pDB286</td>
<td>Prepared by cloning the 1106-base pair KpnI fragment from pDB280 into the KpnI site of pDB279.</td>
</tr>
<tr>
<td>pDB290</td>
<td>The region between the unique EcoRI and EcoRV restriction site within pDB286 was replaced with the synthetic oligonucleotide cassette shown in Fig. 2.</td>
</tr>
<tr>
<td>pDB288</td>
<td>Derived from pDB286; contains a 1.3-kilobase KmR cartridge cloned into the unique EcoRI site present within the nifH gene coding sequence in pDB288.</td>
</tr>
</tbody>
</table>

**FIG. 1.** Physical map of the A. vinelandii nif structural gene cluster and relevant restriction enzyme sites. The 2577-base pair PstI fragment present in pDB122 and the 1106-base pair KpnI fragment present in pDB280 and pDB286 are shown as horizontal bars. The indicated EcoRI site was used for the construction of pDB288, which has a 1.3-kilobase (kb) KmR gene cartridge cloned into that site. The position of the EcoRV site engineered into pDB122 is also indicated.

### RESULTS AND DISCUSSION

A comparison of the primary sequences of Av2 and Cp2 is shown in Fig. 3. There is an overall 65% conservation in sequence identity between these proteins, and this conservation is distributed throughout the length of the respective polypeptides. Particularly strong sequence conservation is located in the amino-terminal region of the polypeptides corresponding to a proposed ATP-binding domain (25) and in regions surrounding the two conserved cysteinyls (residues 97 and 132 using the Av2 sequence as a reference) proposed as the iron-sulfur cluster ligands (26, 27). The most striking difference between the proteins is that Av2 is elongated by 13 residues compared to Cp2. These 13 amino acids include 5 negatively charged residues (4 glutamates and 1 aspartate) and one positively charged residue (lysine). It has been suggested that these differences in size and charge density located at the carboxyl ends of the respective Fe protein sequences could account for the formation of the tight, ineffective complex in heterologous Av1-Cp2 reconstitution experiments (8, 9). We tested this hypothesis by removing the carboxyl-coding 18 codons from the Av2-encoding gene and replacing them with the analogous five codons from the Cp2 sequence. This was accomplished using cloned A. vinelandii nif structural DNA, site-directed mutagenesis, and gene replacement procedures (see “Materials and Methods”).

Because it was not known whether a mutant harboring such an alteration would exhibit a Nif+ or Nif- phenotype, the mutant strain which produces the hybrid Fe protein was isolated in two steps. In the first step a kanamycin resistance-encoding gene cartridge (KmR) was recombined into the A. vinelandii chromosomal nifH gene by transformation using the appropriately constructed recombinant plasmid, pDB288 (see “Materials and Methods”). This strain (DJ316) exhibited a Nif+ phenotype resulting either from inactivation of the nifH gene product or from polarity upon nifDK gene expression. Purified DNA from another hybrid plasmid, pDB290 (Table I, Fig. 2), was then used in a marker rescue experiment by transformation of competent DJ316 cells. This treatment resulted in reciprocal recombination events between the homologous regions located upon the A. vinelandii chromosome and pDB290. The resultant transformants exhibited both KmR and Nif+ phenotypes. One of these transformants was designated DJ319 and studied further. The KmR cartridge is located between codons 270 and 271 of the nifH sequence in pDB290 (Figs. 2 and 3).

A. vinelandii transformations were performed as described by Page and Von Tigerström (22). Detailed descriptions of gene replacement procedures and marker rescue experiments were published previously (23, 24).

The DNA sequence of the insertion fragment was determined by DNA sequence analysis (21) of denatured pDB290 DNA using the synthetic oligonucleotide 5'-ACCGATCCATGGAACGCA-3' as a primer. Plasmid pDB288 was constructed by digesting pDB286 with EcoRI and cloning a purified 1.3-kilobase EcoRI KmR cartridge from pUC4-Kappa (Pharmacia) into that site. The KmR cartridge is located between codons 270 and 271 of the nifH sequence in pDB290 (Figs. 2 and 3).
Hybrid Nitrogenase Iron Protein

Fig. 2. Schematic representation of DNA manipulations for the construction of A. vinelandii strain DJ319. The top line indicates the relevant carboxyl-coding portion of nifH present in the original hybrid M13mp18 clone, pDB20. The synthetic oligonucleotide used to introduce the EcoRV site present in pDB122 (step A) is shown just below the top sequence. The substitution in this oligonucleotide (T) is underlined. The unique EcoRI and EcoRV sites present in the resultant pDB122 clone are boxed. In step B, the plasmid pDB286, which contains the 1,106-base pair KpnI fragment (Fig. 1) from pDB122 cloned into the KpnI site of pDB279, was digested with EcoRI and EcoRV, and the small fragment was removed by gel electrophoresis and electroelution. Two complementary oligonucleotides, 5'-AATTCGGCCTGATGGACCTATG-3' and 5'-CATACACCTTCATCAGGCCG(') (shown between B and C), were annealed and ligated to the EcoRI- and EcoRV-digested pDB286 DNA in step C to form pDB290. The coding sequence for the carboxyl terminus of the hybrid nifH gene is shown at the bottom. The single-letter code is used to denote amino acid residues.

Fig. 3. Comparison of the primary amino acid sequences of Fe proteins from A. vinelandii (upper sequence) and C. pasteurianum (lower sequence). Numbering refers to the A. vinelandii sequence. The complete sequence of both polypeptides is shown. --, packing characters used to obtain optimal alignment; *, identically conserved residues; I, charge differences.

contains 13 fewer amino acids than native Av2 (Figs. 2 and 3), a reduction in the size of the hybrid Av2 produced in DJ319 was predicted. This prediction was confirmed by SDS-PAGE analyses of crude extracts prepared from diazotrophically grown DJ319 and the isogenic wild-type strain (Fig. 4). The hybrid Av2 protein also has a net loss of 4 negatively charged residues when compared to the wild-type Fe protein (Figs. 2 and 3), which should result in a charge change. This feature was confirmed by two-dimensional gel electrophoresis (data not shown). SDS-PAGE analysis of crude extracts revealed that DJ319 accumulated substantially more Av1 and hybrid Av2 polypeptides than did the wild type (Fig. 4). The basis for this result is not known. One possible explanation is that native Av2 has a role in controlling expression of the nitrogenase structural gene cluster and that this function has been eliminated in the hybrid Av2 protein. Another possibility...
doubling times (1.8 h) when the growth medium was supplemented with ammonium acetate.

FIG. 5. Diazotrophic growth of the wild-type A. vinelandii (circles) and strain DJ319 (triangles). Both strains had identical doubling times (1.5 h) when the growth medium was supplemented with ammonium acetate.

FIG. 6. Approximately 10 μg of Fe protein purified from either wild-type A. vinelandii (lane 3) or strain DJ319 (lane 2) was analyzed by SDS-PAGE (12%). Molecular weight markers (circles) and strain DJ319 (lanes 1 and 4) are the same as used in Fig. 4, legend. Electrophoretic mobilities in this figure cannot be directly compared to those in Fig. 4 because different concentrations of cross-linker were used for preparation of the respective gels.

FIG. 7. Comparison of the catalytic activities of purified wild-type Av2 (closed circles) and hybrid DJ319 Av2 (open circles) were determined at the MoFe : iron protein molar ratios indicated. Units are nanomoles of ethylene produced per min/mg of Fe protein. is that the hybrid Av2 protein is less effective in catalysis resulting in continuous nitrogen limitation when DJ319 is grown in the absence of a fixed nitrogen source. Such conditions could cause a perpetual state of nitrogenase derepression in DJ319, resulting in an elevated accumulation of nitrogenase components.

When cultured in a minimal medium supplemented with a fixed nitrogen source, the growth rates of DJ319 and the wild type were identical (data not shown). Although DJ319 was capable of diazotrophic growth, its growth rate was slower than the wild-type strain, indicating a physiological defect related to nitrogenase catalysis (Fig. 5). The slower diazotrophic growth rate observed for DJ319 could arise directly from a defect in nitrogenase catalysis or indirectly from overproduction of the nitrogenase component proteins.

Both the wild-type Fe protein and the hybrid Fe protein were purified (Fig. 6) and their specific activities were determined (Fig. 7). These experiments revealed that the purified hybrid Av2 protein exhibits a maximum specific activity about one-half that of the wild type (Fig. 7). Under conditions where availability of Av2 becomes limiting, at high Av1/Av2 ratios, a defect in the hybrid Av2 protein is apparent. However, under conditions where the Av1/Av2 ratio is less than one, the hybrid Av2 specific activity appears identical to the wild type. If the hybrid Av2-Avl complex exhibited tighter binding and therefore dissociated significantly slower than the wild-type complex, a reduced specific activity of the hybrid Av2 when compared to wild-type Av2 would be expected even at low Av1/Av2 component ratios. Thus, these results demonstrate that the tight, inactive complex, which is formed when Av1 and Cp2 are mixed in heterologous in vitro reconstitution experiments, cannot be accounted for only by differences in the A. vinelandii and C. pasteurianum Fe-protein primary sequences located at their respective carboxyl termini.

Although our results do not eliminate the possibility that the carboxyl end of Av2 plays any role in nitrogenase component-protein association or dissociation, they do show that the terminal 18 amino acids in Av2 are not essential to these processes. Also, neither the truncation of Cp2 when compared to Av2 nor the absence of the concentration of charged residues present at the carboxyl end of Av2, but not present in Cp2, is solely responsible for the formation of the tight, ineffective complex in heterologous Cp2-Avl reconstitution experiments. Nevertheless, it is a reasonable hypothesis that localized charge differences between Av2 and Cp2 are likely to be involved in formation of the catalytically inactive Av1-Cp2 complex. The Av2-Cp2 comparison shown in Fig. 3 reveals that such charge differences are concentrated mainly in the regions between residues 49 to 79 and in the carboxyl one-third of the respective polypeptides. Murrell et al. (29) reported that ADP-ribosylation of Cp2 prevents its formation of the tight complex when reconstituted with Av1. Thus, the ADP-ribosylation site (corresponding to arginine 100 in the Av2 sequence) might be located at or near a component-protein interaction site. Similarly, chemical cross-linking studies have revealed that one Fe protein subunit can be specifically cross-linked to each MoFe protein β-subunit (30). The specific residues involved in such cross-linking include Fe protein residue Glu-112 and MoFe-protein β-subunit residue Lys-399 (31). Although the region encompassing the Av2 Glu-112 residue and the corresponding Cp2 sequence are highly conserved (Fig. 3), some primary sequence differences, which result in charge changes, are recognized. It will, therefore, be interesting to examine this region within Av2 by using the hybrid protein construction approach described in the present study. Our attempts to duplicate in vitro heterologous nitrogenase reconstitution experiments in vivo provide a genetic approach for the identification of component-protein interaction sites. This approach complements the classical cross-linking studies (30, 31) and in vitro modification studies (29) that are also being used to analyze the nature of nitrogenase component-protein interactions.

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REFERENCES


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