Site-directed mutagenesis of the nitrogenase MoFe protein of Azotobacter vinelandii

(iron–molybdenum cofactor/nitrogen fixation/nifD gene/protein engineering)

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ABSTRACT  A strategy has been formulated for the site-directed mutagenesis of the Azotobacter vinelandii nif/DK genes. These genes encode the α and β subunits of the MoFe protein of nitrogenase, respectively. Six mutant strains, which produce MoFe proteins altered in their α subunit by known single amino acid substitutions, have been produced. Three of these transversion mutations involve cysteine-to-serine changes (at residues 154, 183, and 275), two involve glutamine-to-glutamic acid changes (at residues 151 and 191), and one involves an aspartic acid-to-glutamic acid change (at residue 161). All three possible phenotypic responses are observed within this group—i.e., normal, slow, and no growth in the absence of a fixed-nitrogen source. Two-dimensional gel electrophoresis indicates that all mutants accumulate normal levels of the subunits of both nitrogenase component proteins. Whole-cell and crude-extract acetylene-reduction activities indicate substantial levels of Fe protein activity in all strains. In contrast, MoFe protein activities do not parallel the diazotrophic growth capability for all strains. Two strains appear to exhibit altered substrate discrimination. Such analyses should aid in the identification of metalcluster-binding sites and subunit–subunit interaction domains of the MoFe protein and also provide insight into the mechanistic roles of the various prosthetic groups in catalysis.

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Mossbauer and electron paramagnetic resonance spectroscopy (2–6), as well as biochemical and genetic studies (10), have been used to demonstrate that the metal centers located within the MoFe protein are likely to participate in the binding and catalytic reduction of dinitrogen. However, little direct information is available concerning the structures, redox properties, and functions of the individual metal centers within the MoFe protein. Furthermore, there is no information regarding the spatial distribution of the metal centers among or between the four subunits of the MoFe protein. One approach toward addressing these issues is to alter specifically the polypeptide environments of the individual clusters and to determine the spectroscopic, redox, and catalytic consequences that result from such alterations. For example, specific amino acid substitutions at key metal-center ligation points within the MoFe protein should produce altered proteins with strengthened, weakened, or eliminated metallocenter interactions. Observations of these effects could indicate appropriate cluster-binding amino acid residues and also provide information on the mechanistic roles of the individual clusters. For this purpose, we isolated the nif/DK gene cluster from Azotobacter vinelandii and determined its complete nucleotide sequence (11). Here, we describe the formulation of a strategy for the site-directed mutagenesis of the genes that encode the MoFe protein subunits and describe the isolation and characterization of six mutant A. vinelandii strains that produce altered MoFe α-subunit proteins with known amino acid substitutions.

MATERIALS AND METHODS

Oligonucleotide-Directed Mutagenesis. Oligonucleotides used for mutagenesis were synthesized on a Beckman System 1 Plus DNA synthesizer. After deprotection, the oligonucleotides were purified by HPLC (12). Oligonucleotide-directed mutagenesis was performed by the method of Zoller and Smith (13). Template DNA used for mutagenesis was a 1.4-kilobase A. vinelandii nifD-specific EcoRI fragment (11) cloned into the EcoRI site of bacteriophage vector M13mp18 (14). Single-lane dideoxy sequencing (15) was used to screen for the desired mutations. Further DNA sequence analysis using all four reactions confirmed the mutations and established that there were no other mutations within 400 nucleotides surrounding the planned mutation. For each mutant construction, replicative-form DNA carrying a known single-base-pair transversion mutation within the nifD coding region was prepared and used for transformation of A. vinelandii cells. Such DNA, which carries a single transversion muta-
tion within the \textit{nitD} coding sequence, will hereafter be called mutation-vector DNA.

\textbf{Isolation of Mutant Strains.} Transversion mutations carried on mutation-vector DNA were transferred to the \textit{A. vinelandii} chromosome in either one or two steps (Fig. 1), depending on the resultant \textit{Nif} phenotype of the mutant strain. Mutations resulting in a single amino acid substitution within the \textit{nitD}-encoded polypeptide may exhibit one of three \textit{Nif} phenotypes. Namely, such mutant strains will grow normally, at a reduced rate, or not at all in the absence of a fixed-nitrogen source. Mutations that cause slow growth or that have no effect on diazotrophic growth can be introduced into the \textit{A. vinelandii} genome by a marker-rescue procedure where the mutation-vector DNA is used to transform a strain deleted for \textit{nitD} to prototrophy (see Fig. 1). Mutations that result in a strictly \textit{Nif} phenotype can be introduced into the \textit{A. vinelandii} chromosome by the simultaneous transformation of wild-type cells with mutation-vector DNA and purified \textit{A. vinelandii} chromosomal DNA that carries an uncharacterized rifampicin-resistance marker. \textit{Nif} transformants are recovered by selection on rifampicin-containing medium, and the coincident transfer (congression) of the \textit{Nif} marker is identified by scoring transformants in the absence or presence of a fixed-nitrogen source (see ref. 16 for a detailed description of this procedure).

\textbf{Cell Growth and Nitrogenase Derepression.} Wild-type and mutant strains of \textit{A. vinelandii} were cultured on a modified Burk medium (17) that was supplemented with filter-sterilized urea to a final concentration of 10 mM when a fixed-nitrogen source was required. For derepression of nitrogenase synthesis, 11 liters of urea-supplemented Burk medium was inoculated with a 500-ml culture (200 Klett units, no. 54 filter) in a New Brunswick Microferm SF116 fermentor of 12-liter working capacity. All cultures were stirred at 300 rpm at 30°C with an aeration rate of 12 liters/min. When the culture density reached 110 Klett units, cells were concentrated to 1 liter with a Millipore tangential-flow ultrafiltration apparatus. Two liters of sterile nitrogen-free Burk medium were then added to the fermentor, and the culture was concentrated again. A similar second wash was taken to 500-ml final volume. Eleven liters of sterile, nitrogen-free Burk medium was used to transfer cells back to the fermentor and incubated as above for 2.5 hr. Derepressed cells were harvested as above except that the cells were washed with chilled 0.05 M Tris (pH 8.0), then centrifuged at 10,000 \times g for 10 min and stored at -80°C until needed.

\textbf{Two-Dimensional Gel Electrophoresis.} Preparation of extracts for two-dimensional gel electrophoresis was performed as described by Bishop et al. (18). Two-dimensional gel analyses were performed as described by O'Farrell (19).

\textbf{Enzyme Assay.} Frozen cells (13 g) were resuspended in 39 ml of cold 0.05 M Tris (pH 8.0) containing 1 mM Na2S2O4 and passed through a chilled, argon-flushed French pressure cell at 12,000 psi (82.7 MPa), followed by centrifugation at 17,000 \times g for 20 min at 4°C. The extracts were then degassed under argon and the MoFe and Fe protein activities were assayed by acetylene reduction (20), with and without saturating amounts of the purified complementary protein. Specific activities of these purified MoFe and Fe proteins were 2020 and 1700 nmol of acetylene reduced per min per mg of protein, respectively. Protein concentrations were determined by the biuret method (21).

\section*{RESULTS AND DISCUSSION}

\textbf{Rationale for Amino Acid Replacements.} Several indirect approaches have been used in attempts to identify structurally important regions within the MoFe protein. These include (i) comparison of MoFe protein sequences to other metal-center-containing proteins of known structure, in particular ferredoxins (11); (ii) comparison of MoFe protein sequences from widely diverse diazotrophic species (see for example refs. 11 and 22); (iii) comparison of the MoFe protein \(\alpha\) and \(\beta\) subunits (22); and (iv) comparison of MoFe protein subunit sequences to sequences of polyketides that are required for metal-center assembly (for example, the \textit{nitE} and \textit{nitN} gene products, which are required for FeMoco biogenesis (20)).

The above comparisons have shown that there are no typical ferredoxin-like sequences located within the \textit{A. vinelandii} MoFe protein, an observation that is consistent with the unusual redox and spectroscopic properties of the MoFe protein Fe-S centers when they are compared to ferredoxin Fe-S clusters. Interspecies comparisons of MoFe protein subunits reveal homologies that are greatest within the amino-terminal half of homologous subunit polypeptides, and these homologies are largely centered around cysteine residues. There are five cysteine residues that can be considered conserved among all known \(\alpha\)-subunit sequences (residues 62, 88, 154, 183, and 275, using the \textit{A. vinelandii} sequence in ref. 11) and three conserved cysteine residues among all known \(\beta\)-subunit sequences (residues 70, 95, and 153, using the \textit{A. vinelandii} sequence in ref. 11). Significant sequence homology is also found surrounding \(\alpha\)-subunit cysteine residues 62, 88, and 154 when they are respectively compared to \(\beta\)-subunit cysteine residues 70, 95, and 153. These latter homologies have led to the suggestion that there are structural prosthetic group-binding features common to both subunits of the MoFe protein (22). Finally, the products of the FeMoco-specific biosynthetic genes, \textit{nitE} and \textit{nitN}, share structural homology when compared to the MoFe protein \(\alpha\) and \(\beta\) subunits, respectively (20). Importantly, these homologies are also centered around those cysteine residues that exhibit interspecies and, in some cases, intersubunit sequence conservation.

Insights regarding potential metal-center ligands within the MoFe protein can also be gained by considering the extrusion requirements of the metal centers. The Fe-S clusters, which are extruded by thiol treatment, are likely to exhibit cysteine-thiol-type ligation within the MoFe protein. However, the unusual redox and spectroscopic properties of the Fe-S
centers within the MoFe protein raise the possibility that there are also other ligand modes. Thiol reactivity experiments using purified FeMoco have also indicated the potential for a single cysteine-thiol ligand for each FeMoco species (23). In addition, the requirement for N-methylformamide or formamide for the extraction of FeMoco indicates a potential role for amide groups in binding FeMoco to the MoFe protein.

Construction and Expression of Altered MoFe Proteins. In *Klebsiella pneumoniae*, the *nif* genes are clustered and are organized into at least seven transcriptional units (24). The promoter regions for these *nif* gene clusters share a characteristic structure and their activation requires the presence of a positive regulatory element, the product of the *nifA* gene (24). It has been shown that elevation of the copy number of the *nifH* gene promoter unbalances *nif* gene expression (25), presumably by sequestering the available activator molecules. Recent studies (20, 26) indicate that the genetic organization, as well as the mechanisms for regulating *nif* gene expression in *A. vinelandii*, is similar to that determined for *K. pneumoniae*. Thus, to avoid potential deleterious effects that could unbalance the synthesis of *nif*-specific components, it is important that altered *nif* genes are re-incorporated into the chromosome in single copy rather than residing in the form of multicopy plasmids. Such gene-replacement strategies are described in Materials and Methods and are depicted in Fig. 1. Strains constructed in this way are particularly useful, since all of the ancillary *nif*-specific genes and their products are unaffected by this procedure. Consequently, any alterations in the properties of the MoFe proteins prepared from the various mutant strains can be attributed to the introduced mutation rather than as arising from indirect effects on processing or metallocenter-assembly functions.

Characterization of Mutants. The site-directed mutagenesis strategy described above was used to isolate six mutant strains, each of which contains a known transversion mutation resulting in an altered MoFe protein α subunit containing a known single amino acid substitution (Table 1). All three of the anticipated *nif* phenotypes are represented in this group. Namely, mutant strains were isolated that are altered within the MoFe protein α subunit and that individually exhibit a *Nif*+ phenotype (DJ45, DJ64, and DJ56), a *Nif*+ phenotype (DJ62 and DJ63), or slow growth in the absence of a fixed-nitrogen source (DJ55). The diazotrophic growth properties of these mutants are shown in Fig. 2. Strain DJ62 was useful because it exhibits normal diazotrophic growth yet carries a mutation that should lead to a charge change within the MoFe protein α subunit (Glu-191 substituted for Gin-191, Table 1). This feature permitted the proof that strains that carry phenotypically silent mutations are readily recovered by our mutagenesis scheme. This proof was accomplished by demonstrating the appropriate charge shift in the MoFe protein α-subunit polypeptide as judged by two-dimensional gel electrophoresis (Fig. 3). Two-dimensional-gel and/or immunological analysis of crude extracts prepared from nitrogenase derepressed cultures was also used to demonstrate that each of the mutant strains accumulates normal amounts of Fe protein and MoFe protein subunits (data not shown). Thus, active MoFe protein is not required for the full expression of nitrogenase components in *A. vinelandii*. These results further indicate that inactive MoFe protein subunits

Table 1. Mutant strains isolated and characterized in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>nfd</em> codon altered</th>
<th>Codon change</th>
<th>Amino acid substitution</th>
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</thead>
<tbody>
<tr>
<td>DJ45</td>
<td>154</td>
<td>TGC→TCC</td>
<td>Cys→Ser</td>
</tr>
<tr>
<td>DJ55</td>
<td>183</td>
<td>TGC→TCC</td>
<td>Cys→Ser</td>
</tr>
<tr>
<td>DJ56</td>
<td>275</td>
<td>TGC→TCC</td>
<td>Cys→Ser</td>
</tr>
<tr>
<td>DJ62</td>
<td>151</td>
<td>CAG→GAG</td>
<td>Gin→Glu</td>
</tr>
<tr>
<td>DJ63</td>
<td>161</td>
<td>GAC→GAG</td>
<td>Asp→Glu</td>
</tr>
<tr>
<td>DJ64</td>
<td>191</td>
<td>CAG→GAG</td>
<td>Gin→Glu</td>
</tr>
</tbody>
</table>

*Indicates the position of the altered codon within *nfd* in the mutant strain. The initiation ATG codon is number 1.

†Indicates the specific nucleotide change (wild type→mutant) within the altered codon.

‡Indicates the amino acid substitution resulting from the transversion mutation.
Table 2. Component protein activities in mutant and wild-type strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Whole cell, % wild type</th>
<th>Crude extract, spec. act.*</th>
<th>MoFe protein†</th>
<th>Fe protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>100</td>
<td>43.3</td>
<td>53.7</td>
<td></td>
</tr>
<tr>
<td>DJ45</td>
<td>0</td>
<td>14.7</td>
<td>0.1</td>
<td></td>
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<td>DJ55</td>
<td>4</td>
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<td>3.1</td>
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<tr>
<td>DJ56</td>
<td>0</td>
<td>20.9</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>DJ62</td>
<td>70</td>
<td>33.6</td>
<td>38.2</td>
<td></td>
</tr>
<tr>
<td>DJ63</td>
<td>64</td>
<td>18.1</td>
<td>41.7</td>
<td></td>
</tr>
<tr>
<td>DJ64</td>
<td>12</td>
<td>27.3</td>
<td>10.0</td>
<td></td>
</tr>
</tbody>
</table>

All values represent the average of at least three determinations.

*Specific activity: nmol of ethylene formed per min per mg of crude extract protein.

†Determined in the presence of saturation levels of purified A. vinelandii MoFe protein.

‡Determined in the presence of saturation levels of purified A. vinelandii Fe protein.

are not rapidly degraded in A. vinelandii as was suggested for K. pneumoniae (27).

Whole-cell and crude-extract activity measurements (Table 2) show that alteration of the interspecifically conserved $\alpha$-subunit cysteine residues 154 and 275 results in a complete loss of both MoFe protein activity and the capacity for diazotrophic growth. In contrast, alteration of $\alpha$-subunit Cys-183 results in substantially lowered MoFe protein activity, yet the mutant strain retains the capacity for diazotrophic growth. These comparisons are in line with the notion that conserved cysteine residues 154 and 275 play an essential structural or functional role in the MoFe protein, whereas conserved cysteine residue 183 does not. These results do not, however, prove a proposed role for cysteine residues 154 and 275 as metallocluster-binding ligands within the MoFe protein. Rather, the data provide a basis for the further biophysical analyses of MoFe protein prepared from these mutant strains, as well as provide some insight regarding other potential targets for site-directed mutagenesis.

Comparison of mutant strains DJ55 and DJ64 is of particular interest. Although strain DJ64 is incapable of diazotrophic growth (Fig. 2), it retains substantial levels of MoFe protein activity as judged by its acetylene-reduction activity (Table 2). In contrast, DJ55 exhibits a much lower MoFe protein acetylene-reduction activity (Table 2), yet it is still capable of diazotrophic growth (Fig. 2). The difference in substrate utilization shown by these two strains must reside in their ability to recognize or reduce different substrates. Consequently, the further characterization of these strains and the isolation of other mutants with similar phenotypes should prove useful in elucidating the substrate-discrimination and -reduction properties of nitrogenase. In this regard, analytical measurements of the Fe and Mo content and spectrosopic characterization of MoFe protein prepared from these mutant organisms should be particularly useful.

In summary, the above results demonstrate the potential for introducing any site-specific mutation within the genes encoding nitrogenase components. Such mutant organisms are readily constructed irrespective of their Nif phenotype. Importantly, mutants constructed by the procedure described here do not alter the expression of any of the ancillary nif-specific components required for metallocenter assembly, or component maturation. Another important observation is that normal levels of both MoFe protein subunits accumulate in strains altered within the $\alpha$ subunit, indicating that inactive subunit polypeptides are not rapidly degraded in A. vinelandii. Purification and exhaustive biochemical characterization of MoFe protein purified from mutant strains described here should help to identify the role each mutation has in altering enzyme activity. Specifically, we believe that such analyses will provide insights into the roles that each cluster type plays in the catalytic activity of this extraordinarily complex metalloenzyme. Finally, studies using the mutagenesis strategy described here could identify catalytically important residues within the MoFe protein $\beta$ subunit and the Fe protein, and in those gene products involved in FeMoco biogenesis.

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