Nitrogenase catalyzes biological dinitrogen fixation, the reduction of \( \text{N}_2 \) to \( 2\text{NH}_3 \). Recently, the binding site for a non-physiological alkyne substrate (propargyl alcohol, \( \text{HC}≡\text{C}-\text{CH}_2\text{OH} \)) was localized to a specific Fe-S face of the FeMo-cofactor approached by the MoFe protein amino acid \( \alpha\text{-70Val} \). Here we provide evidence to indicate that the smaller alkyl side chain (isoleucine) significantly lowered the capacity of the MoFe protein to reduce dinitrogen, hydrazine, or acetylene.

Biological reduction of dinitrogen (\( \text{N}_2 \)) is catalyzed by nitrogenase, a two-component metalloenzyme. The ideal reaction, summarized here by equation 1,

\[
\text{N}_2 + 8\text{e}^- + 16\text{MgATP} + 8\text{H}^+ \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{MgADP} + 16\text{P},
\]

is catalyzed by nitrogenase. For the molybdenum-dependent nitrogenase, substrate reduction occurs at a complex metallocluster called the FeMo-cofactor, which is contained within the \( \alpha\)-subunit of the nitrogenase component protein called the MoFe protein. The structure of the active site FeMo-cofactor was revealed in 1992 when a high resolution x-ray crystal structure of the MoFe protein was solved (Fig. 1) (1–4). In inspection of the structure suggested the following two candidate sites for substrate binding: 1) at one or more of the iron atoms that comprise the central waist region of the FeMo-cofactor; and 2) at the molybdenum atom (5). The results from studies with small organometallic clusters and from theoretical calculations on the FeMo-cofactor have provided support for both of these incompatible binding models (5–12). Recently, we used a combination of genetic and spectroscopic methods to locate an amino acid residue within the MoFe protein \( \alpha\)-subunit that controls access of substrates to the FeMo-cofactor (13–19). This residue, \( \alpha\text{-70Val} \), is positioned directly above a specific Fe-S face of the FeMo-cofactor comprised of iron atoms 2, 3, 6, and 7 (numbered from the x-ray structure) (Fig. 1) (2). Substitution of \( \alpha\text{-70} \) by alanine was found to allow access of larger alkyl substrates such as propyne (\( \text{HC}≡\text{C}-\text{CH}_2\text{OH} \)) and propargyl alcohol (\( \text{HC}≡\text{C}-\text{CH}_2\text{OH} \)), which otherwise are very poor substrates for the unaltered MoFe protein (15, 18). It was further demonstrated that a propargyl alcohol reduction intermediate can be trapped on the FeMo-cofactor in the substituted MoFe protein (15). Characterization of this trapped intermediate has led to a detailed molecular description of the catalytic adduct bound to a specific iron atom of one Fe-S face of the FeMo-cofactor (15–17).

It remained, however, to establish whether or not this same site is relevant to the binding of \( \text{N}_2 \), the physiological substrate. Here, we report the consequences of substitution of \( \alpha\text{-70Val} \) by amino acids having smaller (alanine) or larger (isoleucine) side chains on the reduction of \( \text{N}_2 \), its semi-reduced form hydrazine, and the non-physiological alkyl substrate acetylene.

**EXPERIMENTAL PROCEDURES**

**Materials and Protein Purification**—All reagents, unless specified otherwise, were obtained from Sigma-Aldrich and were used as received. Hydrazine was obtained as hydrazine dihydrate. *Azotobacter vinelandii* strains DJ995 (wild-type \( \alpha\text{-70Val} \)), DJ1373 (\( \alpha\text{-70Ala} \)), and DJ1310 (\( \alpha\text{-70Val} \)) were grown and nitrogenase proteins were expressed as described previously (13, 14, 20–22). The \( \alpha\text{-70Val} \) (wild-type), \( \alpha\text{-70Ala} \), and \( \alpha\text{-70Val} \) MoFe proteins, each containing a seven-His addition on the \( \alpha\)-subunit, were purified by a zinc affinity purification protocol as described earlier (20). All proteins used were >95% pure as judged by analysis using SDS-PAGE with Coomassie staining. Manipulation of proteins was done in septum-sealed sealed vials under an argon atmosphere, and all gas and liquid transfers used gas-tight syringes.

**Dinitrogen, Acetylene, and Proton Reduction Assays**—Activity assays for dinitrogen reduction were performed in 1-mL liquid volumes in vials with 9 mL of total volume using established protocols (13, 20) for 10 min at 30 °C in assay buffer with a MgATP regenerating system (5 mM ATP, 30 mM phosphocreatine, 100 mM MOPS, pH 7.0, 1.2 mg/ml bovine serum albumin, 0.2 mg/ml creatine phosphokinase, 6 mM MgCl\(_2\), and 9 mM dithionite). Unless stated otherwise, reactions utilized 50 \( \mu \)g of MoFe protein, were initiated by the addition of 500 \( \mu \)g of Fe protein, and were quenched by the addition of 300 \( \mu \)L of 400 mM EDTA. Ammonia was quantified using a liquid chromatographic-fluorescence protocol (24) with some modifications. A 30-\( \mu \)L aliquot of post-reaction solution containing NH\(_3\) was added to 1 mL of a detection solution containing 20 mM phthalic dicarboxaldehyde, 3.5 mM 2-mercaptoethanol, 5% (v/v) ethanol, and 200 mM potassium phosphate, pH 7.3, and allowed to react in the dark for 30 min. A portion of this mixture was then separated by

\[ \text{N}_2 + 8\text{e}^- + 16\text{MgATP} + 8\text{H}^+ \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{MgADP} + 16\text{P}, \]

1 The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; TAPS, 3-(2-hydroxy-1,1-bis(hydroxymethyl)ethylamino)-1-propanesulfonic acid.
high performance liquid chromatography on a C-18 guard column running isocratically, utilizing 50% acetonitrile in 10 mM phosphate buffer at pH 7.3 flowing at 1.8 ml/min. Detection was by fluorescence (λ excitation of 410 nm and λ emission of 472 nm). An NH₃ standard curve was generated using NH₄Cl. Kinetic constants (V max and K m) were determined by non-linear least squares fitting of the data to the Michaelis-Menten equation using the computer program IgorPro.

Acetylene reduction to ethylene (C₂H₄) was determined using the assay mixture described above and the protocols for analysis described previously (25), whereas proton reduction was done as for acetylene, except that they were performed under an argon atmosphere and analyzed using a molecular sieve 5A column and a thermal conductivity detector to quantify hydrogen gas (23).

Hydrazine Reduction Assays—Rates for ammonia formation from the reduction of hydrazine were determined using the fluorescence technique described above for dinitrogen reduction with modifications to lower the interferences from the hydrazine. Assays were run for 15 min with increased concentrations of MoFe protein (200 μg/ml) in a multiple buffer system containing MOPS (75 mM) and TAPS (75 mM). Only 5 μl of the post-reaction solution containing NH₃ was added to 1 ml of the detection solution. The pH of buffer solutions was adjusted following each addition of hydrazine (either to pH 7 or to pH 8). The K m values obtained for wild-type (α-70Val) agree well with those obtained previously by Davis (26).

Dinitrogen Inhibition of Hydrazine Reduction—Rates for hydrazine reduction were monitored as the loss of hydrazine with quantification by the colorimetric assay described previously (27). Dinitrogen inhibition of hydrazine reduction was measured for five starting concentrations of hydrazine (1, 2, 3, 4.5, and 6 mM) under increasing partial pressures of N₂ (0, 0.25, 0.5, 0.75 and 1 atm), with the remaining gas being argon. Reactions were run in a modified assay solution with a MgATP regeneration system (5 mM ATP, 30 mM phosphocreatine, 200 mM MOPS, pH 7.8, 1.2 mg/ml bovine serum albumin, 0.2 mg/ml creatine phosphokinase, 6 mM MgCl₂, and 12 mM dithionite). A total of 200 μg of MoFe protein was used, and the reactions were initiated by the addition of 500 μg of Fe protein and quenched after 10 min by the addition of 300 μl of 400 mM EDTA. Hydrazine was quantified in the quenched samples by adding a portion of the quenched assay solution to 1 ml of hydrazine assay reagent (70 mM dimethylaminobenzaldehyde and 1 μM HCl in ethanol). The solution was mixed and the absorbance determined at 458 nm against a reagent blank. The inverse of the initial rate was plotted against the inhibitor concentration (N₂) in a Dixon plot.

RESULTS AND DISCUSSION

Dinitrogen and Alkyn Reduction Site—Previous studies indicated that propargyl alcohol can access the nitrogenase active site and be trapped there when the MoFe protein α-70Val residue is substituted by alanine. The α-70Val side chain approaches a specific Fe-S face of the FeMo cofactor comprising iron atoms 2, 3, 6, and 7 (Fig. 1) (13–18). It was also shown that the trapped intermediate is a semi-reduced metalloacyclopropene species, most likely bound to iron atom 6 (16, 17). Questions that emerged from this analysis included whether or not other substrates such as acetylene and N₂ are also reduced at this Fe-S face, and, if so, whether this is the only site that is available for the binding of these substrates. These questions are important ones, because non-reciprocity in the mutual inhibition patterns for acetylene and N₂ reduction, as well as kinetic parameters for acetylene reduction, has been interpreted to indicate that there could be multiple and separate substrate binding sites and/or that substrates are able to access a variety of different redox states (28–30).

Here, experiments are presented that aim to establish the location for the binding of N₂, its semi-reduced form hydrazine, and acetylene. If N₂ and alkynes access the same Fe-S face of the FeMo cofactor composed of iron atoms 2, 3, 6, and 7, then substituting an amino acid with a larger side chain (e.g., isoleucine) for α-70Val would be predicted to interfere with the binding and/or reduction of both N₂ and acetylene (Fig. 1). The wild-type MoFe protein, when combined with the Fe protein, MgATP, and an electron source (e.g. dithionite), catalyzes the reduction of N₂ to 2NH₃ with a specific activity of ~600 nmol of NH₃ per minute per milligram of MoFe protein and a K m for N₂ reduction of 0.1 atm (Table I). Under these conditions, ~30% of the electrons delivered to the FeMo cofactor for the reduction of substrates (electron flux) by nitrogenase are utilized to reduce protons to H₂. In the absence of N₂ (under 1 atm of argon), all of the electron flux is used to reduce protons with an overall specific activity of ~2000 nmol of H₂ per minute per milligram of MoFe protein. This latter reaction provides a convenient control to monitor the catalytic integrity of nitrogenase. When valine at position α-70 is substituted by isoleucine, the resulting MoFe protein retains normal electron flux (~2300 nmol of H₂ per minute per milligram) as measured by proton reduction activity (Table I). However, the ability to reduce N₂ is significantly diminished in the α-70Ile MoFe protein, with the specific activity being lowered by 72% (at 1 atm of N₂) as compared with the specific activity for the wild-type MoFe protein (Table I).

| MoFe protein | Argon specific activity a | Dinitrogen | Acetylene | K m | Acetylene |
|--------------|--------------------------|------------|-----------|-----|----------|
| α-70Val (wild-type) | 2092 ± 20 | 640 ± 20 | 600 ± 30 | 0.1 | 200 ± 20 | 1800 ± 25 | 0.005 |
| α-70Ile | 2340 ± 35 | 1940 ± 50 | 170 ± 20 | >1.5 | 2000 ± 60 | 130 ± 10 | >0.6 |

a With nanomoles of H₂.
b With nanomoles of NH₃.
c With nanomoles of C₂H₂.
I and Fig. 2). In addition, the $K_m$ for $N_2$ reduction was dramatically increased by $>15$-fold (Table I).

The $a$-$70^{\text{Val}}$ MoFe protein also showed a $-14$-fold decrease in the specific activity for acetylene reduction and a $>100$-fold increase in $K_m$ when compared with the wild-type MoFe protein (Table I). These results are consistent with the larger side chain of isoleucine preventing access to the iron 2, 3, 6, and 7 face for both $N_2$ and acetylene interaction but not interfering with the reduction of protons or electron flux through nitrogenase. These results indicate that $N_2$ and acetylene access the same Fe-S face (comprising iron atoms 2, 3, 6, and 7) of the FeMo-cofactor.

Hydrazine Reduction Site—Hydrazine, a semi-reduced form of dinitrogen, is also a substrate for nitrogenase, being reduced by two electrons and two protons to yield $2\text{NH}_3$ (26). Furthermore, hydrazine has been detected when a nitrogenase reaction mixture is chemically quenched during $N_2$ reduction, which has been interpreted as evidence that a metal-bound dinitrogen-hydride intermediate is likely to exist during $N_2$ reduction (27).

If the low rate of hydrazine reduction for the wild-type MoFe protein is the result of poor access to the active site and if hydrazine is reduced at the same Fe-S face of the FeMo-cofactor as the $N_2$ and alkynie substrates, its reduction should be improved by the substitution of $a$-$70^{\text{Val}}$ by alanine and diminished by the substitution of $a$-$70^{\text{Val}}$ by isoleucine (Fig. 1). These predictions were verified experimentally. For example, substitution of $a$-$70^{\text{Val}}$ by alanine significantly improves the ability of the MoFe protein to reduce hydrazine with an approximate 2-fold increase of the $V_{\text{max}}$ and an 8-fold decrease in the $K_m$ at both pH 7 and 8 (Fig. 3 and Table II). The differences in activity for hydrazine at different pH values probably reflect the differences in affinity for the hydrazine protonated state. As expected, $N_2$ was found to be an inhibitor of hydrazine reduction by the $a$-$70^{\text{Ala}}$ MoFe protein.

We probed the reduction of hydrazine by nitrogenase MoFe proteins having amino acid substitutions at $a$-$70$ as a way to further establish that this Fe-S face of the FeMo-cofactor is required for the reduction of nitrogenous substrates. For the wild-type MoFe protein, hydrazine is a relatively poor substrate with a $K_m$ of 26 mM at pH 8 and 125 mM at pH 7 (Table II) as compared with the $K_m$ for $N_2$ of 0.05 atm (30 $\mu$M) at 30 °C. The reduction activity for hydrazine by the wild-type MoFe protein is lower than the reduction activity observed for $N_2$ (Table II).

Mechanistic Implications and Conclusions—In this work we demonstrate that the size of the amino acid side chain at
position α-70 in the MoFe protein, which is located directly over a specific Fe-S face of the FeMo cofactor, controls the ability of nitrogenase to reduce N₂, acetylene, and hydrazine. The substitution of isoleucine at this position blocks the reduction of acetylene, N₂, and its semi-reduced form hydrazine, whereas the substitution of alanine has the opposite effect, increasing the reactivity of nitrogenase toward hydrazine and allowing the reduction of short chain alkynes larger than acetylene (18). These and other results (23, 33), support a model in which alkyne substrates, N₂, and hydrazine all interact with a common Fe-S face composed of iron atoms 2, 3, 6, and 7. Although these results indicate that this Fe-S face provides the metal-sulfur surface for substrate binding, they do not exclude the possibility for multiple sites within this face, different binding configurations for different substrates, or different binding configurations at different redox states.

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