Analysis of *Azotobacter vinelandii* Strains Containing Defined Deletions in the *nifD* and *nifK* Genes

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Strains of *Azotobacter vinelandii* which contain defined deletions within the *nifD* and *nifK* genes which encode, respectively, the α and β subunits of the MoFe protein of nitrogenase were analyzed. When synthesized without its partner, the β subunit accumulated as a soluble β_4 tetramer. In contrast, when the α subunit was present without its partner, it accumulated primarily as an insoluble aggregate. The solubility of this protein was increased by the presence of a form of the β subunit which contained a large internal deletion, such that the α subunit could participate in the assembly of small amounts of an $\alpha_2\beta_2$ holoprotein. When synthesized alone, the β subunit was remarkably stable, even when the protein contained a large internal deletion. The α subunit, however, was much more rapidly degraded than the β subunit, both when it was synthesized alone in its native background and when it was synthesized with its β subunit partner in a foreign background. Antibodies raised against purified $\alpha_2\beta_2$ MoFe protein recognized epitopes only on the nondenatured β subunit and not on the nondenatured α subunit. Our findings that all epitopes for the $\alpha_2\beta_2$ tetramer appeared to be on the β subunit, that the β subunit assembled into β_4 tetramers, and that the α subunit alone was very insoluble, combined with the previous finding that the Fe protein binds to the β subunit (A. H. Willing, M. M. Georgiadis, D. C. Rees, and J. B. Howard, *J. Biol. Chem.* 264:8499–8503, 1989) all suggest that the β subunit has a more surface location than the α subunit in the $\alpha_2\beta_2$ tetramer.

Nitrogenase catalyzes the biological reduction of N_2 to NH_3 . Molybdenum nitrogenases from diverse procaryotes are composed of two separately purified proteins called the iron protein (Fe protein), and the molybdenum-iron protein (MoFe protein), both of which are required for substrate reduction (28, 34, 36). The Fe protein from one organism can complex with the MoFe protein from another to yield an active enzyme (13, 14).

The MoFe proteins are $\alpha_2\beta_2$ tetramers with molecular weights of 210,000 to 250,000. The α and β subunits encoded by the *nifD* and *nifK* genes, respectively, are similar in size (29, 40). The arrangement of the subunits is unknown, but information from neutron small-angle scattering (26), electron microscopy (35, 39), and X-ray diffraction studies (43) points toward an aspherical, probably pseudotetrahedral particle with twofold molecular symmetry. Current models suggest six metal centers within the $\alpha_2\beta_2$ tetramer (25, 28, 34, 36), two of which are FeMo cofactor clusters. The FeMo cofactor, at the site of substrate binding and reduction (18), contains about six iron atoms, eight sulfur atoms, and one molybdenum atom arranged in a novel spin-coupled cluster (28, 34, 36) with one molecule of homocitrate (19). The other four metal centers appear to be [4Fe-4S]-type clusters in an unusual (zero) oxidation state (22, 25) with some noncysteine ligation (11, 22, 25, 40); they are not all identical and appear to occupy two different environments (25).

The relationships of the metal centers of the MoFe protein to each other and to the polypeptides are unknown, but much information has been deduced from sequence comparisons (40) and site-directed mutagenesis studies (11). Amino acid sequence information is now available for the α and β

subunits of the MoFe proteins from several organisms. Comparisons show that the α subunit (*nifD*) contains 5 conserved cysteine residues while the β subunit contains 3 (23, 40), giving 16 for the holoprotein. Site-directed mutagenesis has shown that these residues are important for enzyme activity (11). The α and β subunits show sequence similarity in the region of three of the conserved α subunit cysteine residues (e.g., see references 11, 37, and 40) which have been proposed as ligands to the [4Fe-4S]-type clusters (11, 25). The remaining two conserved cysteine residues in the α subunit are proposed to be involved in FeMo cofactor ligation (3, 11). The location of the clusters relative to the surface of the protein is not known, although the FeMo cofactor has been proposed to be buried (28, 31) while the [4Fe-4S]-type clusters may be located near the surface or at subunit interfaces (40). The clusters could also be bridged between subunits, as in the Fe protein (17).

In recent years, two general types of genetic-biochemical approaches have been used in attempts to understand the structural organization and function of the nitrogenase polypeptides and their metal centers. One involves transfer of *nif* genes to the non- N_2 -fixing bacterium *Escherichia coli* (20, 34), while a complementary approach involves construction of defined deletions within the *nif* structural gene region of an N_2 -fixing organism (30). Here we report the analysis of strains of *Azotobacter vinelandii* which contain defined deletions in the *nifD* and *nifK* genes which encode the α and β subunits of the MoFe protein of nitrogenase.

MATERIALS AND METHODS

Materials. The *A. vinelandii* Fe protein and MoFe proteins were purified and analyzed as described elsewhere (7) to give specific activities of ca. 1,900 and 2,500 nmol of H_2 evolved per min per mg of protein, respectively. Antibodies to MoFe protein that had been recrystallized (AbRC) were produced at the University of California, Irvine (32). Antibodies to crystallized MoFe protein (AbC) were raised in New

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Zealand White rabbits at Bethyl Laboratories (Montgomery, Tex.). The immunoglobulin G fraction of the sera was purified by ammonium sulfate precipitation by a published procedure (15). Acrylamide and sodium dodecyl sulfate (SDS) were from Bio-Rad Laboratories (Richmond, Calif.). Antibiotics were from Sigma Chemical Co. (St. Louis, Mo.). DEAE-cellulose 52 was from Whatman, Inc. (Clifton, N.J.). HA-Ultragel was from IBF Biotechnics (Villeneuve-la-Garenne, France). Sephacryl-S200HR, QAE-Sephacryl, and Octyl-Sepharose were from Pharmacia, Inc. (Piscataway, N.J.). ATP, creatine phosphate, creatine phosphokinase, TES [N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], and Tris were from Sigma. $\text{Na}_2\text{S}_2\text{O}_4$ was from EM Science (Cherry Hill, N.J.).

Strains. *A. vinelandii* $\Delta nifK$ -carrying strain CA13 was previously constructed (1) and partially characterized (30). The construction of $\Delta nifD$ -carrying strain DJ100 is described elsewhere (30). To construct *A. vinelandii* DJ75, a previously constructed hybrid plasmid, pDB6 (32), which contains the *A. vinelandii nifHDKTY'* genes, was cleaved with *SphI*, which has a restriction site located at amino acid residue 242 within the *nifK* gene and at amino acid residue 95 within the *nifY* gene (21). The resulting *SphI* fragment was replaced with an *SphI* fragment from pUC4-KISS (Pharmacia) which carries a Kan^r-encoding gene. The resulting plasmid, pDB113, was isolated from *E. coli* and used to transform competent cells of *A. vinelandii* as described previously (2). Double-crossover events (30) resulted in transfer of the *nif* deletion-Kan^r-encoding gene insertion contained within the plasmid to the homologous region on the host chromosome with subsequent loss of the plasmid vector. A double-crossover recombinant which was Kan^r and had a nonrevertible Nif⁻ phenotype was designated DJ75 and retained for further study. All *A. vinelandii* strains were grown and derepressed for nitrogenase synthesis as described elsewhere (30, 32).

To express the MoFe protein polypeptides in *E. coli*, a plasmid designated pDB29 was constructed. For this purpose, a 4.5-kilobase (kb) *Bgl*II fragment of *A. vinelandii nif* DNA, with part of the *nifH* gene and all of the *nifDK* genes (isolated from pDB1), was cloned into the *Bam*HI site of pGW7 (12) downstream of the lambda p_L promoter. This placed expression of the *nifDK* genes under control of the lambda p_L promoter. Because pGW7 contains the temperature-sensitive *cI857* repressor gene, genes controlled by the lambda p_L promoter are expressed only at high temperature. This plasmid was transformed into *E. coli* TB1 to yield strain TB1(pDB29). For a typical experiment, a single colony of TB1(pDB29) was picked and grown aerobically overnight in Luria broth (24) plus 100 μg of ampicillin per ml at 30°C. For aerobic growth, a 0.5-ml sample of this culture was used to inoculate 50 ml of Luria broth plus ampicillin medium in a 250-ml flask. That culture was grown aerobically with shaking (200 rpm) at 30°C until the culture reached the mid-log phase (ca. 4 h). For anaerobic growth, a 1-ml sample of the overnight culture was used to inoculate 50 ml of degassed Luria broth plus ampicillin in a 250-ml flask under argon. When either the aerobic or anaerobic culture reached the mid-log phase (120 Klett units with a 540-nm filter), an equal volume of fresh 65°C medium (degassed when appropriate) was added to bring the culture to 42°C, the temperature required to derepress the lambda p_L promoter (27). The cells were then grown either aerobically or anaerobically, and at the time intervals indicated in Fig. 10 they were degassed and collected anaerobically by centrifugation at $8,000 \times g$ for 10 min.

Extract preparation and purification of protein Z. Cell extracts of *A. vinelandii* OP (wild type), CA13, and DJ75 were prepared anaerobically as described previously (32). For reconstitution experiments involving DJ100, extracts were also prepared by using the same method (31). However, for protein purification with DJ100 extracts, 130 g of cells was first suspended in 1.4 volumes of 50% ethylene glycol–50% 0.05 M Tris hydrochloride (pH 8.0) which was 2 mM in $\text{Na}_2\text{S}_2\text{O}_4$. The suspended cells were then ruptured and centrifuged as previously described (31). This extract was loaded onto a DEAE-cellulose column (5 by 35 cm) pre-equilibrated with 0.025 M Tris hydrochloride (pH 7.4) containing 1 mM $\text{Na}_2\text{S}_2\text{O}_4$ (buffer A). After the column was washed with 1 liter of buffer A containing 0.08 M NaCl, it was developed with a 2.4-liter linear gradient from 0.08 to 0.5 M NaCl. The protein was monitored by A_{405} , and the first major brown peak, which eluted at 0.25 M NaCl, was collected. This protein was diluted 1:2 with buffer A and loaded onto a second DEAE-cellulose column (2.5 by 30 cm). After being washed with 0.08 M NaCl, the column was developed with a 0.08 to 0.36 M NaCl linear gradient. The brown peak eluted at 0.25 M was collected and concentrated on a DEAE-cellulose column (7), and ca. 200-mg (5-ml) samples were run through a Sephacryl S-200 gel filtration column (2.5 by 90 cm) pre-equilibrated with buffer A containing 0.1 M NaCl. The first brown peak which eluted in the void volume was collected and loaded onto a QAE-Sephacryl column (1.5 by 5 cm) which was washed with buffer A and developed with a 0.08 to 0.5 M NaCl gradient. A peak of protein which appeared homogeneous by the criterion of SDS-gel electrophoresis was eluted at 0.25 M NaCl and designated protein Z. Similar results were obtained when the final Q-Sepharose column was run on a smaller scale by fast-protein liquid chromatography (Pharmacia).

For analysis of the α subunit from DJ75, cells were suspended and ruptured as previously described (31) and centrifuged at $8,000 \times g$ for 30 min and the supernatant was used for all of the gels shown. To obtain an elution profile, this extract was loaded onto a DEAE-cellulose column (5 by 35 cm) pre-equilibrated with buffer A and washed with 2 liters of buffer A. Because the α subunit did not stick to DEAE-cellulose, the wash effluent was collected anaerobically and centrifuged at $112,000 \times g$ for 25 min. The supernatant was discarded, and the pellet was suspended in 100 ml of buffer A and shown to contain the α subunit by SDS-gel electrophoresis analysis.

Protein analysis. Published procedures were used for one-dimensional polyacrylamide gel electrophoresis (32), Western immunoblotting (32), native anaerobic gel electrophoresis (31), two-dimensional gel electrophoresis (32), reconstitution assays (30), iron analysis (7, 8), heme staining (16, 38), and cyanogen bromide cleavage of gel-purified proteins (44). N-terminal amino acid sequencing was performed by an Applied Biosystems 478 Sequenator with 120 on-line high-pressure liquid chromatography for identification of PTH (phenylthiohydantoin) amino acids. Visible spectra were recorded by using a Varian series 639 spectrophotometer.

RESULTS

We have previously reported the initial characterization of two Nif⁻ strains of *A. vinelandii* (30). Strain DJ100 contains a defined in-phase deletion in the *nifD* gene which removes the region that encodes α subunit amino acids 104 to 376. It accumulates active Fe protein, FeMo cofactor, and wild-type levels of the β subunit but does not contain the α

FIG. 1. Reconstitution of MoFe protein activity as a function of the ratio of CA13 to DJ100. The indicated volumes of CA13 cell extracts (23.2 mg/ml) were mixed anaerobically with 0.1 ml of DJ100 extract (31 mg/ml) under argon, and the mixtures were incubated for 20 min at room temperature. Variable amounts of the mixed extracts, all containing 3.1 mg of DJ100 extract, were then used to initiate a C_2H_2 reduction assay which was quenched with acid after shaking for 20 min at 30°C (30).

subunit of the MoFe protein (30). Isogenic strain CA13, which contains a deletion in the DNA that encodes β subunit amino acids 136 to 293, accumulates active Fe protein, FeMo cofactor, and the MoFe protein α subunit but does not contain the β subunit (30). Although neither DJ100 nor CA13 had significant MoFe protein activity alone, a small amount of an active holoprotein was assembled in vitro by simply mixing cell extracts of the two strains (Fig. 1; 30). The reconstitution was greatly enhanced by proportionately increasing the amount of CA13 extract relative to that of DJ100 extract. The sigmoidal kinetics indicated that formation of the active holoprotein may take place in a two-step process, for example, by formation of inactive $\alpha\beta$ dimers before formation of the active tetramer. The specific activity shown in Fig. 1 was only 5% of the expected maximum activity. However, addition of excess isolated FeMo cofactor, iron, inorganic sulfide, and/or $[Fe_4S_4(SPh)_4](Et_4N)_2$ or variation of other reaction conditions (i.e., protein concentration, pH, and temperature) resulted in no increase in activity.

Reaction with antibodies. AbRC cross-reacted strongly in an Ouchterlony assay with wild-type and DJ100 extracts, which contain only the β subunit, but they did not cross-react with control strain DJ33, which is deleted for both *nifD* and *nifK* (Fig. 2). Surprisingly, the antibody also did not cross-react with CA13 extracts (Fig. 2), which were shown to contain the α subunit by Coomassie brilliant blue-stained two-dimensional gel electrophoresis (30). However, when purified MoFe protein or the proteins in the wild-type, DJ100, and CA13 extracts were first denatured with SDS, all cross-reacted with the antibody (Fig. 3). The MoFe protein showed two cross-reacting bands corresponding to the α and β subunits, DJ100 showed a single band in the position of the β subunit, and CA13 showed a strongly cross-reacting band in the position of the α subunit.

Surprisingly, two bands which cross-reacted with AbRC

FIG. 2. Ouchterlony double-diffusion assay showing cross-reaction of *A. vinelandii* wild-type (WT) and mutant extracts with AbRC. The gel was 0.8% agarose in 0.01 M Tris hydrochloride (pH 7.4). The center well contained 50 μ l of undiluted AbRC serum, while the outer wells contained 100 μ l of cell extracts (ca. 30 mg/ml) from the designated mutant strains. The gel was incubated aerobically at 37°C overnight, followed by staining with Coomassie brilliant blue.

were seen for CA13 extracts, one in the α subunit position and one corresponding to a protein with an M_r of ca. 36,000. The CA13 in-phase deletion in the *nifK* gene resulted in removal of 157 amino acids and fusion of β subunit amino acid 135 to β subunit amino acid 294 to yield an internally deleted β subunit protein with an M_r of 36,000. Thus, the second cross-reacting band appears to be the internally deleted β subunit protein. It is possible that DJ100 similarly carries a 219-amino-acid residue internally deleted α subunit. However, no cross-reacting protein, or new Coomassie brilliant blue-stained protein, of that size was detected on SDS-gel electrophoresis of DJ100 extracts.

Construction and characterization of $\Delta nifK$ strain DJ75. Because CA13 appears to contain an internally deleted form of the β subunit, a different $\Delta nifK$ deletion strain, DJ75, in which the *nifK* gene was truncated at a codon corresponding to amino acid 242 of the β subunit, was constructed (see Materials and Methods). Denatured extracts from derepressed DJ75 cells showed only one cross-reacting band in the α subunit position on Western blots (Fig. 4) and no new band on stained gels (data not shown). DJ75 extracts had levels of Fe protein and FeMo cofactor comparable to those previously reported for CA13 (30). However, unlike CA13 (Fig. 1), when extracts of DJ75 were mixed with extracts of DJ100, no active holoprotein was assembled.

Analysis of the β subunit from DJ100. Purification of the wild-type MoFe protein involved heating a cell extract to 56°C, followed by centrifugation to give supernatant solutions containing 100% of the MoFe protein activity (7). When derepressed DJ100 extracts were similarly heated, however, all of the β subunit was found in the pellet, causing

FIG. 3. One-dimensional SDS-gel electrophoresis separation of purified MoFe protein (MF) and cell extracts from mutant strains CA13 and DJ100 after Western blotting and reaction with AbRC. The gel was 7.5% acrylamide and contained 0.3 μ g of MoFe protein or 14 μ g of extract protein per lane. Comparison to molecular weight standards (not shown) indicated that the lower band in the CA13 extracts had an M_r of ca. 36,000. This band was also easily seen on stained gels (data not shown).

us to omit the heat step and go directly to centrifugation at $148,000 \times g$ for 1 h (7). Under these conditions, when cells were ruptured in Tris hydrochloride buffer with or without 0.5 M NaCl, the β subunit appeared to be equally distributed between the supernatant and the pellet. The relative amount of the β subunit in the supernatant was increased to an estimated 80% by rupturing in a mixture of 50% ethylene glycol–50% Tris hydrochloride.

Next, we wanted to establish whether the β subunit in

FIG. 4. One-dimensional SDS-gel electrophoresis separation of purified MoFe protein (MF) and cell extracts from mutant strain DJ75 after Western blotting and reaction with AbRC. The gel was 7.5% acrylamide and contained 0.3 μ g of MoFe protein or 14 μ g of extract protein per lane.

FIG. 5. Native anaerobic gel electrophoresis of wild-type (WT) and DJ100 cell extracts after blotting and reaction with AbRC. For the extracts shown, the cells were ruptured in Tris hydrochloride buffer, but the same result was obtained for cells ruptured in 50% ethylene glycol–50% Tris hydrochloride. The gels were run as described previously (31). The gel was 7.5% acrylamide and contained 14 μ g of extract per lane. The purified Fe protein of nitrogenase, which has a native molecular weight of ca. 64,000 (less than the 59,438 of the β subunit monomer), ran off the bottom of the gel.

DJ100 was present as a monomer or as a larger aggregate. The possible presence of a β_4 tetramer was indicated by the Ouchterlony assay results shown in Fig. 2, in which the diffusion distances for the wild-type MoFe protein and the β subunit in DJ100 extracts were the same, showing that the two species were similar in size. This was confirmed by the data in Fig. 5, which shows the results of native anaerobic gel electrophoresis separation of wild-type and DJ100 cell extracts. The β subunit ran as the smaller of the two subunits on denaturing gels (Fig. 3), and the only band that cross-reacted with AbRC on native gels of DJ100 extracts (Fig. 5) was at a position consistent with that expected for a β_4 tetramer.

The properties of this soluble β_4 tetramer and native MoFe protein were further compared by partial purification. Figure 6 is an elution profile showing the separation of wild-type and DJ100 extracts on DEAE-cellulose with monitoring of the A_{405} . In wild-type extracts, the first brown peak contained the MoFe protein, the second contained the Fe protein, and the third contained *A. vinelandii* ferredoxin I (7). Since DJ100 makes only the β subunit of the MoFe protein, the presence of the first large peak in DJ100 extracts (Fig. 6) was initially surprising. As expected, however, peak 2 was shown to contain the Fe protein by activity assays and peak 3 was shown to contain ferredoxin I by Western analysis using anti-ferredoxin I antibodies. When dot blots for all DJ100 fractions were reacted with AbRC, only the fractions found in peak 1 reacted (Fig. 6). There was also no cross-reaction with any proteins that did not initially stick to DEAE-cellulose. When this procedure was repeated numerous times, both the antibody reaction and the size of peak 1 varied dramatically, with the latter ranging from that shown in Fig. 7 to about 75% of the size of the Fe protein peak. Very small amounts of an active MoFe protein holoprotein were reconstituted by addition of this fraction to CA13 cell extracts, confirming the presence of the β subunit. Thus, the behavior of the β_4 tetramer on DEAE-cellulose was similar to that of native MoFe protein.

FIG. 6. Elution profiles for wild-type (WT) and DJ100 cell extracts separated on DEAE-cellulose monitored by A_{405} and dot blotting and reaction with AbC. Extract preparation and column conditions are described in Materials and Methods. The A_{405} pre-peak in the DJ100 profile was absent for wild-type extracts because, unlike the DJ100 extracts, the wild-type extracts were heat treated. This peak is unrelated to the mutation in DJ100. The numbers 1 to 7 for the DJ100 extracts represent fractions which were dot blotted (150 μ g of protein per dot) and reacted with AbC.

Further purification of the β_4 tetramer was, unfortunately, frustrated by the presence of a contaminating protein which was present in the same DJ100 DEAE-cellulose fraction that contained the β_4 tetramer. This fraction was further purified as described in Materials and Methods to yield a light brown protein, protein Z, which appeared to be homogeneous by the criterion of Coomassie brilliant blue-stained SDS-gel electrophoresis (Fig. 7). This protein had the same apparent subunit molecular weight as the β subunit, and its behavior on gel filtration columns was also consistent with its being at least a tetramer. This protein did not, however, yield any active MoFe holoprotein when mixed with CA13 cell extracts.

Because the protein sample shown in Fig. 7 cross-reacted with AbC (Fig. 7), it was further analyzed by N-terminal

FIG. 7. Copurification of protein Z and the β subunit. One-dimensional SDS-gel electrophoresis of protein Z purified from DJ100 extracts and MoFe protein (MF) stained with Coomassie brilliant blue after blotting and reaction with AbC. Protein Z was purified as described in Materials and Methods by using fast-protein liquid chromatography for the final Q-Sepharose column. The gel was 7.5% acrylamide and contained 0.3 μ g of MoFe and 14 μ g of extract. Note that antibody reaction with protein Z was due to a small amount of the β subunit which was also present in the sample. Protein Z from DJ75 did not react with AbRC (e.g., Fig. 4).

FIG. 8. Elution profile of DJ75 cell extracts separated on DEAE-cellulose and monitored by A_{405} . The conditions were as described in Materials and Methods. FdI, Ferredoxin I.

amino acid sequencing. Although the fraction contained a minor amount of the β subunit (accounting for the antibody reaction), the major protein present had the sequence NH_2 -Ala-Tyr-Tyr-Gln-Met-Ala-Phe-Asp, which was not found anywhere in the predicted sequence of the β subunit (2). This result led us to examine further the properties of the protein shown in Fig. 7. Characterization included the following. (i) The protein shown in Fig. 7 and the authentic β subunit, cut out of SDS-gels of purified MoFe protein, were subjected to cyanogen bromide cleavage. This yielded different peptide patterns (data not shown). (ii) The isoelectric point of the major protein species in Fig. 7 was shown to be lower than that of the authentic β subunit by two-dimensional gel electrophoresis (data not shown). (iii) A protein running in the same position, which did not react with AbRC, was purified from DJ75, our $\Delta nifK$ deletion strain, which does not synthesize the β subunit of the MoFe protein. Attempts to separate the β_4 tetramer from contaminating protein Z at later stages of DJ100 purification resulted only in purification of protein Z or mixtures of the two proteins.

Properties of protein Z. Because DJ100 contains both the β subunit and protein Z and because the two copurify, we purified protein Z from DJ75. Figure 8 is the DEAE profile showing three brown peaks corresponding to protein Z, Fe protein, and ferredoxin I. Unlike the DJ100 profile, which has a first peak of varying size, the DJ75 profile consistently showed a first peak smaller than the Fe protein peak. Thus, peak 1 from DJ100 appeared to contain a mixture of the β_4 tetramer and protein Z, whereas the same peak from DJ75 contained only protein Z. (The α subunit in DJ75 does not stick to DEAE-cellulose, as discussed below). This observation is interesting, because the A_{405} peak size is dependent to a large extent upon the presence of iron in a protein so that the larger size of peak 1 in DJ100 extracts is consistent with metalation of the β_4 tetramer.

The visible spectrum of protein Z, as purified to homogeneity from DJ75 extracts, in its dithionite-reduced state is shown in Fig. 9. This spectrum is consistent with the idea that protein Z is a heme-containing, cytochrome-type protein (e.g., see reference 55). Interestingly, very early reports of the crystallization of the MoFe protein from *A. vinelandii* showed an extremely similar spectrum which was attributed to the MoFe protein (9, 10). This spectrum was not obtained

FIG. 9. Visible spectrum of protein Z in the presence of excess $\text{Na}_2\text{S}_2\text{O}_4$ purified from DJ75 cells. The purification procedure was the same as that used to purify protein Z from DJ100 as described in Materials and Methods.

by others (5, 33), and the controversy concerning its origin was never resolved (4, 6). We did not observe the features of this spectrum for our crystallized MoFe protein. Iron analysis of preparations of protein Z gave ca. 1 Fe atom per 59,000 M_r subunit. Although these data are consistent with the notion that the protein contains one heme per subunit, this protein did not react with a heme stain (16, 38) on denaturing gels.

Stability and solubility of the α subunit. *E. coli* TB1 (pDB29) carries the *A. vinelandii nifDK* genes under control of the lambda p_L promoter (Materials and Methods). Figure 10 shows the synthesis of the α and β subunits in that *E. coli* strain, after induction of their expression from the lambda p_L promoter, under aerobic (top) and anaerobic (bottom) growth conditions. In both cases, there was much less of the α than the β subunit present, but the effect was much more dramatic for anaerobically grown cells. The different rates of

FIG. 10. One-dimensional SDS-gel electrophoresis of purified MoFe protein (MF) and ruptured cells of *E. coli* TB1(pDB29) after blotting and reaction with AbRc. The ruptured cells were not centrifuged. This strain was constructed, and *nifDK* expression was induced as described in Materials and Methods. The numbers represent times in hours following the temperature shift. For the top gel, the cells were grown aerobically, and for the bottom gel, they were grown anaerobically as described in Materials and Methods.

degradation under anaerobic and aerobic conditions might be due to the presence of different sets of proteases or an inherent difference between the MoFe proteins produced under the two sets of conditions.

In addition to the decreased stability of the α subunit, this subunit is also much less soluble than the β subunit. Thus, when DJ75 extracts were subjected to the 1-h $148,000 \times g$ centrifugation step, used successfully with DJ100 extracts, all of the α subunit remained in the pellet. Unlike that of the β subunit, the solubility of this protein could not be increased by rupturing the cells in 50% ethylene glycol. In fact, once pelleted, the α subunit remained insoluble in a variety of detergents, including 0.1% Triton X-100, 0.2 mg of Nonidet P-40 per ml, 0.1% sodium deoxycholate, and 1% octyl- β -D-glucopyranoside. Also because of its insolubility, the α subunit did not stick to ion-exchange or hydroxyapatite resins but rather passed through those columns, remaining as a cloudy solution.

DISCUSSION

Stability of the α and β subunits. To understand the structural organization and function of the MoFe protein, we are examining the properties of its α and β subunits when they are synthesized in the absence of one another. This was done by constructing *A. vinelandii* strains with defined deletions in their *nifDK* gene clusters (30). One such strain, DJ100, synthesizes only the β subunit. Analysis of that strain has shown that the levels of the β subunit accumulated in vivo were unaffected by the absence of the α subunit (30; Fig. 2 and 3). The data presented in Fig. 3 further show that even an internally deleted form of the β subunit, which lacked 157 amino acid residues, accumulated at wild-type levels in another strain, *A. vinelandii* CA13. Finally, Fig. 10 shows that the β subunit was also very stable when synthesized in a foreign *E. coli* background. Thus, in vivo, the β subunit is remarkably resistant to degradation.

When synthesized in the absence of its partner, the α subunit is much less stable than the β subunit. This is evidenced by (i) two-dimensional gel electrophoresis characterization (30), (ii) the activity data shown in Fig. 1, (iii) the absence of the internally deleted α subunit in DJ100 compared with the presence of the internally deleted β subunit in CA13 (Fig. 3), and (iv) the degradation of the α subunit in *E. coli* (Fig. 10). This difference in stability may be a general property of the α subunit, even in wild-type *A. vinelandii*. Thus, the DNA sequence of the *A. vinelandii nifHDK* operon shows a putative attenuator between *nifD* and *nifK* which leads to increased levels of *nifD*-specific mRNA (2) and increased initial synthesis of the α subunit relative to the β subunit. Since the two subunits are needed in equal quantities, these observations strongly suggest that the α subunit is turned over more rapidly than the β subunit.

Solubility of the α and β subunits. When synthesized without its partner, the β subunit in DJ100 is present as a soluble β_4 tetramer. This is evident in Fig. 2, which shows that the diffusion distances for wild-type MoFe protein and the β subunit in DJ100 extracts were the same, and in Fig. 5, which shows that the β subunit in DJ100 behaved as a β_4 tetramer on native anaerobic gel electrophoresis. There is known to be significant sequence identity between the α and β subunits of the MoFe protein (11, 37, 40), and if at least some of these conserved regions are located at the subunit interfaces, then formation of a β_4 tetramer should not be surprising. Although this β_4 tetramer has lowered stability toward heat, its behavior on DEAE-cellulose was remark-

ably similar to that of native MoFe protein (Fig. 6). The β_4 tetramer has no C_2H_2 reduction activity, but under the appropriate conditions it can disassemble and reassemble with its α subunit partner from CA13 to form an active holoprotein (Fig. 1). This result suggests, but certainly does not prove, that the β_4 tetramer synthesized by DJ100 is metalated, a view supported by comparison of the DEAE-cellulose elution problems for a strain that synthesizes only the β subunit (Fig. 6) with a strain that synthesizes only the α subunit (Fig. 8).

In addition to its decreased stability, the α subunit is also much less soluble than the β subunit, appearing as a high-molecular-weight aggregate when synthesized alone in DJ75. It is interesting that the aggregated α subunit from DJ75 was unable to assemble with the β subunit from DJ100 to form an active holoprotein, while the α subunit synthesized by strain CA13 was able to complement DJ100. The most likely explanation for this result is that the presence of the internally deleted β subunit in CA13 (Fig. 3) in some way facilitates assembly of the individual α and β subunits into an active holoprotein (Fig. 1), possibly by binding to the α subunit and increasing its solubility.

Contamination by protein Z. Purification of the β_4 tetramer synthesized by DJ100 has unfortunately been frustrated by the presence of a tenacious contaminant which we call protein Z. This protein ran with the β subunit on SDS-gel electrophoresis, was also present as a tetramer, and was copurified with the β_4 tetramer from DJ100. It is a ca. 59,000- M_r subunit protein which appears to contain one heme per monomer. *A. vinelandii* is known to contain numerous cytochromes (42), and a cytochrome oxidase has been suggested to be associated with N_2 fixation in another organism. However, the relationship, if any, of this cytochrome to nitrogenase is unknown. Regardless of its relevance to N_2 fixation, it is important to be aware of the possible presence of protein Z. This is especially true when characterizing altered forms of the MoFe protein from *A. vinelandii* which have not been purified by crystallization, the only method we found able to remove this contaminant from the native protein.

Relative locations of the two subunits. The data discussed above show that antibodies to the MoFe holoprotein recognized the β subunit in its native (Fig. 5) and O_2 -damaged (Fig. 2) forms, as well as in its SDS-denatured form (Fig. 3). However, the same antibodies recognized the α subunit only in its denatured form (Fig. 3 and 4) but not in its native form (data not shown) or O_2 -damaged form (Fig. 2). These data cannot be explained by the lack of solubility of the α subunit, because at least some of the α subunit in CA13 extracts was accessible for reconstruction with the β subunit (Fig. 1) and because no AbRC cross-reaction was seen anywhere on native gels of DJ75 or CA13, even in the high-molecular-weight region, where any aggregated proteins should be present. Another possibility is that any antibodies raised against the MoFe holoprotein in a nondenatured albeit O_2 -damaged state are directed against epitopes only on the β subunit, whereas any antibody raised against MoFe protein that denatured after injection into a rabbit would recognize epitopes on both subunits. This, in turn, suggests that the α subunit of the $\alpha_2\beta_2$ tetramer is much more buried than the β subunit. This view is also consistent with our observations that the β subunit assembles into a β_4 tetramer and that the α subunit is very insoluble, as well as with the prior observation that the Fe protein binds to the β subunit of the MoFe protein (41).

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