Isolation of Two Forms of the Nitrogenase VFe Protein from *Azotobacter vinelandii*†

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ABSTRACT: When Q-Sepharose was used in the purification of the V nitrogenase proteins from *Azotobacter vinelandii*, an increase in resolution was observed that resulted in a separation of the nitrogenase component 1 protein (Av1′) into two forms, labeled $Av1'_A$ and $Av1'_B$. Even though both forms possessed the same enzymatic behavior, $Av1'_A$ exhibited a lower specific activity and migrated during gel filtration with an apparent lower molecular weight than $Av1'_B$. Furthermore, SDS−polyacrylamide gel electrophoresis showed different relative compositions of the two major subunits of both forms, with $Av1'_A$ possessing a trimer ($\alpha\beta_2$) pattern compared to the more typical tetramer ($\alpha_2\beta_2$) pattern found for $Av1'_B$. Metal analysis indicated a V-to-Fe ratio of 1:19 for $Av1'_A$ and 1:15 (or 2:30) for $Av1'_B$, while acid-labile sulfide analysis showed that $Av1'_A$ possessed about half as much sulfide as $Av1'_B$. EPR spectroscopy revealed that both proteins retained the $S = 3/2$ and $S = 1/2$ signals observed in earlier isolations, with an additional $S = 1/2$ signal present in the spectrum of protein A. These results suggest that $Av1'_A$ is an incomplete form of the VFe protein, containing only one cofactor and one P cluster with an additional [Fe_4-S_4]-like cluster. The presence of a V storage protein in *A. vinelandii* was also investigated. Although no V storage protein was found, two V-binding proteins were observed.

Nitrogen fixation is the enzymatic process in which N_2 is reduced to NH_3. Nitrogenase, the enzyme that catalyzes this reaction, is composed of two separable proteins called components 1 and 2. Component 2, the smaller of the two proteins with a molecular weight of about 60 000, is a dimer of two identical subunits bridged by a single 4Fe-4S cluster (Georgiadis et al., 1992). Component 1, also referred to as the MoFe protein, is a tetramer composed of two pairs of subunits in an $\alpha_2\beta_2$ structure with a molecular weight around 240 000. Contained in the α subunit of component 1 is an extractable metal cluster called the FeMo cofactor, or FeMoco, which is widely believed to be at or near the substrate reduction site. The structure of this cofactor recently has been determined by X-ray crystallography and shown to consist of two cuboidal clusters, $MoFe_3S_3$ and Fe_4S_3, bridged by two or three inorganic sulfides for a proposed stoichiometry of $MoFe_7S_9$ (Chan et al., 1993; Kim & Rees, 1992b). In addition to the two cofactors, component 1 contains another pair of metal clusters called P clusters, which have been proposed to be bridged [4Fe-4S] cubanes giving an overall composition of Fe_8S_8 (Chan et al., 1993; Kim & Rees, 1992b). The function of the P clusters is unknown but may serve to shuttle electrons from component 2 to the cofactor in component 1.

Molybdenum is obviously required for the synthesis of the FeMoco. Unfortunately, biological uptake of molybdenum may be problematic due to its low natural abundance (ca. 1.5 ppm in soil) (Mason, 1966). *Azotobacter vinelandii* has circumvented this problem by being extremely efficient in both Mo uptake and accumulation (Pienkos & Brill, 1981). Therefore, in response to iron deprivation, the bacterium excretes a siderophore that strongly binds either Fe or Mo (as MoO_4^{2-}) (Stiefel et al., 1980). Under Mo-deficient conditions, *A. vinelandii* hyper produces a 44 000 protein along with a minor 77 000 protein in the outer membrane, possibly to facilitate Mo transport into the cell (Page & von Tigerstrom, 1982). Once inside the cell, Fe and Mo have been shown to accumulate in the form of bacterial ferritin and a constitutive Mo storage protein, respectively (Pienkos & Brill, 1981; Stiefel & Watt, 1979). Analysis of the purified Mo storage protein suggests that it is a tetrameric structure having a molecular weight of approximately 100 000 and consisting of two subunit types of molecular weights 21 000 and 24 000. In addition to binding Mo (at least 15 per protein), this protein can similarly sequester W (Hales & Case, 1987; Pienkos & Brill, 1981). A Mo storage protein also has been shown to be synthesized in the bacterium *Clostridium pasteurianum*; however, its expression occurs only under the conditions of nitrogen fixation. This latter storage protein differs from the one isolated from *A. vinelandii*, having a molecular weight of 50 000 with a monomeric structure that binds around 6 atoms of Mo per protein (Mortenson & Thorneley, 1979).

It is now well documented (Bishop et al., 1980; Hales et al., 1986a,b) that *A. vinelandii* possesses the ability to synthesize an additional (or alternative) nitrogenase system, called V nitrogenase, that is only expressed in the absence of molybdenum and the presence of vanadium. Like the conventional form of the enzyme, Mo nitrogenase, V nitrogenase contains both component 2 (Fe protein) and component 1 (VFe protein) proteins. The principal physical features that distinguish V nitrogenase from Mo nitrogenase are the metal content, where V is used instead of Mo in the cofactor, and the subunit structure, where the VFe protein is assumed to exist as a hexamer ($\alpha_2\beta_2\delta_2$) instead of a tetramer. The structure of the FeV cofactor (FeVco) in the VFe protein has not been determined, although MCD (Morningstar et al., 1987), EXAFS (Arber et al., 1987; Chen

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et al., 1993; George et al., 1988), and Mössbauer (Ravi et al., 1994) spectroscopic studies strongly suggest that the FeV cofactors, as well as the P clusters, are similar to the analogous metal clusters found in the MoFe protein.

Previous purification (Hales et al., 1986a) of the VFe protein from *A. vinelandii* revealed a variable V content when compared to the MoFe protein and failed to detect the δ subunit. An improved purification procedure is described, which demonstrates the existence of two forms of this protein that differ in their polypeptide and metal composition as well as their EPR spectra. Furthermore, the previously isolated storage protein in *A. vinelandii*, known to bind either Mo or W (Hales & Case, 1987; Pienkos & Brill, 1981), does not appear to have a similar affinity for V. On the other hand, two new proteins were partially purified from crude extract and shown to contain small amounts of loosely bound V.

MATERIALS AND METHODS

Cell Growth. All strains of *A. vinelandii* were grown in a modified Burk's nitrogen-free medium (Strandberg & Wilson, 1968), where MoO_4^{2-} is replaced with 40 μM V_2O_5 (Matheson Coleman & Bell) to induce the expression of the vanadium nitrogenase and any possible vanadium-binding proteins. Three different strains were used: UW, a wild-type strain capable of producing all three nitrogenases; LS10, a strain containing a deletion in *nifHDK*, the genes encoding the structural proteins of Mo nitrogenase and rifampicin resistance; and LS15, a strain identical to LS10 but tungsten resistant for diazotropic growth. Cells were grown at 30 °C in a Nalgen 100 L cylindrical vessel with constant vigorous aeration. Growth was monitored by measurement of absorbance at 630 nm (A_{630}) with a UV−vis spectrophotometer. Under these conditions, cells were harvested during midlog growth ($A_{630} \approx 1.3$) to yield approximately 2 g/L (wet weight) of cells, with a typical component 1 crude extract specific activity of 25 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$.

Protein Purification. The component proteins of V nitrogenase were isolated and purified by using a modification of the previously published methods (Burgess et al., 1980; Hales et al., 1986a). Cells (200−300 g) were lysed by osmotic rupture. Due to the greater heat sensitivity of V nitrogenase compared to Mo nitrogenase, no heat treatment step was used. Following centrifugation of the lysed cells, the resultant crude extract was concentrated to about 200 mL with a Millipore Minitan concentrator and loaded onto an anaerobic DEAE−cellulose (Sigma) column preequilibrated with 0.08 M NaCl buffer (25 mM Tris, pH 7.4). The column was washed with 3 bed volumes of buffer containing 0.1 M NaCl, and the absorbed protein was eluted into 10 mL fractions using a linear salt gradient (0.1−0.4 M NaCl) in 25 mM Tris (pH 7.4). Salt concentrations were monitored using a conductivity meter (Fischer Scientific, Model 152). Fractions containing component 1 (Av1′) activity were pooled, diluted to decrease the salt concentration, and reconcentrated with an Amicon concentrator. Further purification of component 1 was accomplished by loading Av1′ protein onto a 150 mL Q-Sepharose (Sigma) column preequilibrated with 0.1 M NaCl buffer. The Q-Sepharose column was subsequently washed with 0.2 M NaCl buffer, and Av1′ was eluted with a linear salt gradient (0.2−0.5 M NaCl). Av1′ fractions from this column were combined, concentrated, and applied to an S300 (Pharmacia) gel filtration column for further purification. All purification procedures were performed under anaerobic and reducing conditions maintained by the addition of 2 mM sodium dithionite (Aldrich) to all solutions.

Specific Activity Determination. Acetylene reduction was used as a monitor of protein activity (Hales et al., 1986a). Samples of Av1′ were titrated with component 2 [specific activity = 1800 nmol of ethylene produced min^{-1} (mg of protein)$^{-1}$] to determine the maximum specific activity. A Varian gas chromatograph (Model 3700) fitted with a Porapac N column was used to measure ethylene generation. Protein concentration was determined by the biuret method using bovine serum albumin as a protein standard.

Metal and Sulfide Determinations. Vanadium analysis of column fractions was performed using a Perkin-Elmer atomic absorption spectrophotometer (Model 5100) equipped with a graphite furnace. For sample digestion, 3−13 mg of protein was placed in a 7 mL polyethylene vial (Kimble) and diluted to 0.5 mL with double-distilled water, after which 0.25 mL of concentrated nitric acid (Mallinkrodt) was added. Caps were loosely screwed onto each vial and samples were incubated at 70−80 °C for 5 h. Once digested, each sample was diluted with double-distilled water to a final volume of 1 mL and 25% (w/v) nitric acid. Simultaneous vanadium and iron determinations on purified Av1′ were performed using an ICP emission spectrometer (ARL, Model 3400), since it was found that this technique consistently yielded reproducible metal ratios. The detection limit for both V and Fe was 2 ppb. Molybdenum concentrations were determined by using a colorimetric assay (Cardenas & Mortenson, 1974; Hales & Case, 1987).

Acid-labile sulfide analysis was performed by using the modified technique of Chen and Mortenson (1977).

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). The method for conducting SDS−PAGE has been described elsewhere (Laemmli, 1970). Stacking gels containing 3% acrylamide and running gels containing either 8% or 15% acrylamide were prepared from stocks of 30% (by weight) acrylamide (Amresco) and 0.8% (by weight) *N*,*N*′-bis-(methyleneacrylamide) (Bio-Rad). All samples were treated with SDS (Life Technologies Inc.) prior to loading on gels. Gels were stained with Coomassie Brilliant Blue R-250 (Amresco), and protein bands were quantified with a video densitometer (Bio-Rad, Model 620). Molecular weight standards (Bio-Rad) were run along with samples and used to estimate the molecular weights of protein bands separated on gels.

EPR Spectroscopy. Component 1 fractions were concentrated with an Amicon concentrator (YM30 ultrafiltration membranes), transferred into quartz EPR tubes, and frozen in liquid nitrogen under anaerobic conditions. EPR spectra were recorded at cryogenic temperatures (10 K, 10 mW) on a Bruker ER 300D spectrometer interfaced to a Bruker 1600 computer for data storage and manipulations. An Oxford Instrument ER-900 helium flow cryostat positioned in a TE_{102} cavity was used to reach low temperatures. Temperature was monitored and controlled by using an Oxford Instrument Model ITC4 temperature controller with a digital readout.

RESULTS

VFe Protein. Previous purification of the VFe protein (Av1′) of V nitrogenase from *A. vinelandii* consistently yielded proteins with variable V and Fe analyses. In an attempt to decrease this variability, we recently modified our purification scheme by following the previously employed

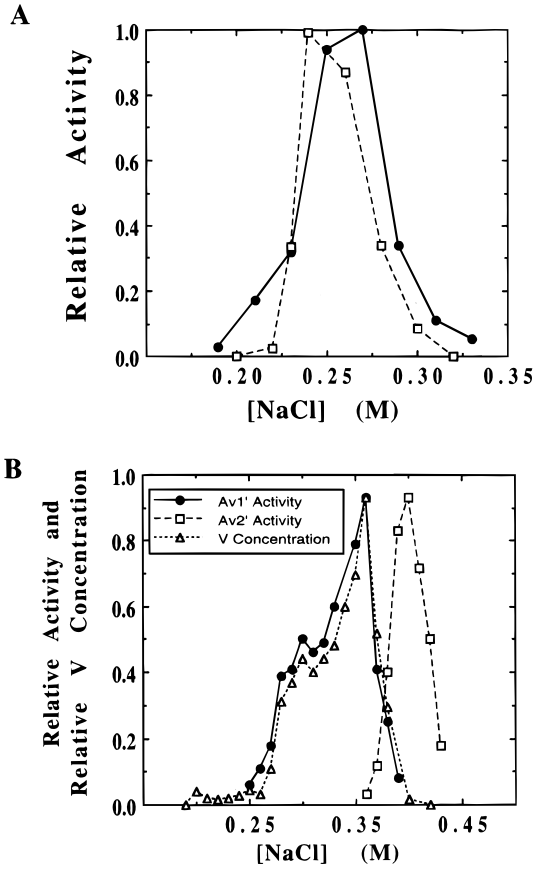

FIGURE 1: Elution profiles of component 1 (●) and component 2 (□) activity from DEAE−cellulose (panel A) and Q-Sepharose (panel B) for the V nitrogenase from *A. vinelandii*. Activity is expressed as the relative rate of acetylene reduction. The improved separation ability of the Q-Sepharose results in the resolution of the VFe protein into two active peaks (A and B). Relative vanadium concentration (△) for eluted fractions during the purification of Av1′ on a Q-Sepharose column was also determined. Vanadium concentration was determined by atomic absorption as described in Materials and Methods. Note that the activity per [V] ratio is the same for $Av1'_A$ and $Av1'_B$.

ion exchange fractionation step on DEAE−cellulose with a Q-Sepharose anion exchange resin. The use of this latter resin greatly improved the separation of Av1′ and the Fe protein (Av2′), which coelute on the DEAE resin (Figure 1). Unexpectedly, when fractions from the Q-Sepharose column were assayed for component 1 acetylene reduction activity, two maxima were observed, suggesting two different forms of Av1′ (Figure 1B). The form of Av1′ that elutes at lower salt concentrations will be referred to as $Av1'_A$ or form A (typically representing about 35% of both forms), while the form eluting at higher salt concentrations will be termed $Av1'_B$ or form B. The expression of the VFe protein was found to be independent of either the concentration or the form of vanadium (e.g., VO^{2+}, VO_4^{2-}, V_2O_5) used in the medium. Furthermore, both forms were found to be expressed by all strains of *A. vinelandii* investigated. Comparison of the acetylene reduction capabilities of two forms at this early stage of purification consistently showed the specific activity of $Av1'_B$ to be greater (at least 25%) than that of $Av1'_A$. One interpretation of the profile in Figure 1B is that Av1′ exists in two different protein compositions. Supporting this interpretation, gel filtration demonstrated that the two forms of Av1′ migrated with different apparent molecular weights, with $Av1'_B$ migrating with a molecular weight commensurate with the complete protein and the less active form ($Av1'_A$) possessing the smaller molecular weight.

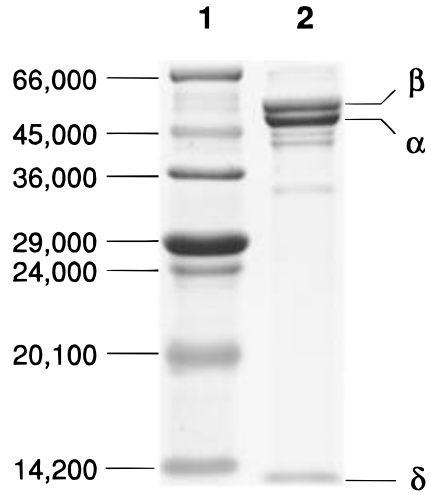

FIGURE 2: SDS−15% polyacrylamide electrophoretic gel of $Av1'_B$ showing the α, *β*, and *δ* subunits ($Av1'_A$ gave similar results). To see the small *δ* subunit band, Av1′ was overloaded on the gel, resulting in the appearance of protein contamination bands at around 45 000. Molecular weight markers used were the following: bovine albumin, 66 000; egg albumin, 45 000; phosphate dehydrogenase, 36 000; carbonic anhydrase, 29 000; trypsin inhibitor, 20 100; α-lactalbumin, 14 200.

The fact that the two forms of Av1′ chromatograph upon gel filtration with different apparent molecular weights suggests differences in their subunit compositions. The subunit composition of the VFe protein is thought to be $\alpha_2\beta_2\delta_2$, compared to the $\alpha_2\beta_2$ structure of the MoFe protein. While the *δ* subunit has been detected in preparations of purified VFe protein isolated from *Azotobacter chroococcum* (Robson et al., 1989), its presence previously had not been observed in preparations of Av1′. However, by using 15% gels for SDS−PAGE of Av1′, we can now demonstrate the presence of all three subunits (Figure 2) in both forms A and B. To quantify the α and *β* subunits of both forms, densitometric traces of 8% gels were deconvoluted into Gaussian peaks for better analysis (Figure 3). Integration of these traces showed an approximately 27% difference in the integrated intensities of the α and *β* subunits for form B, with the α subunit being greater. When interpreting this result, it is important to remember that two-dimensional gels have shown that the α subunit migrates faster than the *β* subunit on SDS−PAGE, despite the larger molecular weight of the α subunit (Joerger et al., 1990). The faster migration of the α subunit has also been shown to occur (Robson et al., 1989) with the VFe protein from *A. chroococcum*. Because of the higher molecular weight of form B on the elution profile, it is believed to be the "normal" enzyme with the proposed $\alpha_2\beta_2$ composition for the two major subunits. This means that the *β* subunit stains 27% less than the α subunit. Similar Gaussian analysis of form A (Figure 3) showed a reversal in the staining pattern with the *β* subunit staining around 27% greater than the α subunit. By taking into account the difference in staining ability of the two subunits, these results imply that the *β* subunit is at a concentration approximately twice that of the α subunit, suggesting an $\alpha\beta_2$ subunit ratio for $Av1'_A$ (Table 1).

To perform a similar determination on the relative concentration of the *δ* subunit in the two forms of Av1′ proved to be much more difficult. While this subunit is observable in SDS−PAGE gels of both forms of the protein, the low and irreproducible intensity of this subunit among various preparations has prohibited an exact determination

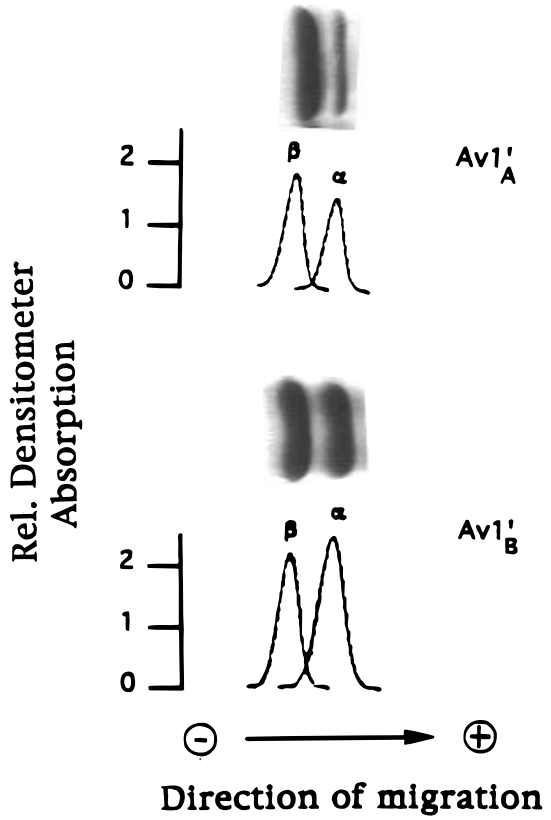

FIGURE 3: Enlarged SDS−8% polyacrylamide gels of Av1′$_A$ (A) and Av1′$_B$ (B) showing the different densities of the α and β subunits. Below each gel is the Gaussian deconvolution of the densitometer trace. Arrow indicates the direction of electrophoresis.

Table 1: Summary of Analyses of Av1′$_A$ and Av1′$_B$

protein	relative activity[a] (%)	composition[b]	metal content[c] (V:Fe)	sulfide content[d]	epr g factors $S = 1/2$[e]	epr g factors $S = 3/2$[f]
Av1′$_A$	75	$\alpha\beta_2$	1:19	19 ± 3	2.05, 1.94 2.03, 1.93, 1.89	5.5
Av1′$_B$	100	$\alpha_2\beta_2$	2:30	34	2.05, 1.94	5.5

[a] Acetylene reduction activity relative to Av1′$_B$, the complete form of the protein, which has been assigned a value of 100% [specific activity of Av1′$_B$ is 245 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$, while that of Av1′$_A$ is 184 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$]. [b] Composition of the major α and β polypeptides; the δ subunit, which was found to be present in both Av1′$_A$ and Av1′$_B$, could not be quantified. [c] Relative metal content from ICP determinations (error ± 1 Fe) assuming 1 V per Av1′$_A$ and 2 V per Av1′$_B$; actual quantitation determined from AA and EPR spectroscopies was 0.7 ± 0.2 V per Av1′$_A$ and 1.4 ± 0.2 V per Av1′$_B$. [d] Acid-labile sulfide content relative to Av1, assuming Av1′$_B$ possesses 34 sulfides. [e] Av1′$_A$ exhibits two different $S = 1/2$ signals; spin quantitation of each of these signals is about 10% of the $S = 3/2$ cofactor signal. [f] Average g factor for two inflections of the $S = 3/2$ cofactor signal.

of the amount of the δ subunit in either form. The source of this irreproducibility is unknown, but suggests a loose binding of the δ subunit to Av1′ similar to that observed for component 1 of nitrogenase-3 from *A. vinelandii* (Eady, 1991).

The crystal structure (Kim & Rees, 1992a; Rees, 1993) of the MoFe protein shows that the cofactors (FeMoco) are contained inside the α subunits. Because the VFe protein also contains cofactors (FeVco) (George et al., 1988; Morningstar & Hales, 1987; Smith et al., 1988), which are similar to FeMoco (Morningstar & Hales, 1987), it is important to determine how the lack of an α subunit in Av1′$_A$ affects its cofactor concentration. If the VFe protein lacks

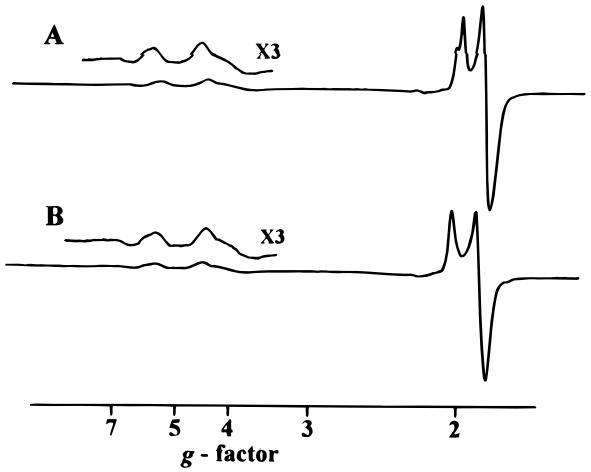

FIGURE 4: EPR spectra of Av1′$_A$ (A) and Av1′$_B$ (B) recorded at 10 K. Both spectra contain $S = 3/2$ and 1/2 signals centered at $g = 5.5$ and 1.94, respectively. Protein concentrations of Av1′$_A$ and Av1′$_B$ samples were 87 and 60 mM, respectively. Spectrometer settings: frequency, 9.47 GHz; modulation amplitude, 12 G; microwave power, 10 mW.

an α subunit, we would expect the cofactor concentration to be lower and, thus, a lower V concentration. Figure 1 compares component 1 activity and vanadium concentration for fractions eluted from the Q-Sepharose column, showing a direct correlation between these two quantities for all of the active fractions and, therefore, for both Av1′$_A$ and Av1′$_B$. As expected, the vanadium concentration dictates the activity of the component 1 protein.

The determination of the absolute metal content of nitrogenase has always been difficult, sometimes underestimating the actual amount by as much as 40% (Eady, 1980). Because of this, attempts at determining the V-to-Fe ratio in the two protein forms through separate quantitations of the V and Fe concentrations will result in values with high uncertainties. On the other hand, we have found that the ratio of different metals in a protein can be accurately and reproducibly determined by using ICP emission spectroscopy. By using this technique, the V-to-Fe ratio (Table 1) of Av1′$_B$ was found to be 1:15 (or 2:30), which is analogous to that found in purified MoFe protein (two cofactors containing 7 Fe and 1 Mo each and two P clusters possessing 8 Fe each), while the ratio in Av1′$_A$ was 1:19.

A similar problem exists when quantifying the absolute amount of acid-labile sulfide of nitrogenase (Eady, 1980), where analysis of the normal MoFe or VFe proteins yields values of about 20 S^{2-} per protein compared to the known amount of 34 for the MoFe protein (Smith & Eady, 1992). Therefore, the best way to estimate the amounts of sulfide in forms A and B is to compare the ratios of the quantified sulfide (albeit low) for fixed concentrations of the two proteins with that of the MoFe protein, which is used as a standard. By using this procedure, 10 different measurements of forms A and B of Av1 at set concentrations were assayed; it was found that the ratio of detected sulfide in Av1′$_B$-to-Av1′$_A$ was 1:0.55 ± 0.09. In other words, as expected, Av1′$_A$ has approximately half (or 19 ± 3) of the acid-labile sulfide found in Av1′$_B$ (assumed to be 34).

The EPR spectra of the two forms differed slightly. Both forms (Figure 4) exhibited the presence of the $S = 3/2$ and $S = 1/2$ states centered at $g = 5.5$ and 1.94, respectively, previously observed by us in earlier preparations of Av1′

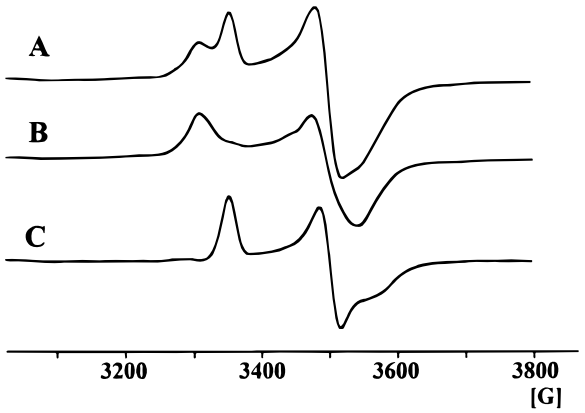

FIGURE 5: Expanded EPR spectra of purified Av1′$_A$ (A) and Av1′$_B$ (B) in the $g = 2$ region. Difference spectrum (C) obtained by computer subtraction of the spectrum of Av1′$_B$ from that of Av1′$_A$. Spectrometer settings were the same as described in legend to Figure 4, with the exception of a modulation amplitude of 8 G. Spectra were recorded at 10 K.

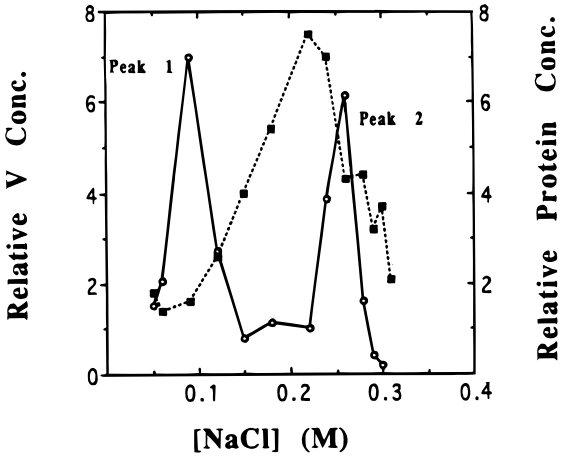

FIGURE 6: Q-Sepharose elution profile of the vanadium-containing fraction (0.1 M NaCl) from the DEAE−cellulose column. A shallow salt gradient (0.0−0.3 M NaCl) revealed two peaks of high vanadium with no Av1′ activity. Symbols: ○, vanadium concentration; ■ protein concentration.

(Hales et al., 1986a). Through analogy with the MoFe protein, the $S = 3/2$ signal has been assigned (Morningstar & Hales, 1987) to the cofactor (spin quantitation of 0.9 spins per V), while the origin of the $S = 1/2$ signal is unknown. The only difference between the spectra of Av1′$_A$ and Av1′$_B$ occurs in the $g = 2$ region. When the $g = 2$ region of these spectra (Figure 5) are expanded, form B is found to contain only the single axial $S = 1/2$ signal with $g_{\|} = 2.05$ and $g_{\perp} = 1.94$ normally observed in Av1′. Form A, however, exhibits an additional signal that can be seen more easily by subtracting the axial signal of form B from the composite signal of form A, revealing a narrow, slightly rhombic signal with $g_z = 2.03$, $g_y = 1.93$, and $g_x = 1.89$ (Table 1).

Vanadium-Binding Proteins. While the vanadium concentration of the various fractions eluted during the purification of Av1′ was monitored two new proteins, containing vanadium but lacking component 1 activity, were found. Specifically, extract from cells of strain UW or LS15 (both gave identical results) grown in the presence of V_2O_5 was concentrated by using pressure dialysis, a technique that significantly decreases the concentration of non-protein-bound inorganic vanadium compounds. The extract was applied to a equilibrated DEAE−cellulose column washed with 0.1 M NaCl buffer (the remaining column was treated with a 0.1−0.5 M NaCl gradient and produced no V-containing proteins other than Av1′$_A$ and Av1′$_B$). The initial 0.1 M NaCl wash fraction was found to contain vanadium but lacked component 1 activity. This fraction was applied to a Q-Sepharose column equilibrated with buffer containing no salt and washed with 3 bed volumes of buffer, after which a 0.0−0.3 M NaCl gradient was applied. The fractions collected were simultaneously analyzed for protein, salt, and vanadium concentrations. This procedure revealed the presence of two V-containing elution peaks (Figure 6) at about 0.08 and 0.25 M NaCl.

Because *A. vinelandii* had been shown to synthesize a Mo storage protein, it is possible that one of these new peaks corresponds to this protein, which is now storing V. To investigate this possibility, it was necessary to determine whether the Mo storage protein eluted from Q-Sepharose similar to either of the V-containing peaks. Wild-type strain UW of *A. vinelandii* was grown on medium containing 20 μM Mo, the cells were lysed, and the crude extract was chromatographed on a Q-Sepharose column. By using colorimetric techniques to monitor Mo, the Mo storage protein was found to elute at 0.12 M NaCl, obviously different from either V peak. These results demonstrate that the V peaks are not the previously characterized Mo storage protein and suggest that the Mo storage protein is unable to bind V. It should be noted that separate experiments showed that neither of these peaks corresponded to the elution of simple inorganic VO_x ions, all of which tightly bind to Q-Sepharose and elute at salt concentrations greater than 0.5 M.

Vanadium was found to be only loosely bound to the proteins of peaks 1 and 2. For example, when comparing the V content of the 0.1 M wash fraction from the initial DEAE−cellulose column to the total vanadium eluted from the Q-Sepharose column, there was a 75% loss in vanadium. The majority of this vanadium was observed to be lost during the concentration step of the 0.1 M wash fraction prior to chromatography on the Q-Sepharose column. Because of this, an exact metal analysis could not be carried out, and peaks 1 and 2 could not be further purified. Therefore, all characterizations were performed on the Q-Sepharose peaks. At this stage of purification, the V concentration of both peaks was determined to be less than 1 V per polypeptide.

By using SDS−PAGE, the polypeptide composition of peaks 1 and 2 was investigated. Peak 1 contained no dominant polypeptides, but exhibited four primary bands (data not shown) ranging in molecular weight from 23 800 to 33 000, with the greatest intensity in the band at 23 800. Peak 2, on the other hand, consisted of a major band (>90% total protein) having an apparent molecular weight of approximately 53 000 and a mobility intermediate to the α and β subunits of Av1′ (Figure 7).

DISCUSSION

The presence of multiple forms of nitrogenase component 1 is very interesting but not unprecedented. In the early purification of the MoFe protein from Mo nitrogenase, an inactive form of the protein was isolated. Although this protein retained the $\alpha_2\beta_2$ tetramer composition of the active MoFe protein, it possessed only about half the iron and approximately 0.5 Mo and was devoid of the $S = 3/2$ EPR spectrum of the protein-bound cofactor, but exhibited an $S = 1/2$ signal nearly identical to that observed in Av1′$_B$ (Palmer et al., 1972; Zumft & Mortenson, 1973).

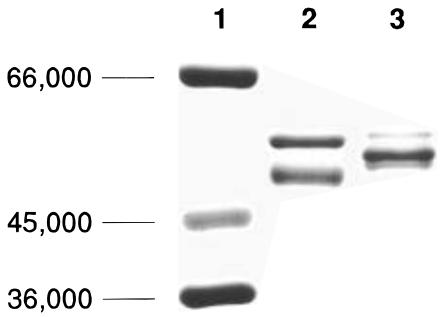

FIGURE 7: SDS−8% polyacrylamide gel of peak 2: lane 1, molecular weight markers (bovine albumin, 66 000; egg albumin, 45 000; phosphate dehydrogenase, 36 000); lane 2, Av1′ α and β subunits; lane 3, V-binding protein. Peak 2 fraction (lane 3) shows a dominant band with an apparent molecular weight of 53 000.

Purification of component 1 from the third nitrogenase enzyme, nitrogenase-3, from *A. vinelandii* (Chisnell et al., 1988; Eady et al., 1993) also showed the presence of two forms of the enzyme. Amino acid analysis of the separated peptides has determined the composition of each form to be $\alpha_2\beta$ and $\alpha_2\beta_2$ (the δ polypeptide was not quantified). The X-ray structure of the MoFe protein shows (Kim & Rees, 1992a) the polypeptide arrangement to be $\alpha\beta\beta\alpha$, in which the α subunits bind to adjacent β subunits but do not interact with each other. The $\alpha_2\beta$ composition of nitrogenase-3 suggests the arrangement $\alpha\beta\alpha$, where the second α subunit has replaced the missing β subunit. Considering that the α and β subunits have similar secondary and tertiary structures, each consisting of the same three domains of α helices and β strands, this replacement may not be that extreme.

The $\alpha\beta_2$ composition of Av1′$_A$ suggests the more conservative peptide arrangement $\alpha\beta\beta$ arising from the absence of one of the terminal α subunits. Regardless of the peptide arrangement, it is obvious that both alternative enzyme systems synthesize an active form of component 1, which lacks a full complement of subunits. Why different forms of component 1 are synthesized only by the alternative systems and have not been observed in purified MoFe protein is not known. One simple explanation may be that these secondary forms are also processed by the Mo nitrogenase genes but that they are less temperature stable than the normal MoFe protein and, therefore, denatured during the 60 °C heat step, a purification step not used on either of the alternative component 1 proteins due to their greater temperature instability. Another possibility is that the α and β subunits are more loosely bound to each other in the alternative enzymes than in the MoFe protein and, therefore, dissociate more easily during purification. This latter possibility is consistent with our observation that the subunits of the VFe protein of *A. vinelandii* appear to dissociate slowly upon dilution to <10 μM.

The metal composition and EPR spectra of Av1′$_A$ suggest that it possesses only one cofactor and, therefore, should have a lower activity than Av1′$_B$, i.e., the specific activity of Av1′$_A$ (1 cofactor per $\alpha\beta_2$) should be only 67% of that of Av1′$_B$ (2 cofactors per $\alpha_2\beta_2$), a value close to the observed 75%. However, in spite of the differences in polypeptide and cofactor compositions, the enzymologies of Av1′$_A$ and Av1′$_B$ are the same. This is expected since both the P clusters and cofactors on the opposite $\alpha\beta$ dimers of the protein are separated by about 70 Å. Our results show that incomplete construction of one $\alpha\beta$ dimer of the enzyme does not affect the catalytic behavior of the other. Even though the enzymology of Av1′$_A$ appears to be unaffected by the missing subunit and cofactor, its redox profile is dramatically affected. Stepwise oxidation of Av1′$_A$ shows [see Tittsworth and Hales (1996)] an unusual redox gating midway through the titration that is not observed during the oxidation of either Av1′$_B$ or Av1.

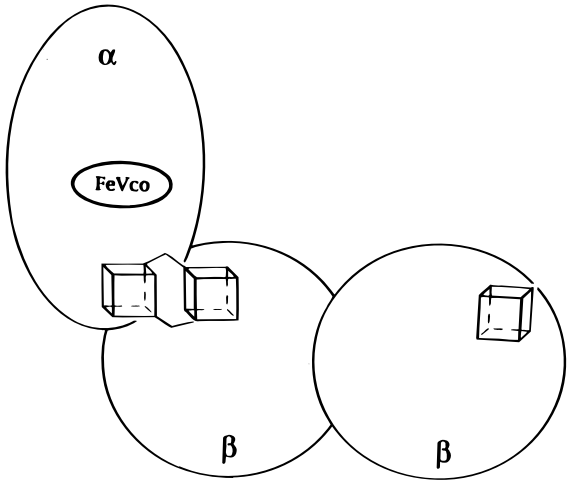

FIGURE 8: Proposed structure of Av1′$_A$, where one α subunit and half of a P cluster are missing, resulting in a 4Fe-4S metal cluster in one of the β subunits.

Comparative V and Fe metal analyses of Av1′$_A$ show it to possess approximately four more Fe atoms beyond that required for the single cofactor and P cluster. Sulfide analysis similarly shows that Av1′$_A$ may contain a little more than half the acid-labile sulfide found in Av1′$_B$, suggesting that the additional iron and sulfide are associated with another FeS cluster. The EPR spectrum of this form strengthens this argument and shows an additional signal with $g_{av} < 2$ (Figure 5) characteristic of reduced ferredoxin. Because the ligation of the normal Fe_8S_8 P cluster is shared equally by three cysteine thiols on both the α and β subunits, the absence of the α subunits negates the ability of Av1′$_A$ to form two complete P clusters, but does not eliminate the potential for the formation of a [4Fe-4S]-like cluster with the remaining three cysteine residues on the β subunit. Therefore, we propose that the second $g = 2$ signal in the EPR spectrum of Av1′$_A$ arises from a single $[4Fe\text{-}4S]^+$-type cluster bound to the second β subunit in a position normally occupied by the second P cluster (Figure 8). If such a cluster, in fact, is present, its structure may be different from that found in a normal [4Fe-4S] ferredoxin, which requires four cysteine residues. The low-spin concentration of this cluster (typically 10−20% of the $g = 5.5$ cofactor signal) suggests that all of the [4Fe-4S] clusters are not EPR observable, possibly because they have a low redox midpoint potential and, therefore, are not all reduced to the $[4Fe\text{-}4S]^+$ state in the presence of 2 mM dithionite (a similar situation exists with the $S = 1/2$ axial signal in Av1′$_B$). While it has been shown that the cofactor is synthesized outside of the component 1 protein before insertion into it, the mechanism of P cluster synthesis is unknown. The presence of the [4Fe-4S] cluster in the $\alpha\beta_2$ protein now suggests a possible mechanism for the synthesis of P clusters in the holoprotein. In this mechanism, ferredoxin-like [4Fe-4S] clusters may be formed separately on the α and β subunits during their initial

synthesis. Subsequent binding of these two polypeptides during the formation of component 1 would induce the coalescence of these [4Fe-4S] clusters into a single P cluster.

The absence of a detectable V storage protein in *A. vinelandii* is very interesting. This bacterium has been shown (Hales & Case, 1987; Pienkos & Brill, 1981) to be able to accumulate Mo (or W) in a Mo storage protein whose expression occurs under all conditions of growth and is not dependent on nitrogenase synthesis. In contrast, Mo storage in *C. pasteurianum* (Mortenson & Thorneley, 1979) is repressed by a fixed source of nitrogen, while *Klebsiella pneumoniae* (Pienkos & Brill, 1981) does not accumulate Mo under any growth conditions. Presumably the uptake and storage system in *A. vinelandii* allows Mo to accumulate inside the cell in times of plenty, such that in the event of exhaustion of extracellular Mo the stored Mo can be used to synthesize the MoFe protein. To our knowledge, this is the only species that has the ability to do this. Our results show that this constitutive Mo storage protein does not appear to bind vanadium. Because of the higher natural abundance of vanadium relative to molybdenum, the absence of a V storage protein may arise simply because there is no need to sequester V in most environments. Another possibility is that *A. vinelandii* may store only Mo and not V, thus favoring the synthesis of the more efficient Mo nitrogenase.

Although a V storage protein was not observed in the cell extract of *A. vinelandii* grown in the presence of high V concentrations, V binding to two different proteins was detected. The low vanadium concentration of each of these proteins (less than 1 V per polypeptide following the initial ion exchange column) precludes their definition as storage proteins, but does not negate their possible involvement with V transport or cofactor processing. Unfortunately, the generally loose binding of V in both proteins prevented detailed structural and spectroscopic investigations. The only *vnf* gene products with molecular weights close to the apparent molecular weight of peak 2 (53 000) are the α (53 874) and β (52 772) subunits of the VFe protein, whose association with this protein was eliminated by their differing SDS−PAGE mobilities (Figure 7), and the proteins of *vnfN* (48 701) and *vnfE* (51 089), proteins previously associated with cofactor construction. Although we do not know whether the V-binding protein in peak 2 corresponds to any of these polypeptides, it is interesting to note that the only *vnf* gene products with molecular weights close to that of peak 2 are those known to be associated with cofactor construction or binding.

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