Characterization of a Tungsten-Substituted Nitrogenase Isolated from *Rhodobacter capsulatus*[†,‡]

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ABSTRACT: In the phototrophic non-sulfur bacterium *Rhodobacter capsulatus*, the biosynthesis of the conventional Mo-nitrogenase is strictly Mo-regulated. Significant amounts of both dinitrogenase and dinitrogenase reductase were only formed when the growth medium was supplemented with molybdate (1 μM). During cell growth under Mo-deficient conditions, tungstate, at high concentrations (1 mM), was capable of partially (~25%) substituting for molybdate in the induction of nitrogenase synthesis. On the basis of such conditions, a tungsten-substituted nitrogenase was isolated from *R. capsulatus* with the aid of *anfA* (Fe-only nitrogenase defective) mutant cells and partially purified by Q-sepharose chromatography. Metal analyses revealed the protein to contain an average of 1 W-, 16 Fe-, and less than 0.01 Mo atoms per $\alpha_2\beta_2$-tetramer. The tungsten-substituted (WFe) protein was inactive in reducing N_2 and marginally active in acetylene reduction, but it was found to show considerable activity with respect to the generation of H_2 from protons. The EPR spectrum of the WFe protein, recorded at 4 K, exhibited three distinct signals: (i) an $S = 3/2$ signal, which dominates the low-field region of the spectrum ($g = 4.19, 3.93$) and is indicative of a tungsten-substituted cofactor (termed FeWco), (ii) a marginal $S = 3/2$ signal ($g = 4.29, 3.67$) that can be attributed to residual amounts of FeMoco present in the protein, and (iii) a broad $S = 1/2$ signal ($g = 2.09, 1.95, 1.86$) arising from at least two paramagnetic species. Redox titrational analysis of the WFe protein revealed the midpoint potential of the FeWco ($E_m < -200$ mV) to be shifted to distinctly lower potentials as compared to that of the FeMoco ($E_m \sim -50$ mV) present in the native enzyme. The P clusters of both the WFe and the MoFe protein appear indistinguishable with respect to their midpoint potentials. EPR spectra recorded with the WFe protein under turnover conditions exhibited a 20% decrease in the intensity of the FeWco signal, indicating that the cofactor can be enzymatically reduced only to a small extent. The data presented in the current study demonstrate the pivotal role of molybdenum in optimal N_2 fixation and provides direct evidence that the inability of a tungsten-substituted nitrogenase to reduce N_2 is due to the difficulty to effectively reduce the FeW cofactor beyond its semi-reduced state.

The biological fixation of molecular nitrogen (N_2)—one of nature's most fundamental processes—is mediated by the enzyme system nitrogenase, which is present in a small number of bacteria and archaea. Nitrogenase is composed of two oxygen-labile metalloproteins: dinitrogenase and dinitrogenase reductase. Three genetically distinct but related types of nitrogenase systems (*nif*, *vnf*, and *anf*)[1] have been proven to occur in nature (for review, see refs *1* and *2*). The most common, and best characterized system, is the conventional molybdenum-containing nitrogenase (*nif*-system), which is the only system known to be present in all N_2-fixing microorganisms. The two types of alternative (i.e., molybdenum-independent) nitrogenase systems found in diazotrophs are (i) the vanadium-dependent nitrogenase (*vnf*) and (ii) the iron-only nitrogenase (*anf*), which lacks both molybdenum and vanadium. A third alternative, but completely *nif*/*vnf*/*anf*-independent nitrogenase system, has been recently reported to occur in *Streptomyces thermoautotrophicus* (*3*). This enzyme appears to derive the electrons required for N_2 reduction from superoxide oxidation coupled to CO oxidation.

The dinitrogenase component (MoFe protein) of the classical nitrogenase system is an $\alpha_2\beta_2$ heterotetramer (M_r ~240 kDa) and contains two sets of two unique metal−sulfur clusters: the FeMoco, which is comprised of molybdenum/iron/sulfur as well as homocitrate in a ratio of 1:7:9:1 and an Fe_8S_7 cluster, termed P cluster (*4*−*8*). The FeMoco is believed to be the site of substrate reduction (*9*, *10*), whereas the P cluster appears to mediate electron as well as proton transfer to FeMoco (*8*, *11*, *12*).

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[1] Abbreviations: *nif*, nitrogen fixation; *vnf*, vanadium dependent nitrogen fixation; *anf*, alternative nitrogen fixation; EPR, electron paramagnetic resonance; FeMoco, iron−molybdenum cofactor; FeWco, iron−tungsten cofactor; ICP−MS, inductively coupled plasma-mass spectrometry; K_M, Michaelis constant; $Rc1^{Mo}$, MoFe protein of *R. capsulatus*; $Rc2^{Fe}$, Fe protein of the Fe-only nitrogenase of *R. capsulatus*; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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The second protein component, dinitrogenase reductase (Fe protein), which is present in all *nif*, *vnf*, and *anf* nitrogenases, is a homodimer (M_r ~64 kDa), containing two nucleotide (MgATP or MgADP) binding sites, and one conventional Fe_4S_4 cluster, bridging the two subunits (*13*). During the catalytic cycle, the Fe protein forms a complex with the MoFe protein, thereby facilitating the transfer of one single electron from its Fe_4S_4 cluster to dinitrogenase, an event that is accompanied by the concomitant hydrolysis of two molecules of MgATP (*14*−*16*). Apart from serving as the physiological electron donor to the MoFe protein, the Fe protein is also involved in FeMoco biosynthesis and maturation of the cofactorless apodinitrogenase (*17*, *18*).

The discovery of two functional alternative nitrogenase systems led to speculations about the role of molybdenum and vanadium in Mo- and V-dependent nitrogen fixation, respectively. In view of the similarities between molybdenum and tungsten, several attempts have been made to replace the molybdenum center in the cofactor of the conventional nitrogenase by tungsten, to gain an insight into the role of the heterometal in the enzyme. Although the effect of tungstate, added to cultures of N_2-fixing microorganisms, on nitrogenase expression and activity has been investigated to some extent (*19*−*22*), only one report by Hales and Case provided conclusive evidence for tungsten-incorporation into the cofactor of *Azotobacter vinelandii* nitrogenase (*23*). However, the isolated tungsten-containing *A. vinelandii* protein has been shown to contain considerable amounts of molybdenum and to be active in N_2 fixation. Metal analyses have revealed active protein preparations to contain one W- and one Mo-center per protein tetramer. Hence, the activity observed has been solely attributed to the presence of FeMoco. The contamination with FeMoco in these protein preparations was likely based on the difficulty to remove traces of molybdenum from the growth medium in this organism because of the presence of a highly specific Mo-storage protein (*24*). Further characterization of the tungsten-substituted nitrogenase from *A. vinelandii* has not been pursued. More importantly, a Mo-free tungsto-nitrogenase has yet to be isolated and characterized.

This report pertains to the purification and partial characterization of a tungsten-substituted nitrogenase, isolated from an *anfA* mutant of the phototrophic non-sulfur bacterium *Rhodobacter capsulatus*. Using this particular strain of this organism, we were able to (i) minimize the accumulation of molybdenum in cells cultivated under Mo-deficient conditions (in view of the absence of a Mo-storage protein) and (ii) overcome the expression of the Fe-only nitrogenase (*anf*-regulated) under conditions of Mo-deprivation (*25*, *26*). Furthermore, since *R. capsulatus* is devoid of the *vnf*-system, only *nif*-genes are expressed under growth conditions favorable to tungsten incorporation into the cofactor.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The organisms used were *R. capsulatus* wild-type strain B10S (*27*) and a spectinomycin-resistant *anfA* insertion mutant (*25*), which was generated from B10S.

Growth Medium and Culture Conditions. The composition of the growth medium and the culture conditions were as those reported previously (*28*) but with some minor modifications. The standard growth medium consisted of KH_2PO_4 (final concn: 7.4 mM), $MgSO_4$ (0.8 mM), NaCl (1.7 mM), $CaCl_2$ (0.35 mM), $MnCl_2$ (18 μM), EDTA (60 μM), ferric citrate (0.75 mM), L-lactate (12 mM), and L-serine (5 mM) as the sole nitrogen source. Under nitrogen-limiting growth conditions, which guarantee the expression of nitrogenase with maximal specific activities, a serine concentration of 2.3 mM was used. In cases where molybdenum had to be strictly omitted from the growth medium (e.g., for the isolation of the tungsten-containing nitrogenase), L-lactate (supra pure, from Sigma-Aldrich) was used, and the stock solution of ferric citrate (38 mM) was purified by an activated-carbon method (*29*, *30*). The medium was further supplemented with thiamine (80 μg/L) and biotin (8 μg/L), and the pH was adjusted to 6.8. Spectinomycin was added to the medium (final concn: 10 μg/mL) for the cultivation of *anfA* cells, whereas no antibiotic was used in the case of the wild-type strain. Cultures were purged with argon for 20 min prior to their cultivation under phototrophic anaerobic conditions at 35 °C.

In experiments designed to investigate the influence of molybdate and/or tungstate in the growth medium on nitrogenase activity and expression, the medium was supplemented with Na_2MoO_4 and/or Na_2WO_4 in required amounts (see Results section). For the isolation of the tungsten-substituted nitrogenase from the *anfA* mutant, a final concentration of 1 mM Na_2WO_4 in the growth medium was used.

Preparation of Cell-Free Extracts and Purification of Nitrogenase. The protocol employed for the preparation of cell-free extracts as well as the isolation and purification of the conventional nitrogenase was similar to that described earlier (*30*, *31*). All steps were carried out under strictly anaerobic conditions. Buffers were flushed with O_2-free argon and contained $Na_2S_2O_4$ (final concn: 4 mM). Prior to their centrifugation (13 000*g*, 1 h, 4 °C), the cell suspension was supplemented with $Na_2S_2O_4$ (4 mM) to guarantee reducing conditions during the centrifugation step. After resuspension, the cells were disrupted by lysozyme treatment as reported previously (*32*). The resulting extract was supplemented with $Na_2S_2O_4$ (2 mM) and subsequently centrifuged (200 000*g*, 1 h, 4 °C) to remove all cell debris and membrane particles. The supernatant was referred to as crude extract.

The dinitrogenase component was isolated and partially purified by one Q-sepharose chromatography step. The column (internal diameter: 2.5 cm) contained 60 mL of gel and was cooled to 8 °C with a cryostat. Tris-buffer (50 mM, pH 7.6) containing NaCl (150 mM), glycerol [10% (v/v)], and sodium dithionite (4 mM) served as the equilibration buffer and was used throughout the entire purification procedure. The cell-free extract was applied to the Q-sepharose column, followed by the stepwise elution with 50−60 mL of NaCl solutions (in equilibration buffer) of increasing concentrations (200/250/300/350/400 mM). The dinitrogenase component was eluted with 300 mM NaCl, whereas the Fe protein was recovered with 350 mM NaCl. The dinitrogenase component was finally concentrated to approximately 1 mL by anaerobic ultrafiltration as described earlier (*32*).

Assay of Nitrogenase Activity (C_2H_2 Reduction). The whole-cell assay was performed as reported previously (*28*).

Prior to the anaerobic transfer of the cell suspension into Ar-pregassed test tubes, Ti(III)-citrate was added at a final concentration of 0.25 mM to remove residual oxygen. The assay was initiated by the injection of 250 μL of C_2H_2 (~5% of the gas phase) and terminated after 1 h. Assays with cell-free extracts or isolated dinitrogenase were performed as documented earlier (*32*).

Protein Determination. The protein content was determined by the Biuret method. The whole-cell procedure was carried out as described by Schmidt et al. (*33*), whereas the protein content of extracts and isolated protein preparations was determined by the method of Beisenherz et al. (*34*).

SDS−PAGE. The purity of protein components and molecular masses of the subunits were routinely analyzed by Tris-glycine SDS−PAGE, according to the method of Laemmli (*35*). The stacking gel contained acrylamide [4.8% (w/v)] and bisacrylamide cross-linker [0.13% (w/v)], whereas the resolving gel was comprised of 12.5% (w/v) acrylamide and 0.33% (w/v) bisacrylamide. The protein samples were supplemented with dithioerythritol (20 mM) prior to their denaturation. Soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), and bovine albumin (66 kDa) were used as protein standards.

Immunoassays. Antisera, containing monospecific, polyclonal antibodies against purified dinitrogenase ($Rc1^{Mo}$) and dinitrogenase reductase ($Rc2^{Fe}$), respectively, were employed in all immunoassays. Antibodies against the iron protein of the iron-only nitrogenase ($Rc2^{Fe}$) were used to detect the iron protein of the conventional nitrogenase since antibodies against $Rc2^{Mo}$ were not available. In this context, it is important to note that both Fe proteins ($Rc2^{Mo}$, $Rc2^{Fe}$) have been shown to be serologically related (*30*).

Single Radial Immunodiffusion Assays. Single radial immunodiffusion assays were performed according to the method of Mancini et al. (*36*), using the conditions described by Catty and Raykundalia (*37*) with some modifications. The assay was carried out in 1% (w/v) agarose gels, which were prepared as follows: agarose was dissolved in hot diethyl barbiturate/acetate buffer (50 mM, pH 8.6) containing sodium 5,5-diethyl barbiturate (22 mM), sodium acetate (22 mM), and sodium azide (6 mM). After the addition of polyethylene glycol 6000 [3% (w/v)], aliquots of 11.5 mL were withdrawn from the mixture, placed into glass test tubes, cooled to 56 °C in a water bath, and supplemented with the required amount of antiserum (0.5 mL of anti:$Rc1^{Mo}$). The gel (12 mL) was evenly poured onto 7.5 × 9 cm glass plates (0.18 mL/cm^2) and subsequently allowed to solidify at room temperature. A punch (∅ 3 mm) was employed to cut out circular wells (4 × 5 well pattern).

Antigen samples (containing dinitrogenase) were prepared as follows: an appropriate amount of cell-free extract was supplemented with SDS (35 mM) and boiled for 10 min. Triton X-100 [2% (v/v)] was subsequently added, and the wells were filled with antigen samples (4.5 μL each). A range of standard antigen solutions (purified dinitrogenase of known concentration) was always included in the assay for calibration. The loaded gel was stored in a damp storage box for 24 h at 4 °C to allow for the formation of stable precipitation rings. The amount of dinitrogenase present in the samples was deduced from the diameter of the precipitation rings as documented in the literature (*37*).

Western Immunoblot Analysis. Western immunoblot experiments were conducted essentially by the method of Towbin et al. (*38*), using the protocol described previously (*32*).

Metal Analyses. The Mo, W, Re, and Fe content of cell-free extracts and purified protein preparations was quantified by inductively coupled plasma−mass spectrometry (ICP−MS), using a Perkin-Elmer/Sciex ICP−QMS Elan 6000 (Concord, Ontario, Canada). Protein samples were prepared in nitric acid (2%). External calibration of the ICP−mass spectrometer was achieved using standard metal solutions (blank, 0.05, 0.1, 1, 10, 25, 50, 100 μg/L) prepared in nitric acid (2%). Rhodium (10 μg/L) was added as an internal standard to all samples and all calibration solutions to compensate instrumental drift and signal depression from matrix ions. The calibration curves were calculated by linear regression.

EPR Measurements. EPR (X band) spectra were recorded on a Bruker ECS 106 spectrometer equipped with an ECS 041 MR Bruker microwave bridge and an Oxford Instruments ESR 900 helium flow cryostat. All spectra were recorded at 9.45 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 1 mT, a conversion time of 0.04 s, and a time scale of 0.08 s. All other experimental conditions (temperature, microwave power, and receiver gain) are given in the Results section.

Redox Titrations. Redox titrations were performed with the aid of a combined platinum−Ag/AgCl electrode (PT 4800-M5-S7/80, Mettler Toledo, Steinbach, Germany) as described previously (*39*). All potentials were quoted relative to the standard hydrogen electrode. Prior to the redox titration, the protein samples were subjected to buffer exchange by gel filtration on Sephadex G-25 equilibrated with HEPES buffer (50 mM, pH 7.4) containing $Na_2S_2O_4$ (1 mM). It is pertinent to note that the reducing agent was not entirely removed from the protein preparations in view of their extreme lability in the presence of trace amounts of oxygen. For the sake of direct comparison, MoFe and WFe protein samples were treated under analogous conditions. The final sample solution (3 mL) containing a number of redox mediators (*39*) and 2 mg of protein per mL was adjusted to the desired redox potentials by the stepwise addition of a $K_3[Fe(CN)_6]$ solution as oxidant and $Na_2S_2O_4$ as reductant. After equilibration (i.e., after a constant potential of the solution had been achieved), which usually required 2 min of incubation, 170-μL samples were withdrawn from the titration solution with a gastight syringe, placed in an Ar-pregassed EPR tube and immediately frozen in liquid nitrogen.

The relative concentration of paramagnetic species was determined by measuring the amplitude of the $g = 3.67$ zero-crossing feature in the EPR spectrum for quantitation of the FeMoco signal and that of the $g = 3.93$ resonance for quantitation of the FeWco signal. The quantitation of the $S = 1/2$ signal of the one-electron-oxidized P cluster was achieved similarly, using the $g = 1.96$ zero-crossing feature.

RESULTS

Influence of Tungstate on the Catalytic Activity of Nitrogenase. Tungstate has been shown to influence nitrogenase activity and expression, as well as molybdate-uptake in

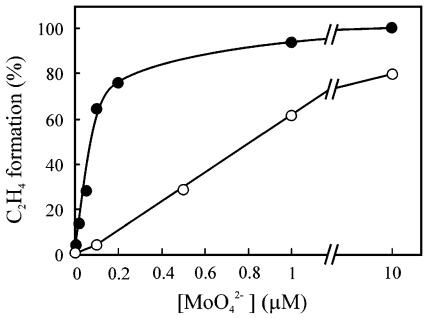

FIGURE 1: Dependence of nitrogenase activity on the MoO_4^{2-} concentration in the growth medium in the absence and presence of WO_4^{2-}. *anfA* cells were cultivated under anaerobic, phototrophic conditions in the presence of serine (final concn: 5 mM). The molybdate concentration was varied as indicated in the figure. The formation of C_2H_4 was measured with whole cells of the late log phase as outlined in the Materials and Methods section. 100% activity corresponded to 2500 nmol C_2H_4 produced mL^{-1} h^{-1} (i.e., approximately 100 nmol/mg min). ●, absence of WO_4^{2-}; ○, presence of WO_4^{2-} (final concn: 10 μM).

Table 1: Influence of Tungstate in the Growth Medium on the Catalytic Activity of Nitrogenase in *anfA* Cells

cultivation in the presence of[a]		acetylene reduction to ethylene[b]	
MoO_4^{2-} (μM)	WO_4^{2-} (μM)	nmol h^{-1} mL^{-1}	%
1		1770	100
1	1	1450	82
1	10	1240	70
1	100	920	52
1	500	445	25
1	1000	90	5
<0.005[c]		125	7
<0.005[c]	1000	4	0.2

[a] Cells were cultivated under anaerobic, phototrophic conditions in the presence of serine (5 mM), and their activity (production of ethylene from acetylene) was determined in the late log growth phase. [b] The precision of the acetylene reduction rate determinations was within the range of ±10%. [c] Cells were grown without added molybdate. The residual Mo concentration in the medium, resulting from Mo impurities in chemicals, was estimated (by ICP−MS) to be less than 5 nM.

various organisms (*19*−*22*, *40*). In view of the lack of a comparative study on the effect of tungstate (and molybdate as reference) in the growth medium on nitrogenase activity of whole cells of *R. capsulatus*, we addressed this aspect by cultivating the *anfA* mutant of this organism with varying concentrations of molybdate in the absence and presence (final concentration 10 μM) of tungstate. The mutation in *anfA* renders the cells incapable of expressing the iron-only nitrogenase of *R. capsulatus* (*25*, *26*). Serine, which has previously been shown not to repress nitrogenase synthesis (*28*), was generally used as the source of nitrogen instead of N_2 to guarantee cell growth also in the absence of molybdate.

As indicated in Figure 1, the presence of tungstate in the medium significantly altered the dependence of nitrogenase activity (C_2H_2-reduction assay) on the Mo concentration. In the absence of tungstate, half of the maximal activity (1250 nmol ethylene produced per hour per mL cell suspension) was observed with cells supplemented with 0.08 μM MoO_4^{2-}. However, when cells were grown at this molybdate concentration in the presence of tungstate (10 μM), their activity was found to be marginal. To achieve 50% activity with WO_4^{2-}-containing cultures, a concentration of ca. 0.7 μM MoO_4^{2-} was required. Hence, in the presence of tungstate, the concentration of MoO_4^{2-} in the growth medium needs to be increased almost 10-fold to reach activities comparable to those observed in its absence (Figure 1).

When cells of the *anfA* mutant were cultivated at a constant concentration of MoO_4^{2-} (1 μM) and the concentration of tungstate in the growth medium was varied (0−1 mM), a strong inhibitory effect on nitrogenase activity was only observed with a >100-fold excess amount of tungstate over molybdate (Table 1). At a concentration of 1 mM tungstate (1000-fold excess), the activity was found to be ∼5% of that recorded in its absence. Cultures to which neither molybdate nor tungstate was added exhibited a similarly low activity (7%). This residual activity appears to arise from traces of Mo impurities (3−4 nM) brought into the medium by other chemicals. A Mo-starved but tungstate (1 mM)-supplemented culture showed only marginal nitrogenase activity (∼0.2%).

It is interesting to note that acetylene saturation curves recorded for both the highly active Mo cells and the marginally active Mo-starved W cells were almost identical (data not shown) and yielded similar K_M values (∼0.55 ± 0.07 mM). This result suggests that in W cells acetylene reduction to ethylene is due to the presence of traces of FeMoco-containing nitrogenase molecules rather than to the catalytic activity of a tungsten nitrogenase.

Influence of Tungstate and Molybdate on the Expression of the Nitrogenase Component Proteins. An essential prerequisite for the isolation of a tungsten-substituted nitrogenase from *R. capsulatus* concerns the expression of component proteins in a MoO_4^{2-}-depleted growth medium supplemented with WO_4^{2-}. Hence, the aspects that needed to be considered in the studies designed to achieve expression of the tungsten−nitrogenase are (i) Is the biosynthesis of MoFe and Fe protein Mo-regulated (i.e., is Mo essential not only for the production of a catalytically active, cofactor-containing MoFe protein but also for the biosynthesis of the cofactorless apo-MoFe protein as well as the Fe protein) and (ii) if Mo serves as an inducer for the expression of these proteins, can tungstate also fulfill this role?

In this context, it is interesting to note that while the Mo regulation (repression) of the alternative iron-only nitrogenase has been extensively studied (*28*, *31*, *41*, *42*), virtually nothing is known regarding the effect of molybdate on expression of the conventional Mo-nitrogenase in *R. capsulatus*. Hence, the production of nitrogenase was examined at different MoO_4^{2-} and WO_4^{2-} concentrations in the growth medium, using SDS−PAGE and subsequent Western immunoblot analysis for the unequivocal identification of the MoFe protein.

In the first set of experiments, cells of the *anfA* mutant were cultivated in the presence of MoO_4^{2-} at various concentrations, and the cell-free extracts were analyzed by SDS−PAGE (Figure 2A) and Western immunoblots (Figure 2B). The amounts of detectable α (56 kDa)- and β (53 kDa)-subunits of the MoFe protein were found to increase with increasing concentrations of MoO_4^{2-} in the growth medium. In particular, Western blot analysis revealed the presence of significant amounts of MoFe protein subunits only when cells were cultivated in a growth medium supplemented with MoO_4^{2-}. Thus, Mo-starved cells were found to express extremely low amounts of MoFe protein (weak bands of the

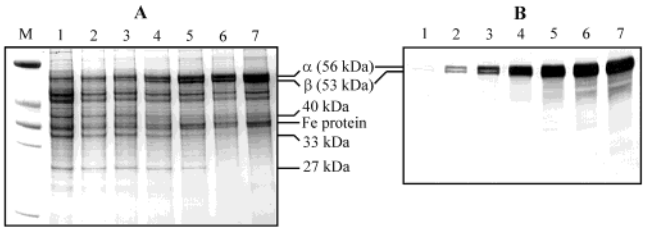

FIGURE 2: Analysis of the influence of MoO_4^{2-} on MoFe protein expression by SDS−PAGE (A) and Western immunoblot (B). *anfA* cells were cultivated in the presence of serine (2.3 mM) as well as MoO_4^{2-} in various amounts and harvested after 20 h of growth. After extract preparation, the soluble proteins were electrophoresed on two SDS−polyacrylamide gels under the same conditions. One of the gels was stained with Cooamassie brilliant blue (A), whereas the other gel was used for immunoblot analysis (B). For each sample, 30−40 μg of extract protein was electrophoresed. The protein samples for Western blot analysis were each diluted 1:5. M, protein marker (only in SDS gel); lane 1, cultivation in the absence of added MoO_4^{2-} (presence of traces of Mo impurities); lane 2, 0.01 μM MoO_4^{2-}; lane 3, 0.02 μM MoO_4^{2-}; lane 4, 0.05 μM MoO_4^{2-}; lane 5, 0.1 μM MoO_4^{2-}; lane 6, 0.2 μM MoO_4^{2-}; and lane 7, 1 μM MoO_4^{2-}.

subunits in Figure 2B, lane 1), presumably because of trace amounts of Mo impurities present in the growth medium.

It is pertinent to note that the weak band detected with the mobility corresponding to that of the α-subunit in lanes 1−3 in the SDS gel (extracts of cells cultivated in the absence or at very low MoO_4^{2-} concentrations) is likely to arise from a non-nif protein, in view of the following observations: (i) The protein could not be detected in Western blots using antibodies against the MoFe protein, (ii) SDS−PAGE performed with extracts prepared from wild-type cells grown in the presence of NH_4^+ (40 mM, i.e., under conditions of total nitrogenase repression) exhibited a pattern of bands identical with that obtained with extracts from Mo-starved cells grown on serine, and (iii) the mobility of the additional weak band is distinct from that of the α subunit band when SDS−PAGE is performed in Tris-tricine (data not shown).

The amount of MoFe protein present in the extracts, estimated by radial immunodiffusion (Materials and Methods), was in agreement with the data obtained from Western blots (Table 2). The MoFe protein content of cells cultivated under Mo-deficient growth conditions was ca. 6% of that of cells grown under optimal expression conditions (at 0.2−1 μM MoO_4^{2-}). This result is in excellent agreement with the residual activity determined with Mo-deficient cultures (Table 1).

In analogous electrophoresis and immunoblot experiments with antibodies against the dinitrogenase reductase, it was demonstrated that the formation of this component protein showed a very similar dependency on the MoO_4^{2-} concentration in the medium (data not shown). This result suggests that both nitrogenase components are co-regulated.

SDS−PAGE analysis of extracts from Mo-starved cells and those grown at low concentrations of molybdate (Figure 2A) revealed several bands corresponding to proteins with molecular weights of 27, 33, and 40 kDa, respectively. Since these proteins failed to react with antibodies raised against the MoFe protein, the possibility of them being proteolytically degraded MoFe protein fragments appears remote. Furthermore, since these proteins appear to be expressed only at low concentrations of molybdate, it is not inconceivable that they are part of the high-affinity molybdenum-transport

Table 2: Quantification of the MoFe Protein in Extracts of *anfA* Mutant Cells Cultivated in the Absence and Presence of MoO_4^{2-} and WO_4^{2-}

cultivation in the presence of[a]		MoFe protein content/ total protein content (%)[b]
MoO_4^{2-} (μM)	WO_4^{2-} (μM)	
<0.005		2.5
0.01		5
0.02		8
0.05		15
0.1		20
0.2		40
1.0		40
<0.005	1000	10
1.0	1000	15

[a] Cells were grown, and extracts were prepared as described in the legends of Figures 2 and 3. [b]The quantitation of the MoFe protein in extract preparations was achieved by radial immunodiffusion as outlined in the Materials and Methods section. The MoFe protein content was divided by the total protein content in the extracts (determined by the Biuret method, ref *34*). The error of these determinations was estimated to be <12%.

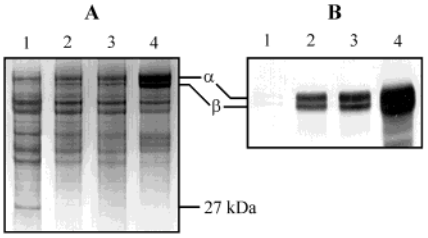

FIGURE 3: Analysis of the influence of WO_4^{2-} on MoFe protein expression by SDS−PAGE (A) and Western immunoblot (B). *anfA* cells were cultivated in the presence of serine (2.3 mM) as well as molybdate and/or tungstate in various amounts and harvested after 20 h of growth. After extract preparation, the soluble fractions were subjected to SDS−PAGE as outlined in the legend of Figure 2. Lane 1, cultivation in the absence of added MoO_4^{2-} (application of 30 μg of protein); lane 2, 1 mM WO_4^{2-} (26 μg of protein); lane 3, 1 μM MoO_4^{2-} + 1 mM WO_4^{2-} (28 μg of protein); and lane 4, 1 μM MoO_4^{2-} (40 μg of protein).

(Mod) system in *R. capsulatus*, which has been shown to be negatively regulated by MoO_4^{2-} (*41*). In fact, preliminary studies on Mo-uptake defective mutants revealed at least the 27 kDa protein to be a component, presumably the periplasmatic MoO_4^{2-}-binding protein ModA, of the Mod system (data not shown).

In the second set of experiments, the influence of WO_4^{2-} on the expression of nitrogenase structural genes was investigated. Of four cultures, two control cultures, one grown under Mo-deficient conditions and the other in the presence of 1 μM MoO_4^{2-}, were included in the study. Of the other two cultures, one contained only WO_4^{2-} (1 mM), while the second was grown on both WO_4^{2-} (1 mM) and MoO_4^{2-} (1 μM). The soluble fractions of the cell-free extracts were subjected to SDS−PAGE (Figure 3A) and Western immunoblot analysis (Figure 3B). In the case of the extract of Mo-deficient cells, only extremely weak α- and β-bands were detectable (Figure 3B, lane 1), whereas the extract of W-grown cells displayed relatively strong subunit bands (Figure 3B, lane 2), indicating that WO_4^{2-} also functions as an inducer for nitrogenase expression. However, the ability of WO_4^{2-} to induce nitrogenase synthesis is far less pronounced than that of MoO_4^{2-} (Figure 3, compare lanes 2 and 4). Quantitative radial immunodiffusion analysis (Table

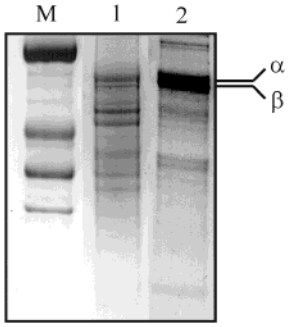

FIGURE 4: SDS−PAGE of the partially purified tungsten-substituted dinitrogenase. M, protein marker; lane 1, cell-free extract of cells grown in the presence of tungstate (1 mM); and lane 2, Q-sepharose eluate (300 mM NaCl fraction).

2) revealed that although the dinitrogenase/total protein ratio in WO_4^{2-}-grown cells was four times lower than that observed in cultures supplemented with MoO_4^{2-} (>0.2 μM), it was four times higher than that in cells cultivated in the absence of WO_4^{2-} and MoO_4^{2-}.

To exclude the possibility that the stimulation of nitrogenase synthesis in Mo-deficient medium was caused by traces of MoO_4^{2-} present in Na_2WO_4, a tungstate solution (1 mM) was analyzed by ICP−MS. Such analysis revealed the solution to contain MoO_4^{2-} at a concentration of only 5 nM. Hence, the induction of nitrogenase synthesis noted in the presence of tungstate appears to be due to the anion's stimulatory action and not to be attributable to trace amounts of extraneous Mo introduced into the growth medium by impure chemicals.

In conclusion, tungstate is capable of partially replacing molybdate in the induction of nitrogenase synthesis. It inhibits the latter's inducer activity, however, when present at high concentrations (compare lanes 3 and 4 of Figure 3B, and Table 2).

Purification and Characterization of the Tungsten-Substituted Nitrogenase. The ability of WO_4^{2-} to induce nitrogenase expression under Mo-deficient growth conditions provided the basis for the isolation and characterization of a tungsten−nitrogenase from *R. capsulatus*. The tungsten-substituted dinitrogenase component was isolated from *anfA* cells grown in Mo-deficient medium in the presence of WO_4^{2-} (final concn: 1 mM) and partially purified by chromatography on Q-sepharose. The majority of the dinitrogenase component was eluted with 300 mM NaCl, whereas the Fe protein (dinitrogenase reductase) was recovered in the 350 mM NaCl fraction. There was no difference between the elution profiles of crude extracts obtained from the *anfA* mutant grown in the presence of WO_4^{2-} and of extracts from the wild-type strain grown in the presence of MoO_4^{2-}. However, the yield of the dinitrogenase component from WO_4^{2-}-grown cells was relatively low (10−12 mg from a 6-L culture), owing to a diminished expression of the dinitrogenase component (see Table 2). SDS−PAGE analysis (Figure 4) revealed the dinitrogenase component to be the predominant protein (with an estimated purity of approximately 70%) and to be virtually devoid of the Fe protein.

In an attempt to obtain homogeneous protein preparations and to determine the quaternary structure of the tungsten-substituted dinitrogenase, the 300 mM NaCl fraction was subjected to gel filtration on Sephacryl S-200. The dinitrogenase component was recovered from the above gel filtration matrix at an effluent volume indicative of the protein's presence in the tetrameric state (M_r ~200−250 kDa). Significant further purification of the protein was, however, neither achieved by gel filtration on Sephacryl S-200 nor by other methods such as chromatography on Octyl-Sepharose. More importantly, such procedures tended to result in a substantial destabilization of the dinitrogenase component, as indicated by the partial loss of tungsten and iron. Thus, the W-containing protein appears to be more labile than the MoFe protein, and its clusters may be destroyed through the influence of extreme dilution (as in the case of the gel filtration) and/or high salt concentrations (employed in chromatographic procedures involving Octyl-Sepharose). In light of these observations, the preparation recovered from Q-sepharose was used for the subsequent investigations.

ICP−MS analysis of the (partially) purified dinitrogenase component revealed the presence of 1 (±0.15) W, 16 (±2) Fe, and less than 0.01 Mo atoms per protein tetramer. These values were estimated on the basis of the dinitrogenase concentration (determined by radial immunodiffusion) in the sample used for metal analysis. Since previous studies have shown that the material recovered from Q-Sepharose with 300 mM NaCl is free of other iron-containing proteins (*32*), the detection of 1 W and 16 Fe atoms appears to be indicative of the presence of one FeW cofactor (WFe_7S_9) and one P cluster (Fe_8S_7) molecule on average per tetramer. Hence, the designation of this protein component as a tungsten dinitrogenase or WFe protein appears justified. It is pertinent to note that the incorporation of just one single FeW cofactor molecule into a tungsten-substituted dinitrogenase has also been reported for the *A. vinelandii* protein (*23*). However, in the latter case the second cofactor binding site was occupied by the FeMo cofactor (the relevant dinitrogenase preparation contained equal amounts of W and Mo), whereas in the *R. capsulatus* preparations significant amounts of molybdenum, and thus, of the FeMoco were absent.

C_2H_2-reduction assays performed with concentrated tungsten−dinitrogenase samples revealed the protein to be only marginally active (1 nmol ethylene formed per min per mg of protein). Thus, the protein, in agreement with the in vivo activities, was approximately 1000-fold less active than the original FeMoco-containing enzyme (*30*). N_2 reduction was not detected.

Despite such negligible catalytic activities, the tungsten−nitrogenase exhibited relatively high rates of H_2 evolution. Under an Ar atmosphere in the absence of an added substrate and at a 2-fold molar excess of the Fe protein, a specific activity of 102 nmol H_2/min/mg of protein was determined. After appropriate corrections for the contribution from the protein impurities associated with the 300 mM NaCl fraction, this value corresponds to approximately 25% of the H_2-evolving activity reported for the native MoFe protein (*30*). Furthermore, H_2 production was not inhibited by acetylene, a finding that is in accordance with it not being a substrate of the tungsten−nitrogenase. In contrast, the H_2 evolution catalyzed by the Mo nitrogenase of *R. capsulatus* was almost completely inhibited in an atmosphere containing 20% (v/v) acetylene (*30*).

EPR Spectroscopic Characterization of the Tungsten−Substituted Nitrogenase. Strong support for the incorporation

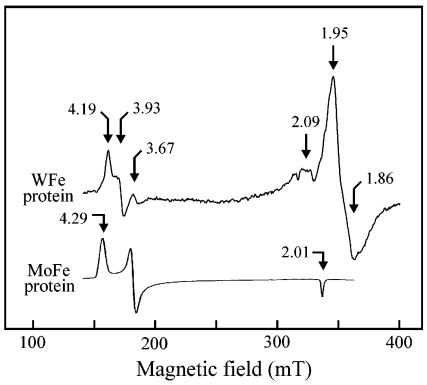

FIGURE 5: EPR spectrum of the WFe protein. The protein sample contained 20 mg of dinitrogenase protein per mL (in 300 mM NaCl elution buffer, pH 7.6). The spectrum was recorded at 4 K with a microwave power of 20 mW, a receiver gain of 2.5×10^5, and 5 scans. The other experimental conditions are given in the Materials and Methods section. For comparison, the low field spectrum of the FeMo cofactor (bound to the MoFe protein isolated from B10S) is presented below the main spectrum.

Table 3: *g* Values of EPR-Active Species in Tungsten-Substituted Dinitrogenase Preparations from *R. capsulatus* and *A. vinelandii*

	R. capsulatus	*A. vinelandii*[a]
	g values	
FeWco ($S = 3/2$)	4.19, 3.93, (2.01)[b]	4.22, 3.95, (2.01)[b]
FeMoco ($S = 3/2$)	(4.29),[c] 3.67, (2.01)[b]	4.33, 3.68, (2.01)[b]
$S = 1/2$ signal	2.09, 1.95, 1.86	2.10, 1.92, 1.85

[a] *g* values taken from ref *23*. [b] Value of the resonance, given in parentheses, was undetectable because of interference with the $S = 1/2$ signal. [c] This resonance was unresolved because of the low FeMoco concentration in the sample and interference with the FeWco signal.

of tungsten into the cofactor of the W-containing nitrogenase is derived from the EPR spectrum of the $Na_2S_2O_4$-reduced WFe protein, which significantly differs from that of the MoFe protein (*39*). As shown in Figure 5, two dominant EPR signals, an $S = 3/2$ and an $S = 1/2$ signal, were detectable in the spectrum of the WFe protein. The $S = 3/2$ signal ($g = 4.19$ and 3.93) is indicative of the presence of an FeWco. The putative third $g_{(x)}$ value of this rhombic signal at high field, which is expected to be situated near $g = 2.01$, is completely obscured by the $S = 1/2$ signal (Figure 5). The $S = 3/2$ signal of the WFe protein resembles that of the MoFe protein ($g = 4.29$, 3.67, 2.01), the spectrum of the latter species also being shown in the figure for comparison. The small peak at $g = 3.67$ within the WFe protein spectrum indicates the presence of a slight contamination of the WFe protein with the FeMo cofactor. This observation is consistent with the demonstration of residual catalytic activities in such dinitrogenase preparations and the low Mo content determined by ICP−MS. The *g* values of the FeW cofactor signal, which are in closer proximity than the *g* values of the FeMo cofactor signal, reflect a significantly higher rhombicity of the W-containing cluster. A very similar FeWco-associated signal has been observed in the case of the tungsten-substituted *A. vinelandii* dinitrogenase (see Table 3). Because of the higher molar Mo/W ratio in the latter case, the FeMoco signal was, however, significantly stronger than that of the FeWco (*23*).

The FeMo cofactor of the $Na_2S_2O_4$-reduced MoFe protein is present in the semi-reduced state. Under enzymatic turnover conditions (i.e., in the presence of MgATP, dinitrogen, and the Fe protein), the MoFe protein is further reduced, and thus, capable of reducing dinitrogen. During turnover at a molar excess of the MoFe protein (low electron flux), the intensity of the FeMoco EPR signal has been shown to be lowered by approximately 2/3 of its original intensity because of the reduction of 2/3 of all cofactor molecules, leading to an EPR silent integer spin state (*39*, *43*). When the WFe protein was subjected to similar turnover conditions, the FeWco signal intensity decreased only slightly (~20%), whereas the small FeMoco signal disappeared almost completely from the EPR spectrum (Figure 6). The drastic reduction of the FeMoco signal led to a purer FeWco spectrum, showing a more pronounced peak at $g = 3.93$. The results presented above demonstrate that (a) the peak at $g = 3.67$ observed in the spectrum of the dithionite-reduced sample is in fact due to an FeMoco contaminant and (b) under the turnover conditions applied, the FeWco cannot be as readily reduced enzymatically to an EPR-silent state as the FeMoco.

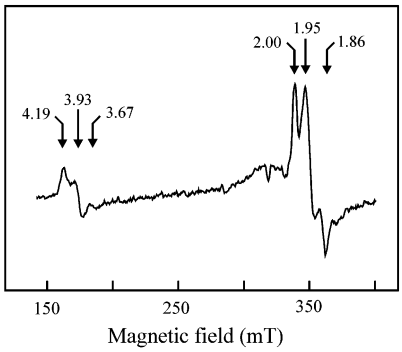

FIGURE 6: EPR spectrum of the WFe protein under turnover conditions. The sample contained 15 mg/mL tungsten-substituted dinitrogenase protein, 0.5 mg/mL Fe protein (isolated and purified from B10S), ATP (10 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), $Na_2S_2O_4$ (6 mM), 200 μg of creatine kinase, and N_2 in HEPES buffer (100 mM, pH 7.6). The reaction was initiated by the addition of ATP and incubated for 2 min at room temperature under N_2 atmosphere. The spectrum was recorded as outlined in the legend of Figure 5.

The broad $S = 1/2$ signal located in the high field region ($g = 1.8-2.1$) is the most prominent signal in the EPR spectrum of the *Rhodobacter* WFe protein (Figure 5) and differs fundamentally from conventional P cluster signals (*12*, *39*, *44*). A very similar signal has also been observed with the analogous W-containing protein from *A. vinelandii*, which was termed S3 signal and was tentatively assigned to an Fe−S cluster (*23*). It is important to note that in the case of the *Rhodobacter* protein, the line shape of this signal in the turnover spectrum was different from that depicted in Figure 5 (dithionite-reduced state). Instead of one broad peak, the $S = 1/2$ signal showed three resolved peaks at $g = 2.00$, 1.95, and 1.86 (Figure 6). As the high field region of the turnover spectrum has not yet been published for the *A. vinelandii* protein, it remains uncertain whether a change in line shape would be also observable in that case.

For further characterization of the signals in the EPR spectrum of the dithionite-reduced WFe protein, the temperature and microwave power dependencies of signal intensities were investigated. As presented in Figure 7A, the intensity of the FeWco signal decreased with increasing temperatures. Maximal signal intensity was observed at 4 K. In a comparative study, the maximum intensity of the

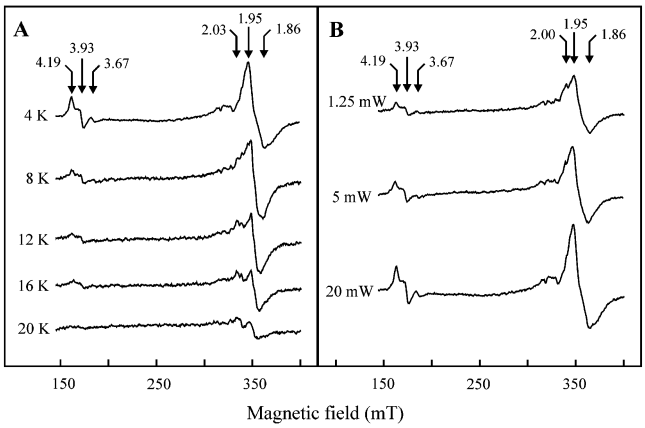

FIGURE 7: Temperature and microwave power dependence of EPR signals of the WFe protein. The protein sample contained 20 mg of dinitrogenase protein per mL (in 50 mM Tris/HCl buffer, pH 7.6, + 4 mM $Na_2S_2O_4$). Temperature dependence (A): The spectra were recorded at temperatures indicated in the figure with a microwave power of 20 mW and a receiver gain of 10^5. Microwave power dependence (B): The spectra were recorded with 1.25, 5, and 20 mW at 4 K and a receiver gain of 2.5×10^5, and 5 scans.

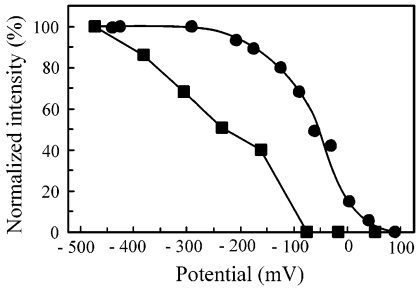

FIGURE 8: Redoxtitration of the FeWco signal from the WFe protein and the FeMoco signal from the native MoFe protein. The redox titration was performed as described in the Materials and Methods section. The spectra of the tungsten-substituted protein (2 mg/mL) were recorded at 4 K with 20 mW and a receiver gain of 2.5×10^5. The data collected from the redoxtitration of the native enzyme (*39*) are included in this figure. The $g = 3.93$ resonance was used for the determination of relative intensity of the FeW cofactor signal. ■, FeWco (values at potentials > -100 mV were arbitrarily set to zero since the amplitude of the FeWco signal could not be determined accurately because of noise); ●, FeMoco.

FeMoco signal, measured with the native enzyme, was also found to be at 4 K. However, the intensity of the FeMoco signal decreased more rapidly with increasing temperatures compared to that of the FeWco signal (data not shown). Power saturation studies revealed the FeWco signal to be only slightly saturated at powers >5 mW (Figure 7B). In contrast, saturation effects of the FeMoco signal of the native enzyme were clearly observable at 5 mW (data not shown). In conclusion, both microwave power and temperature dependence of the intensities of the FeMoco and FeWco EPR signals were similar but not identical.

Maximal intensity of the broad $S = 1/2$ signal was also observed at 4 K (Figure 7A). At higher temperatures, a resonance centered at $g = 2.03$ appeared in the spectrum, indicating that this signal arises from at least two different EPR-active species. In contrast to the FeWco signal, the $S = 1/2$ signal was easily saturated at microwave powers as low as 1 mW.

For the characterization of the redox behavior of the FeW cofactor, the tungsten-substituted nitrogenase from *R. capsulatus* was subjected to redox titrational analysis. The data obtained were compared to those collected with the FeMo cofactor of the unaltered MoFe protein (*39*). The redox titration curves of both cofactors ($S = 3/2$ signals) are presented in Figure 8. The midpoint potential (E_m) of the transition of the semi-reduced to the oxidized state of the FeMoco present in the Mo enzyme of *R. capsulatus* has been determined to be approximately −50 mV. Maximal intensity of the $S = 3/2$ FeMoco signal was reached at potentials lower that −300 mV. In the range from −470 (lowest possible redox potential under the applied conditions) to −300 mV, the signal intensity was constant. This observation is in marked contrast to the results obtained with the FeWco present in the tungsten-substituted nitrogenase. With increasing redox potentials (starting from −470 mV), the intensity of the FeWco signal was found to continuously decrease. At potentials higher than −100 mV, the intensity of the FeWco signal could not be reliably assessed because of a low signal-to-noise ratio. When the oxidized sample (+50 mV) was rereduced to −470 mV, the original signal intensity of the FeWco was fully restored. Thus, the FeWco can be reversibly oxidized and reduced in the range from −470 to +50 mV.

It is important to note that the recorded FeWco signal intensities do not appear to obey the Nernst relationship. The origin of this phenomenon is presently unknown. Since it has been assured that a redox equilibrium was established at the time of sample collection (see Materials and Methods), the non-Nernst character of the redox curve is not a consequence of a nonequilibrium titration.

The negative slope of the redox curve at low potentials suggests that maximal intensity of the FeWco signal could not be obtained in the course of the redox titration. Hence, the relative signal intensity at −470 mV was arbitrarily set to 100%. On the basis of this definition, the calculated midpoint potential was approximately −200 mV (50% of the measurable signal intensity). Compared to the E_m of the FeMoco (−50 mV), the midpoint potential of the FeWco is thus shifted by at least 150 mV to more negative potentials. These observations provide the first direct evidence for the FeWco not undergoing reduction as readily as the FeMoco.

During the course of the redox titration, an $S = 1/2$ signal indicative of the P cluster in its one-electron-oxidized state ($g = 2.06, 1.96, 1.84$) was observed at 16 K in the range between −300 to +50 mV. The g values as well as the redox behavior appeared to be indistinguishable from those of the P clusters present in the FeMoco-containing dinitrogenase (ref *39*, data not shown). In conclusion, the replacement of Mo by W in the cofactor dramatically influences its redox behavior, whereas that of the P clusters appears unaffected.

A reliable redox titrational analysis of the broad $S = 1/2$ signal (see Figure 5) was not possible under the experimental conditions employed because of an overlap by the P cluster signal as well as by a $g = 2.01$ radical signal arising from the electron mediators present in the sample.

Can Molybdenum Be Replaced by Rhenium? On the basis of the chemical similarities between Re and Mo, it has been speculated that a Re-containing nitrogenase (Re as part of a FeReco) may be (i) formed under suitable conditions and (ii) capable of reducing dinitrogen (*45*). Especially the potential of both Mo and Re to participate in multielectron-transfer processes, a crucial feature for nitrogenase function,

makes the above-noted hypothesis attractive (*45*, *46*). However, preliminary studies on cells of the *anfA* mutant of *R. capsulatus* revealed that neither cell growth nor in vivo nitrogenase activity was significantly influenced by the presence of $KReO_4$ (up to 1 mM) in the growth medium. Because of the lack of any significant effects of perrhenate on nitrogenase activity, the ability of *Rhodobacter* cells to assimilate this anion was investigated.

Three cultures of the *anfA* mutant were cultivated, one in the presence of ReO_4^- (10 μM) and the others in the absence and presence of MoO_4^{2-} (1 μM), respectively. Prior to cell disruption by NaOH, the cells were subjected to treatment with acetone to remove both the photosynthetic and the carotinoid pigments that would interfere with protein determination (*33*), as well as the *R. capsulatus*-specific slimy capsula, which has been found to adsorb molybdate, and to a smaller extent, also perrhenate (data not shown). Following cell disruption and removal of cell debris by centrifugation, the Mo and Re content of the supernatant was determined by ICP−MS. These studies revealed that cells grown in the absence of supplemented Mo contained ~10 nmol Mo/g of protein, whereas the Mo content of cells cultivated in the presence of molybdate (1 μM, i.e., at optimal concentration) was estimated to be ~350 nmol/g of protein. In contrast, the Re content of cells grown in a perrhenate-containing medium (10 μM) was found to be less than 0.1 nmol of the metal ion per gram of protein. These results indicate (i) the inability of *Rhodobacter* cells to accumulate the perrhenate ion, an observation consistent with the lack of any significant effects of this anion on both cell growth and nitrogenase activity and (ii) the ability of *Rhodobacter* cells to effectively assimilate molybdate from traces of this anion present in the growth medium (note that the Mo content of Mo-deprived cells is 2 orders of magnitude greater than the Re content of Re-grown cells). In conclusion, investigations on the replacement of Mo by Re in nitrogenases using whole cells of *R. capsulatus* appear to be futile by virtue of their inability to assimilate perrhenate ions. Apparently, the transport system(s) for molybdate and tungstate are not effective in the uptake of perrhenate. Future studies may therefore be directed toward the in-vitro incorporation of Re into the cofactor using cell-free extracts. Alternatively, whole cells of other diazotrophic organisms such as those of *A. vinelandii* may be employed.

DISCUSSION

The inhibitory effect of tungstate on the growth of diazotrophic organisms has been well-documented. For instance, it has been demonstrated that WO_4^{2-} inhibits MoO_4^{2-} uptake in *A. vinelandii* (*19*) and that tungsten is incorporated into nitrogenase, rendering the protein inactive (*20*, *22*). Several spontaneous W-resistant mutants have been reported to grow diazotrophically in the presence of WO_4^{2-} (*23*, *47*, *48*); however, a recent study has revealed that the ability of these mutants to fix nitrogen is due to the expression of an alternative nitrogenase and that W tolerance is based on a defect in one of the genes encoding the Mo-transport system (*48*).

Direct evidence for the incorporation of tungsten into nitrogenase (i.e., the cofactor) has so far been only provided in the case of *A. vinelandii* (*23*). The isolated tungsto−protein, however, has been shown to contain molybdenum and tungsten in equal amounts. The difficulty to impose Mo-depleted conditions on *A. vinelandii* cells and hence to isolate a Mo-free tungsten-substituted nitrogenase may have been due to the presence of a high-affinity Mo-storage protein. Similar studies with other organisms have not been reported, and more importantly, a Mo-free tungsto−nitrogenase has yet to be isolated.

In view of the lack of a Mo-storage protein and the availability of an *anfA* mutant, which is incapable of expressing the Fe-only nitrogenase under conditions of Mo deprivation, *R. capsulatus* was chosen in the current investigations to isolate and characterize a tungsten-substituted nitrogenase. To optimize the conditions for its production in this organism, the influence of both WO_4^{2-} and MoO_4^{2-} on nitrogenase activity and expression was investigated. The results presented in this study demonstrate that WO_4^{2-} inhibits nitrogenase activity in a competitive manner, a finding previously documented for *A. vinelandii* to arise from the competition of both anions for oxoanion transport into the cell (*19*). The inability of ModA, the periplasmatic MoO_4^{2-}-binding protein component of the high-affinity molybdenum-transport system, to discriminate between MoO_4^{2-} and WO_4^{2-} appears to be the basis for such competition and is mainly due to the similar size of both anions (*49*). Recently, a transport protein (TupA), which is highly specific for tungstate but not for molybdate, has been identified in *Eubacterium acidaminophilum* (*50*). Although the molecular basis for such specificity is currently unknown, the observation of this phenomenon demonstrates that discrimination between both anions is also possible on the oxyanion level.

The production of a tungsten-substituted nitrogenase requires the structural genes encoding the protein to be expressed under Mo-deficient growth conditions in the presence of WO_4^{2-}. While MoO_4^{2-} is not required for the synthesis of nitrogenase proteins in *K. pneumoniae* (*51*), it appears to be essential for the expression of the Mo-dependent nitrogenase structural genes in *A. vinelandii* (*52*). In the current study, MoO_4^{2-} was found to be an absolute necessity for nitrogenase synthesis in *R. capsulatus*. Although disconcerting at first glance in view of the lack of the MoFe protein in cells cultivated under Mo-deficient conditions, the observation of WO_4^{2-} being capable of inducing nitrogenase synthesis in *R. capsulatus* (in the absence of MoO_4^{2-} in the growth medium) served as the basis for the isolation and characterization of a tungsten-substituted nitrogenase from this organism.

Metal analysis of the isolated protein indicated that (i) molybdenum is not incorporated into the cofactor in significant amounts, (ii) on average only one cofactor binding site is occupied by an FeW cofactor molecule, while the second one appears to be empty, and (iii) on average only one P cluster molecule is present in the protein tetramer. It is interesting to note that the incorporation of just one single FeWco molecule has also been reported for the tungsten-substituted *A. vinelandii* nitrogenase (*23*), although in this case the second cofactor binding site has been demonstrated to be occupied by an FeMoco molecule. The observations recorded in the present study on the *Rhodobacter* nitrogenase may be construed to indicate that only one FeWco and one P cluster molecule are incorporated into the WFe protein.

Alternatively, half of the protein molecules may be fully assembled ($\alpha_2\beta_2$ tetramers harboring two P cluster and two FeWco molecules), whereas the other half may be devoid of any cluster species. Because of the increased lability of the WFe protein (see section on enzyme purification and characterization) and partial denaturation of incompletely assembled (with respect to cofactor insertion) WFe protein molecules, the latter possibility appears to be more plausible.

The most compelling evidence for tungsten incorporation into the cofactor of the *R. capsulatus* dinitrogenase derives from its EPR spectrum. A dominant $S = 3/2$ signal at $g = 4.19, 3.93$ (~2.01; obscured), which distinctly differs from that displayed by the FeMoco ($g = 4.29, 3.67, 2.01$), was observed in the spectrum. This signal has also been detected in the EPR spectrum of the tungsten-substituted *A. vinelandii* nitrogenase and has been proposed to arise from the FeW cofactor (*23*). The assignment of the $S = 3/2$ signal to the FeWco in the *R. capsulatus* spectrum, however, is not solely based on a comparison with the *A. vinelandii* spectrum. Studies with several synthetic Mo/Fe/S and W/Fe/S clusters (with $S = 3/2$) have revealed the EPR signals recorded with the tungsten-containing species to be narrower (less rhombic) than those measured with the analogous Mo clusters (*53*−*55*). This observation is also in agreement with theoretical considerations (*55*).

As regards the broad $S = 1/2$ feature in the EPR spectrum, the observation of its average g value being less than 2 (see Table 3) suggests that this signal derives from a reduced Fe/S cluster. Whether such a signal originates from an incompletely assembled cofactor and/or from a decomposition product of the FeWco (note that the temperature dependence depicted in Figure 7 indicated the presence of at least two EPR-active species in the spectrum) still remains to be established. As this type of spectrum has never been observed with MoFe proteins but only with W-containing dinitrogenases (present work and ref *23*), it is likely that the $S = 1/2$ signal is related to the FeW cofactor rather than the P cluster. The possibility of a mixture of signals of both FeWco and P cluster fragments can, however, not be excluded at the present time.

Activity measurements performed with the tungsten-substituted *R. capsulatus* nitrogenase revealed the protein to be only marginally active in mediating C_2H_2 reduction. The noted residual activity may be due to the incorporation of the FeMoco into some protein molecules. However, the possibility of the FeW cofactor serving as the site of C_2H_2 reduction to a very small extent cannot be completely excluded at the present time. N_2 reduction was not observed.

The notion that an FeWco-containing dinitrogenase should be inactive because of the difficulty to sufficiently reduce the cofactor is widespread (*45*). This view derives support mainly from basic knowledge on Mo/W chemistry (recently reviewed in ref *56*) and from comparative studies on W-containing complexes (*53*, *54*) as well as tungsten-substituted forms of the Moco-containing enzymes, sulfite oxidase (*57*) and DMSO reductase (*56*), which generally possess more negative midpoint potentials than their Mo counterparts. For instance, the midpoint potentials of the Mo and W forms of DMSO reductase have been shown to differ by ~325 mV (*56*). The first concrete evidence that also the midpoint potential of the FeWco is lower than that of the FeMoco was obtained from the redox titrational data and the turnover experiments presented in the current study. The replacement of Mo by W in the cofactor was found to result in a decrease of the E_m value by at least 150 mV. At redox potentials > −470 mV, only a portion of all FeWco molecules appears to be in the semi-(dithionite)reduced state. This result is in accordance with the observation that, despite the vast excess of tungsten over molybdenum in the protein (W/Mo > 100), the intensity of the FeWco EPR signal is only 5-fold of that noted with FeMoco. In agreement with the above considerations, the amplitude of the FeWco signal, detected with the *A. vinelandii* protein, was significantly smaller than that of the FeMoco signal, although both cofactors have been reported to be incorporated into the protein in equal amounts (*23*). Furthermore, under turnover conditions (i.e., in the presence of the Fe protein, ATP, and N_2), the intensity of the FeWco signal ($S = 3/2$) was only slightly (~20%) diminished, whereas that of the FeMoco signal was reduced to approximately 1/3 of its original intensity. This result confirms that the FeWco is less prone to enzymatic reduction than the FeMoco.

Despite the low midpoint potential of the FeWco and the negligible catalytic activities with respect to C_2H_2 and N_2 reduction, the tungsten-substituted nitrogenase was found to effectively reduce protons to hydrogen at a rate approximately 25% of that noted in the case of the native FeMoco-containing MoFe protein (*30*). This unprecedented observation suggests that (i) the W-substituted nitrogenase is indeed a true enzyme in that it catalyzes the reduction of protons to hydrogen and (ii) the generation of H_2 is uncoupled from the reduction of C_2H_2 or N_2. As regards the latter aspect, the uncoupling of H_2 evolution from N_2 and C_2H_2 reduction has so far not been observed. The above-mentioned slight (but not negligible) reduction of the intensity of the FeWco signal under turnover conditions may be a reflection of ~20% of the cofactor molecules being reduced to a state from which proton, but not C_2H_2 or N_2 reduction, can occur. The observation that the WFe protein reduces protons but not C_2H_2 or N_2 is in line with the generally accepted Lowe−Thorneley mechanism for the nitrogenase catalytic cycle (*58*−*61*), in which two consecutive coupled electron and proton-transfer events lead to the generation of a two-electron reduced MoFe protein species (E_2H_2). E_2H_2 is proposed to undergo further reduction with concomitant proton transfer to form E_3H_3, which is capable of binding N_2 upon the release of one H_2 molecule. However, under conditions of low electron flux (e.g., at a MoFe protein/Fe protein molar ratio of >5:1), E_2H_2 may be reoxidized to E_0 upon the release of H_2 before it can undergo further reduction to E_3H_3 (*62*, *63*). The catalytic competence of the WFe protein with respect to H_2 evolution may thus be a reflection of the protein being partly reduced, at least, to its E_2H_2 stage. Slow and less effective reduction of the cofactor may also form the basis for the observed low N_2/C_2H_2 but relatively high H^+ reduction rates in the case of the vanadium and in particular the iron-only nitrogenases (*30*, *64*).

Although the site of N_2 binding and reduction remains a subject of debate, models (mainly based on theoretical calculations) in which the substrate binds to the unusual trigonal prismatic FeS core of the cofactor rather than to the Mo atom itself have been favored (see ref *65* for review). It is pertinent to note, however, that a very recent high-resolution crystallographic analysis of the MoFe protein (*66*)

has revealed the presence of a light atom [presumably nitrogen (*66*, *67*)], ligated to the six Fe atoms of the trigonal prism. Such a feature would allow for a tetrahedral coordination environment around the six Fe atoms instead of the earlier accepted trigonal arrangement. In view of these findings, considerable caution appears warranted in the interpretation of theoretical calculations on the mode of N_2 binding since these are based on the FeMoco having coordinatively unsaturated (trigonal) Fe atoms. Nonetheless, the marked difference in the catalytic profiles and the redox properties of the MoFe and WFe proteins recorded in the present study clearly underscore the importance of the type of heterometal atom in the cofactor of nitrogenase for catalytic function. A recent density functional theory (DFT) study on the association of N_2 to the Mo center after dissociation of one of the carboxyl groups of homocitrate (albeit still based on the trigonal coordination of the six central Fe atoms) has revealed that binding and partial reduction of the substrate is more likely to occur on Mo than on the central Fe atoms (*68*). If one of the crucial steps in the catalytic mechanism is indeed the partial dissociation of homocitrate from Mo with subsequent binding of N_2 to the heterometal atom, as proposed by Grönberg et al. (*69*), then the replacement of Mo by W in the cofactor would likely lead to a decrease in the rate of the metal−oxygen-(homocitrate) bond cleavage because of the higher activation energy required to break a W−O bond relative to a Mo−O bond (*56*) and may thus result in significantly diminished activities.

Although the above considerations as well as the observations reported in the current study appear to suggest that functioning W nitrogenases do not exist in nature, it is interesting to note that the methanogen *Methanococcus thermolithotrophicus* is capable of fixing nitrogen in the presence of tungstate (*70*). The nitrogenase of this organism differs significantly from all known nitrogenase systems in that it is able to catalyze N_2 reduction at high temperatures (∼60 °C). Furthermore, the α subunit of the *Methanococcus* dinitrogenase component exhibits only ca. 40% similarity (on the amino acid level) with that of Mo nitrogenases of several other organisms (*71*).

The relatively recent discovery of tungsten as an essential trace element for some hyperthermophilic microorganisms has led to intensive investigations in the field of tungstoproteins in the past decade. In all naturally occurring W-containing enzymes investigated so far, the tungsten center has been shown to be coordinated to a molybdopterin moiety, which is common to all molybdoenzymes, except for nitrogenase (*72*, *73*). Tungstoenzymes generally catalyze redox reactions at very low potentials, with the exception of acetylene hydratase and trimethylamine oxidoreductase (*74*). The advantage of tungsten over molybdenum in the cofactor appears to be related to its ability to (i) function at high temperatures and (ii) catalyze redox reactions at very low potentials. The requirement of high temperatures for optimal N_2 fixation in *M. thermolithotrophicus* may indicate that, in accordance with the observation that most tungstoenzymes are found in (hyper)thermophilic organisms, its nitrogenase is a functional tungsten-containing enzyme. Since the dinitrogenase component of *M. thermolithotrophicus* has never been isolated and characterized, the type of heterometal atom in the cofactor, involved in the reduction of N_2 in this organism, is still unknown.

In light of these considerations, the question arises as to whether the tungsten-substituted nitrogenase of *R. capsulatus* can also function (at least partly) at temperatures distinctly higher than 30 °C (the temperature used to assess its catalytic activity). The effect of temperature on the catalytic competence of the *R. capsulatus* WFe protein, however, has yet to be investigated in detail. Furthermore, based on the observations that (i) the concentration of WO_4^{2-} near hydrothermal vents (black smokers) is approximately 1000-fold as compared to its average concentration in the sea (*73*) and (ii) a bio-available form of Mo in these anaerobic environments may essentially be absent (*75*) and based on the notion that life might have evolved in such niches, it is not inconceivable that tungsto−nitrogenases may have been operative as an ancestral version of today's Mo-nitrogenases. Whether nitrogenases indeed served to fix N_2 in these early stages of evolution or fulfilled a different purpose (e.g., the removal of toxic cyanide ions) still remains a matter of controversy (*76*).

In summary, the present studies have revealed that a tungsten-substituted FeWco-containing dinitrogenase can be isolated from *R. capsulatus*. The protein appears to be inactive in mediating C_2H_2 and N_2 reduction but shows considerable activity in generating H_2 from protons. While the inability to reduce the former two substrates may be attributed to the significantly lower midpoint potential of the FeWco, the observed H_2 evolution indicates that the cofactor can be, at least partly, enzymatically reduced. Since the role of molybdenum in nitrogenase has yet to be identified, comparative studies on both the MoFe and the WFe protein may prove particularly useful in addressing this aspect, which has for so long puzzled the scientific community. Future studies on the WFe protein will therefore aim at gaining insights into such aspects as the temperature-dependence of its activities (including those with substrates other than C_2H_2, N_2, H^+) as well as the pH dependence of the redox potential of the cofactor.

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