Inorganica Chimica Acta, *151* (1988) 227–232

A Facile Method of Isolation for the Iron Molybdenum Cofactor of Nitrogenase and Bacterial Ferritin from Extracts of *Azotobacter vinelandii*

SAMUEL M. LOUGH, DELORIA B. JACOBS, SUSAN F. PARKER, JOHN W. McDONALD* and GERALD D. WATT*

Battelle-Kettering Laboratory, Yellow Springs, Ohio 45387, U.S.A.

(Received August 27, 1987)

Abstract

An alternative method has been developed for the isolation of both the iron molybdenum cofactor of nitrogenase (FeMoco), a small molecular weight Fe–Mo–S cluster which is the putative nitrogen-reducing site of the enzyme, and bacterioferritin, an iron storage protein similar to other ferritins, but containing heme prosthetic groups. Previously the isolation of these two species, the characterization of which is of significant current interest, has been dependent on the purification of the nitrogenase enzyme from *Azotobacter vinelandii*. Our new procedure eliminates the use of the anaerobic column chromatography necessary to obtain pure nitrogenase components, involving instead the heat and RNAase/DNAase treatment of crude extracts of ruptured cells followed by sedimentation (150 000 × *g* for 18 h) of both the 'nitrogenase complex' and bacterioferritin. The redissolved pellet from this centrifugation yields the pure crystalline bacterioferritin on addition of Mg^{2+} and cooling, the iron content of the protein being higher by this method than in previous reports. Likewise, denaturation by acid/base treatment of this protein mixture yields a precipitate which can be extracted with either *N*-methylformamide or *N*,*N*-dimethylformamide containing dithionite ion to yield solutions of FeMoco, as evidenced by UW 45 reconstitution and EPR spectral criteria. Unfortunately, preparations of FeMoco obtained by this method have a variable, but consistently low, Fe/Mo ratio and additional visible spectral features, indicating that they are significantly less pure than that those generated from purified nitrogenase. The aqueous supernatant from the denaturation also yields bacterioferritin, but with a lower iron content than that from the direct crystallization method.

Introduction

Azotobacter vinelandii, a bacterium easily grown in large quantities under aerobic conditions, is a commonly-exploited source of a variety of metalloproteins. These entities include an unusual 3-Fe ferredoxin [1] which has been the subject of much recent interest, two 2-Fe ferredoxins discovered some years ago by Beinert *et al.* [2], and, perhaps most importantly, the nitrogenase enzyme system [3, 4]. *A. vinelandii* contains an unusually high content of nitrogenase, an enzyme which has been shown by intensive study over the past 20 years to consist of two component proteins, both of which are necessary for activity. One of these components, the molybdenum iron protein ([MoFe]) contains, in addition to a number of iron–sulfur moieties, a biologically-unique iron–molybdenum–sulfur cluster which has been implicated [5] as the substrate-reducing site of the enzyme. As initially shown by Shah and Brill [6], under the proper conditions this Fe–Mo–S core, designated the iron molybdenum cofactor of nitrogenase (FeMoco), can be extracted in intact form from the protein into organic solvents where it has been the subject of extensive physicochemical characterization [3, 4], although its exact structure has remained elusive. In addition, *Azotobacter vinelandii* contains an unusual bacterial ferritin (bacterioferritin) which was first isolated in 1973 by Bulen and coworkers [7] as a by-product of nitrogenase purification, and which more recently has come under intensive study in our laboratory [7, 8] with regard to a comparison of its composition and properties [9] with those of the more-common mammalian ferritins [10].

Our requirement for relatively large quantities of both FeMoco and *A. vinelandii* bacterioferritin to facilitate further chemical and biochemical studies of these species has prompted us to develop a modified procedure for their isolation. This methodology, which excludes the need for the rather time-consuming anaerobic column chromatography involved in the purification of the nitrogenase components, is described herein along with characterization data for the two isolated species.

Experimental

Azotobacter vinelandii OP was grown and harvested as previously described by Bulen and LeComte

*Authors to whom correspondence should be addressed.

0020-1693/88/$3.50

[11]. For our procedures, no difference was noted between fresh cells and those which had been stored for up to 4 months at −80 °C. The total iron and molybdenum contents of FeMoco and the iron and phosphate content of bacterioferritin were determined by literature methods [11, 12]. For FeMoco preparations from 'redissolved pellet' (*vide infra*) the actual Mo associated with FeMoco was estimated from the electronic spectra of thiomolybdates elicited by oxidative decomposition using the previously described method [13]. EPR measurements at liquid helium temperatures [14], the preparation of tetraethylammonium dithionite ($[NEt_4]_2[S_2O_4]$) [15] and the purification of *N*-methylformamide (NMF) and *N*,*N*-dimethylformamide (DMF) for FeMoco extraction [15a] were carried out as previously described. UV–Vis spectra were recorded on a Cary 118C spectrophotometer. Anaerobic chromatography on both DEAE and Sephachryl and nitrogenase activity determinations were carried out by the literature [12] methods, as was the growth of the UW 45 mutant of *A. vinelandii* [12]. FeMoco solutions were assayed by the published method [6] for their effectiveness at reconstituting the ability of UW 45 crude extracts to catalyze the reduction of acetylene by sodium dithionite. Pure [MoFe], [Fe], and FeMoco used in control experiments and assays were prepared by the method of Burgess *et al.* [12].

Sedimentation of A. vinelandii Bacterioferritin and Nitrogenase

Whole cells of *A. vinelandii* (600 g wet weight) were suspended in pH 8 tris buffer (600 ml) and ruptured by passage (2 times) through the modified Manton-Gaulin Homogenizer described by Bulen and LeComte [11]. The ruptured cell suspension was quickly placed in a 3-necked flask and deoxygenated on a Schlenk manifold by repeated vacuum/argon cycles. DNAase (10 μg/ml) and RNAase (10 μg/ml) (both reagents from Sigma) were added and the mixture stirred for 10–15 min at ambient temperature. The vessel was then placed in a 85 °C water bath and the temperature of the cell suspension raised to 56 °C at which time it was removed from the water bath and stirred for 5 min. While maintaining anaerobic conditions, the resulting mixture was centrifuged at 150000 × *g* for 1 h, the pellet from this treatment discarded, and the supernatant further centrifuged at 150000 × *g* for 18 h. This treatment resulted in the formation of three well-defined fractions: (1) a dark brown supernatant which was readily decanted and discarded; (2) a viscous, pinkish–red layer which could be removed by longer-term inversion of the tubes followed by gentle washing with pH 7.4 tris buffer; this fraction was also discarded; (3) a dark brown pellet which was resuspended under anaerobic conditions in 75–80 ml of pH 7.4 tris buffer for use in further steps. This solution, designated the 'redissolved pellet' in subsequent sections, can either be used immediately or can be frozen and stored at −80 °C for up to 1 month with no discernable changes.

Isolation of Bacteriferritin

A 2.0 M solution of $MgCl_2$ in water was added to the redissolved pellet to a final concentration of 0.02 M, and the mixture kept at 5 °C for 24 h during which time red crystals of bacterioferritin formed. The initial solid material, which was separated by decanting after low speed centrifugation, was then recrystallized 3 times by dissolution in pH 7.2 tes buffer, addition of Mg^{2+} as before, and cooling.

Isolation of FeMoco

The procedure is essentially that of Shah and Brill [6] as modified by Burgess *et al.* [12], except that the starting material for the previous work was [MoFe] which had been purified by DEAE chromatography and crystallization. Portions of the redissolved pellet (1.0 ml) were placed in 12 ml centrifuge tubes and diluted to 6.0 ml with water containing 1.0 mM $Na_2S_2O_4$ and citric acid (1.0 ml of 0.10 M) was added. After 3 min, the solution was treated with Na_2HPO_4 (1.0 ml of 0.20 M) resulting in the formation of a precipitate and a light orange supernatant. The separated supernatant yielded bacterioferritin on treatment with Mg^{2+}, but the iron content of the protein was significantly less than that for samples produced by direct crystallization from the resuspended pellet as described above.

The precipitate that formed after citrate/phosphate denaturation of the redissolved pellet was washed once with DMF (2.5 ml) which had been treated with aqueous $Na_2S_2O_4$ (30 μl of 0.1 M) and then was treated either with NMF (2.5 ml) containing 1.0 mM $Na_2S_2O_4$ and 2.0 mM Na_2HPO_4 or DMF (2.5 ml) containing 2.0 M $[NEt_4]_2[S_2O_4]$. The greenish–brown supernatant layers from these extractions were pooled and evaporated under vacuum to the desired concentration (usually about 0.1 mM in Mo). Samples for EPR and UW 45 reconstitution analysis were frozen and stored at 77 K after addition of excess dithionite.

Results and Discussion

For a number of years, the isolation of nitrogenase from the bacterium *Azotobacter vinelandii* in our laboratory [12] and in others [17–19] has involved: (1) treatment of the ruptured cells with DNA- and RNAase (to break up nucleic acids) followed by a heat step (to precipitate easily-denatured proteins); (2) the use of anaerobic anion exchange chromatography on DEAE to separate the enzyme into its

component proteins as well as remove other proteinaceous material, nucleotides, etc.; (3) the further purification of each individual component, the molybdenum iron protein ([MoFe]) by crystallization and the iron protein ([Fe]) by additional gel chromatography on Sephachryl or Ultragel. [MoFe] has in turn been the source of the iron molybdenum cofactor of nitrogenase (FeMoco) [6], a low molecular weight Fe–Mo–S cluster which can be extracted into organic solvents [6, 14, 16] from the acid/base denatured protein and which, when bound to the native protein, represents the site of substrate reduction [5]. Our studies of FeMoco, designed to understand the composition and structure of this cluster, have thus ultimately been dependent on a supply of pure [MoFe], a restriction we have attempted to overcome by eliminating the most time-consuming parts of the protein isolation procedure, *i.e.*, the anaerobic DEAE chromatography, and thus obtain FeMoco from bacterial material which has undergone minimal purification. To realize this goal, we exploited the fact that the 'nitrogenase complex' of Bulen and LeComte [11] can be sedimented to a tractable pellet by prolonged high speed centrifugation of *A. vinelandii* crude extracts. A valuable offshoot of this project has been the finding that the bacterial ferritin first discovered by Bulen and coworkers in 1973 [7] and later characterized more extensively in this laboratory [8, 9] is cosedimented with the nitrogenase complex under these conditions. We find that the ferritin can be more readily obtained from this material than via its standard methods [7] of isolation as a byproduct either from 'nitrogenase complex' purification by protamine sulfate precipitation or from nitrogenase component separation on DEAE.

The preparation of extracts of whole cells of *A. vinelandii* was identical to that of Burgess *et al.* [12], *i.e.*, treatment of ruptured cells with enzymes to fragment nucleic acids and with heat to remove additional proteins. These steps take less than 8 h and allow the centrifugation (*ca.* 18 h at 150 000 × *g*) of the remaining solution to be started on the first day. While these parameters have not been investigated in detail, we speculate that neither the exact centrifugation speed nor the length of the spin is critical. Our choice of 18 h was a matter of overnight convenience, and the speed was the maximum allowable with the required rotor. Bulen and LeComte reported [11] that the 'nitrogenase complex' could be sedimented, albeit in the presence of 0.02 M Mg^{2+} which might encourage aggregation, in 2 h at 205 000 × *g*. Our treatment results in the formation of a well-defined dark brown pellet which is distinct from the other phases: (1) a dark brown easily-decanted supernatant and (2) a viscous red fraction which sediments next to the pellet but can be readily separated by tube inversion and gentle washing with buffer. These decantable fractions were discarded and their make-up was not investigated further. The pellet was redissolved in pH 7.4 tris buffer and this solution used as the source of FeMoco and bacterioferritin.

While the total composition of the pellet from the above centrifugation was not investigated in detail, the presence of nitrogenase was confirmed by activity and EPR spectral studies. By itself, the redissolved pellet had the capability of reducing 110 nmol C_2H_2/min/mg protein, an activity which was increased to 340 units by addition of excess [Fe] which had been prepared in a separate procedure [12]. These nitrogenase activities are much lower than those reported [12, 17–19] for the recombined purified component proteins, *i.e.*, *ca.* 2500 units based on [MoFe], but they are similar in magnitude to those for the 'nitrogenase complex' (in the absence of added [Fe]) prepared [11] by Bulen and LeComte (90 units) [11] and later studied in more detail by Watt *et al.* [20] (250–320 units). Like the 'nitrogenase complex' of Bulen and LeComte [11] and a similar entity studied some years later by Veeger *et al.* [21], our redissolved pellet elutes as a single active colored band from a gel column. In a separate control experiment with purified proteins, the gel column separates a mixture of purified [MoFe] and [Fe] demonstrating that they are indeed strongly bound together in the complex. One of the 2-Fe proteins of Beinert and coworkers [2] was also previously implicated [20–22] as a key component in the 'nitrogenase complex', and indeed we find that chromatography of our redissolved pellet on DEAE results in the elution of a band which has a visible spectrum qualitatively consistent with that of this ferredoxin. This anion exchange chromatography also results in the separation of [MoFe] and [Fe], additional evidence that the nitrogenase components are present in the pellet of the prolonged centrifugation. This biochemical evidence thus is consistent with the facile sedimentation of basically the same nitrogenase complex which has previously [11, 20] been isolated by sequential protamine sulfate precipitation of *A. vinelandii* extracts. However, its purity would appear to be lower than that of samples obtained after this latter procedure was optimized [20], and no attempts were made to quantitiate the relative or absolute amounts of [MoFe], [Fe], and other metalloproteins. Addition of Mg^{2+} to the redissolved pellet yields crystals of bacterioferritin (*vide infra* for characterization data), confirming the presence of this protein in the sedimented fraction. Additional species in the pellet, the presence of which was indicated by additional bands in the aforementioned DEAE chromatography of this fraction, were not identified.

The composition of the redissolved pellet can also be probed by an examination of the EPR spectros-

copy of that solution at 10 K. In the presence of excess dithionite ion, [MoFe] is known [3] to exhibit an $S = \frac{3}{2}$-type EPR spectrum with g values at 2.01, 3.76, and 4.29, while both [Fe] [3] and the aforementioned ferredoxin [2] give rise to resonances in the $g = 2$ region, the former from a $[Fe_4S_4]^+$ chromophore and the latter from a $[Fe_2S_2]^+$ unit. Indeed, as shown in Fig. 1a, the spectrum of the redissolved pellet contains resonances at $g = 3.8$ and 4.3 conclusively demonstrating the presence of [MoFe], along with a pattern in the $g = 2$ region which can be rationalized as due to the superposition of the spectra of [MoFe], [Fe], and other EPR-active Fe–S proteins [2]. As well as confirming that the redissolved pellet contains nitrogenase, this EPR probe may provide a useful short cut to the spectroscopic characterization of nitrogenases from other less-studied organisms, if the enzyme can be concentrated by centrifugation as for *A. vinelandii*.

Denaturation (by slight variation of the technique of Shah and Brill [6]) of the redissolved pellet yields a precipitate which can be extracted with either NMF [6, 16] or DMF [14] to yield solutions which show the characteristic [3] EPR spectrum of FeMoco (g values at 4.5, 3.6, 2.0; Fig. 1b) and are capable of reconstituting the inactive nitrogenase of the UW 45 mutant of *Azotobacter vinelandii* (*vide infra* for numerical activities). These are the two most commonly-employed criteria for FeMoco and conclusively show that this moiety can be obtained in two working days with whole cells of *A. vinelandii* as the starting material, whereas isolation of [MoFe] for use as precursor to FeMoco requires one to two additional days of anaerobic chromatography and involves techniques which are more susceptible to experimental error and more equipment-intensive than the simple high-speed centrifugation described herein.

However, as shown in Table I, the iron to molybdenum ratios of FeMoco solutions obtained in this way are consistently much lower (Fe/Mo = *ca.* 2) than those found for FeMoco from purified [MoFe], *i.e.* Fe/Mo = 6–8 [3, 4]. This result suggests that another form of molybdenum is carried along through the abbreviated purification procedure and is extracted from the denatured pellet into NMF or DMF. The amount of Mo which is actually part of FeMoco in these preparations can be estimated from a comparison of their visible spectra with that of

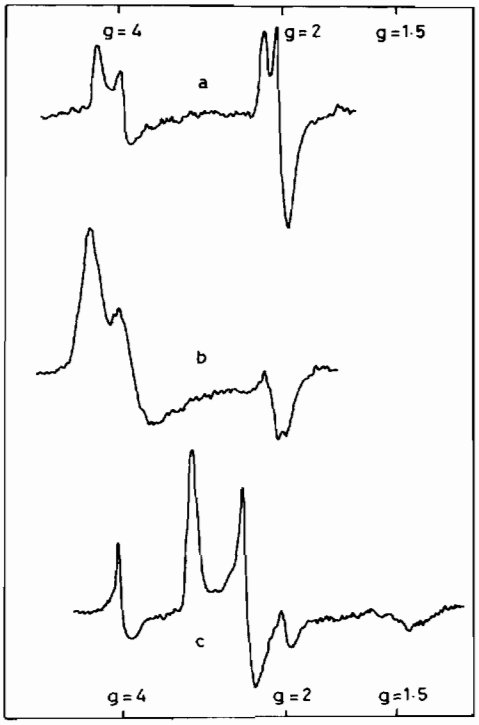

Fig. 1. EPR spectra of (a) 'redissolved pellet' in pH Tris 7.4 buffer at 15 K; (b) FeMoco in NMF from 'redissolved pellet' at 4 K; (c) apobacterioferritin in pH 7.5 TES buffer at 15 K. The microwave power for all spectra was 3.7 mW and the microwave frequency was 9.15 gHz.

TABLE I. Molybdenum, Iron, and FeMoco Composition and UW 45 Reconstitution Activity of several NMF and DMF Extracts of Denatured Redissolved Pellet

Sample	[Mo] (mM)	[Fe] (mM)	Fe/Mo	[FeMoco] (mM)[a]	UW 45 Activity[a]
1 NMF	1.333	2.540	1.9	0.179[b]	65
2 NMF	1.133	2.360	2.1	0.165[b]	80
3 NMF	2.293	1.846	0.8	0.183[b]	25
4 NMF	1.160	3.460	3.0	0.17[c]	113[c]
5 NMF	0.603	2.247	3.7	0.086[c]	157[c]
6 DMF	0.240	0.377	1.6	0.034[c]	197[c]
7 DMF	0.132	0.430	3.2	0.019[c]	192[c]

[a]Units are nmol C_2H_2 reduced per nmol FeMoco per min. [b]Determined from the solution absorbance at 800 nm and the molar absorptivity of FeMoco at that wavelength of 1900 cm^{-1} M^{-1}. [c]These solutions were active in UW 45 reconstitution studies but their visible spectra were not recorded. FeMoco contents were estimated as 1/7 of the total Mo based on the average difference in these values in samples where both $[Mo]_{total}$ and [FeMoco] (from its absorbance at 800 nm) were known. Specific activities are based on this estimated [FeMoco].

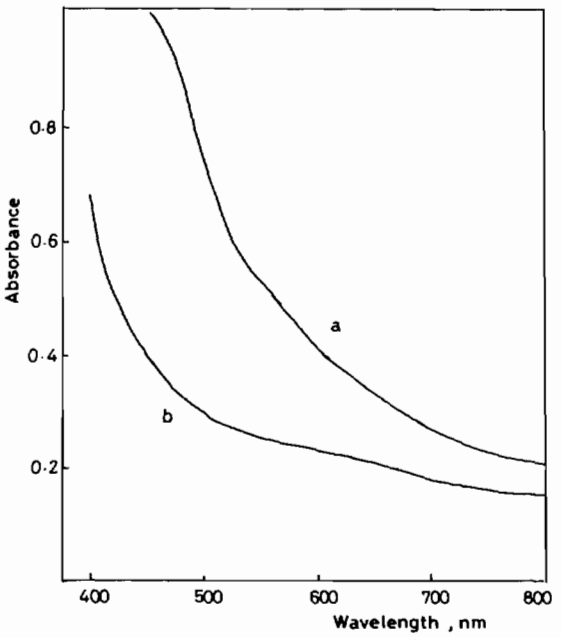

Fig. 2. Visible spectra of FeMoco in NMF: (a) from redissolved pellet, [Mo] = 1.13 mM, [FeMoco] = 0.179 mM; (b) from purified [MoFe], [Mo] = [FeMoco] = 0.127 mM.

FeMoco prepared from purified [MoFe]. Figure 2 shows that the spectrum of FeMoco contains a shoulder at 625 nm, but, unfortunately, FeMoco solutions obtained from denaturation of the redissolved pellet contain varying amounts of impurities which show some absorbance at this wavelength (Fig. 2). We therefore used the absorbance at 800 nm (where impurities are less likely to absorb), in conjunction with the experimentally-determined† molar absorptivity for FeMoco at this wavelength (1900 cm^{-1} M^{-1}) to determine the actual [FeMoco] in these solutions which are characterized by both low Fe/Mo ratios and species which absorb in the visible region. Table I presents the total Mo content, the concentration of FeMoco, and the UW 45 reconstitution activity based on this latter value for three NMF extractions of the denatured redissolved pellet. Calculated on this basis, the reconstitution activities are significantly lower than those typically observed for FeMoco samples prepared from purified [MoFe], indicating either that the FeMoco in these solutions is defective or (perhaps more likely) that the method of evaluating [FeMoco] is in error. It is certainly possible that the impurities in solutions of FeMoco from redissolved pellet absorb significantly at 800 nm, resulting in an overestimation of [FeMoco] and a concomitant lower specific activity. Attempts are currently in progress to determine the nature of and to remove the impurities in solutions of FeMoco obtained from the redissolved pellet.

The aqueous supernatant after acid/base denaturation of the redissolved pellet in the above FeMoco preparation was orange–red in color, leading us to suspect the presence of bacterioferritin. Indeed, addition of Mg^{2+} to this solution (the usual method [7–9] of precipitation for this species) resulted in the formation of red crystals which, when redissolved, showed the characteristic visible spectrum of bacterioferritin (*vide infra*). However, the average iron content of these preparations was 800 Fe/bacterioferritin (apoprotein M_r = 420 000 [8, 9]), significantly lower than the average for samples of this protein previously obtained as a byproduct of nitrogenase purification, *i.e.*, *ca.* 1150 Fe/bacterioferritin [9]. A possible cause of this lower value is the loss of iron during the somewhat harsh denaturation conditions, a possibility which suggested the addition of Mg^{2+} directly to the redissolved pellet prior to addition of acid/base. This latter method did, in fact, provide significantly better (3–8 fold) yields of bacterioferritin with an average of 1700 Fe/protein, an iron content more than double that for bacterioferritin isolated after denaturation and higher than that obtained previously [9]. Thus, this latter procedure for the isolation of bacterioferritin is not only much less time consuming and generally easier than previous methods which are linked to nitrogenase isolation [7], but also provides purer material.

The fully-oxidized bacterioferritin prepared in this way (*ca.* 300 mg/kg wet cells) exhibits the characteristic visible spectrum of this species (Soret band at 417 nm) which changes on reduction with dithionite to one with bands at 558, 527, and 425 nm consistent with the presence of a reduced *b*-type cytochrome as previously noted [7, 8]. Removal of the core iron from these samples of bacterioferritin [9], yields the apoprotein which, in its oxidized form, exhibits an EPR spectrum (Fig. 1c) with *g* values at 2.84, 2.29, and 1.43, consistent with the presence of low spin Fe(III) in a heme environment [23, 24]. The oxidized holo bacterioferritin, containing *ca.* 1800 Fe(III) core atoms/protein and 12 Fe(III) heme units [7–9], exhibits effectively the same EPR spectrum as its apo analog.

Unfortunately, the Mg^{2+}-treated redissolved pellet, which must stand at 5 °C for *ca.* 24 h to allow crystallization of bacterioferritin, did not prove to be a consistent source of FeMoco on subsequent denaturation and organic solvent extraction. Possible reasons for this problem include the presence of excess Mg^{2+} and/or the decomposition of nitrogenase components with time, but whatever the explanation,

†The molar absorptivity was determined from a single FeMoco sample from purified [MoFe] and therefore may be only approximate. Experiments are in progress to accurately quantitate the electronic spectrum of FeMoco from a number of replicate samples.

FeMoco and bacterioferritin are best prepared in separate procedures using the redissolved pellet as the common starting material.

Summary and Conclusions

The methodology described herein for preparation of bacterioferritin offers significant advantages over previous techniques, requiring only the easily-grown *A. vinelandii* and an ultracentrifuge for its implementation. Its exploitation should facilitate the further characterization and study of this species to be more readily carried out in laboratories which do not routinely isolate the nitrogenase enzyme. Although FeMoco can also be readily prepared by this general methodology, its purity is significantly less than that prepared from purified [MoFe]. Further purification of these FeMoco solutions therefore will be required in order to fully realize the benefits of the revised procedure.

Acknowledgement

This work was partially supported by Grants 85-CRCR-1-1639 (to J.W.M.) and 84-CRCR-1-1509 (to G.D.W.) form the USDA/SEA Competitive Research Grants Office and N00014-86-K-0102 from the Office of Naval Research (to G.D.W.). This is Contribution No. 924 from the Battelle-Kettering Laboratory.

References

1 T. V. Morgan, P. J. Stephens, F. Devlin, C. D. Stout, K. A. Melis and B. K. Burgess, *Proc. Natl. Acad. Sci. U.S.A., 81*, 1931 (1984), and refs. therein.
2 (a) Y. I. Shethna, D. V. Der Vartanian and H. Beinert, *Biochem. Biophys. Res. Commun., 31*, 862 (1968); (b) D. V. Der Vartanian, Y. I. Shethna and H. Beinert, *Biochim. Biophys. Acta, 194*, 548 (1969).
3 W. H. Orme-Johnson, *Ann. Rev. Biophys. Biophys. Chem., 14*, 419 (1985).
4 B. K. Burgess, in C. Veeger and W. E. Newton (eds.), 'Advances in Nitrogen Fixation', Martinus Nijhoff/Dr. W. Junk, The Hague and Pudoc, Wageningen, 1984, P. 103–114.
5 T. R. Hawkes, P. A. McLean and B. E. Smith, *Biochem. J., 217*, 317 (1983).
6 V. K. Shah and W. J. Brill, *Proc. Nat. Acad. Sci. U.S.A., 74*, 3249 (1977).
7 W. A. Bulen, J. R. LeComte and S. Lough, *Biochem. Biophys. Res. Commun., 54*, 1274 (1973).
8 E. I. Stiefel and G. D. Watt, *Nature (London), 279*, 81 (1979).
9 G. D. Watt, R. B. Frankel, G. C. Papaefthymiou, K. Spartalian and E. I. Stiefel, *Biochemistry, 25*, 4330 (1986).
10 G. D. Watt, R. B. Frankel and G. C. Papaefthymiou, *Proc. Natl. Acad. Sci. U.S.A., 82*, 3640 (1985) and refs. therein.
11 W. A. Bulen and J. R. LeComte, *Methods Enzymol., 24*, 456 (1972).
12 B. K. Burgess, D. B. Jacobs and E. I. Stiefel, *Biochem. Biophys. Acta, 614*, 196 (1980).
13 W. E. Newton, S. F. Gheller, B. Hedman, K. O. Hodgson, S. M. Lough and J. W. McDonald, *Eur. J. Biochem., 159*, 111 (1986).
14 G. D. Watt and J. W. McDonald, *Biochemistry, 24*, 7226 (1985).
15 (a) S. M. Lough, D. L. Jacobs, D. M. Lyons, G. D. Watt and J. W. McDonald, *Biochem. Biophys. Res. Commun., 139*, 740 (1986); (b) S. M. Lough and J. W. McDonald, *Inorg. Chem., 26*, 2024 (1987).
16 S.-S. Yang, W.-H. Pan, G. D. Friesen, B. K. Burgess, J. L. Corbin, E. I. Stiefel and W. E. Newton, *J. Biol. Chem., 257*, 8042 (1982).
17 V. K. Shah and W. J. Brill, *Biochim. Biophys. Acta, 305*, 445 (1973).
18 C. E. McKenna, C. W. Huang, J. B. Jones, M.-C. McKenna, T. Nakajima and H. T. Nguyen, in W. H. Orme-Johnson and W. E. Newton (eds.), 'Nitrogen Fixation', Vol. I, University Park Press, Baltimore, Md., 1980, pp. 223–235.
19 A. Braaksma, H. Haaker, H. J. Grande and C. Veeger, *Eur. J. Biochem., 121*, 483 (1982).
20 S. Lough, A. Burns and G. D. Watt, *Biochemistry, 22*, 4062 (1983).
21 G. Scherings, H. Haaker and C. Veeger, *Eur. J. Biochem., 77*, 621 (1977).
22 C. Veeger, C. Laane, G. Sherings, L. Matz, H. Haaker L. Van Zeeland-Wobbers, in W. E. Newton and W. H. Orme-Johnson (eds.), 'Nitrogen Fixation', Vol. I, University Park Press, Baltimore, Md., 1980, pp. 111–137.
23 R. Aasa, S. P. J. Albracht, K.-E. Falk, B. Lanne and T. Vanngard, *Biochim. Biophys. Acta, 422*, 260 (1976).
24 C. P. S. Taylor, *Biochim. Biophys. Acta, 41*, 137 (1977).