Pergamon

Tetrahedron, Vol. 53, No. 35, pp. 11971-11984, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00710-2

Nitrogenase Iron-Molybdenum Cofactor Binding Site: Protein Conformational Changes Associated with Cofactor Binding

Jon K. Magnuson[a] Timothy D. Paustian[b], Vinod K. Shah[b], Dennis R. Dean[c], Gary P. Roberts[b], Douglas C. Rees[d] and James B. Howard[a*]

[a]Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

[b]Departments of Bacteriology and Biochemistry and Center for the Study of Nitrogen Fixation, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

[c]Department of Anaerobic Microbiology, Virginia Polytechnical Institute and State University, Blacksburg, Virginia 24061

[d]Department of Chemistry, California Institute of Technology, Pasadena, CA 91125

Abstract Formation of active nitrogenase MoFe-protein requires the assembly and insertion of a unique molybdenum containing Fe:S cluster, the FeMoco. The protein acceptor is held in an open conformation by the binding of a small molecular weight protein, γ. Using selective alkylation of the protein cysteines, conformational changes associated with γ binding and cofactor insertion were evaluated and a model for the change presented. Reversible oxygen damage to the precursor is identified and evaluated in terms of the chemistry of dithionite used in the buffer as an oxygen scavenger. © 1997 Elsevier Science Ltd.

INTRODUCTION

Dinitrogen reduction is catalyzed by a two protein component enzyme, nitrogenase, composed of the Fe-protein and the MoFe-protein (reviewed in [1-4]). The protein metallocenters serve as the sites of electron transfer and substrate reduction; hence, knowledge of their structures and chemistry portend possibilities for development of novel catalysts as well as understanding the fundamental chemistry of the reaction. The crystal structure of the MoFe-protein revealed two unique Fe:S clusters, heretofore unrecognized, the 8Fe:7S, P-cluster and the Mo:7Fe:9S:homocitrate, FeMo- cofactor (FeMoco) (Figure 1)[5-9]. The P-cluster is located at the interface between an α/β protein subunit pair with three bridging and terminal cysteinyl ligands from each subunit (total of six cysteinyl ligands for the eight iron atoms)[9]. Even more remarkable is the FeMoco which is found in the α-subunit and is so named because it can be removed intact by extraction with polar organic solvents such as N-methylformamide from acid denatured protein[11]. The cofactor structure has a core of six, trigonally coordinated iron atoms connected by bridging inorganic sulfur; an iron atom with conventional tetrahedral coordination; and a molybdenum coordinated by homocitrate. Contrary to other Fe:S clusters which usually have one protein ligand per metal, the FeMoco has only two protein ligands for

the eight metals, a cysteinyl ligand (αCys-275) to the tetrahedral iron atom and a histidyl ligand (αHis-422) to the molybdenum atom[5-7].

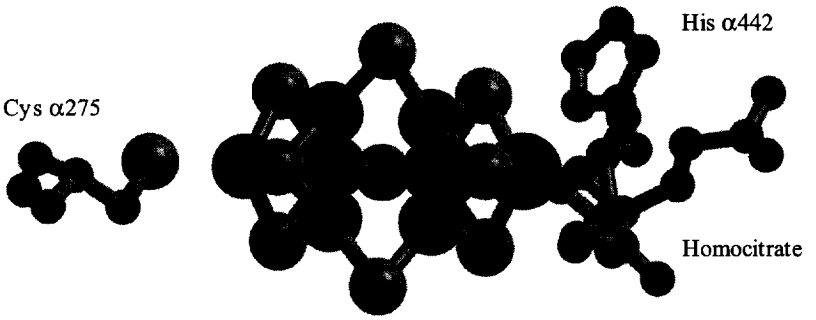


Figure 1. FeMoco from *A. vinelandii* MoFe-protein[7]. Presentation prepared with the program MOLSCRIPT[10].

The MoFe-protein clusters have proven to be some of the most unusual members of the Fe:S family and have defied chemical synthesis. Nevertheless, substantial progress has been made in elucidating the biosynthesis of these clusters and these studies have been instrumental in understanding broader questions of how metal clusters are constructed *in vivo*. FeMoco biosynthesis requires proteins or enzymes encoded by the nitrogenase specific genes, *nifB, nifQ, nifE, nifN, nifH and nifV* [12-23]. Mutations in any of these genes can result in the MoFe-protein lacking a biologically functional FeMoco but retaining P-clusters. The cofactor deficient forms of the MoFe-protein from *A. vinelandii*, will be referred to as desFeMoco-Av1. Catalytic activity can be restored to desFeMoco-Av1 by insertion of FeMoco isolated from acid denatured MoFe-protein or generated by *in vitro* enzymatic biosynthesis. The individual roles of these gene products have been partially identified. For example, NifV is a homocitrate synthase and NifB generates a new Fe:S cofactor that serves as the iron and sulfur donor. FeMoco appears to be assembled on a protein scaffolding, NifE/N[19], with the subsequent transfer to desFeMoco-Av1 mediated by one of several related, small molecular weight proteins, called γ in the *A. vinelandii* system.

Perhaps most enigmatic is the role of NifH, the Fe-protein, in FeMoco synthesis. At least two roles have been identified for the Fe-protein, first as a participant in the assembly of the cofactor on NifE/N, and second as a required aid in the transfer of FeMoco to desFeMoco-Av1. It is the role of Fe-protein in FeMoco

transfer that has been most difficult to assess with early investigations giving seemingly contradictory results. This has been resolved by the recognition that desFeMoco-Av1 has different properties depending upon the genetic background from which it was isolated[20-23]. Namely, desFeMoco-Av1 isolated from strains deleted in *nifB* (no Fe:S donor for the cofactor synthesis) can be readily reconstituted with FeMoco while desFeMoco-Av1 isolated from strains deleted in *nifH* (that is, missing the Fe-protein) cannot be. However, the latter form of desFeMoco-Av1 can be reconstituted if Fe-protein, MgATP and the FeMoco transfer protein, γ, are included. The proteins from these two genetic backgrounds have been partially characterized and were found to differ in their subunit compositions; desFeMoco-Av1 from *nifB* is the hexamer, $\alpha_2\beta_2\gamma_2$, while desFeMoco-Av1 from *nifH* is the tetramer, $\alpha_2\beta_2$. Thus, the function of Fe-protein in cofactor insertion is to induce the tight binding of γ. In the *in vivo* synthesis, γ is lost during the subsequent cofactor insertion since the active MoFe-protein as isolated is a tetramer.

In this paper, we investigate the structural changes in desFeMoco-Av1 resulting from the binding of γ by the $\alpha_2\beta_2$ form. Chemical modification of the cofactor ligand, αCys-275, was used as a probe of accessibility to the cofactor binding site in the various forms of desFeMoco-Av1. The results indicate that the γ component serves to hold desFeMoco-Av1 in an open conformation and receptive to the cofactor attachment.

RESULTS

Cysteines and Metal Clusters of Av1 are Buried. Cysteinyl ligands of Fe:S clusters in several proteins have been identified by chemical modification of the thiols exposed during chelation of the iron. This method was successfully employed with both beef heart aconitase[24] and Fe-protein from *A. vinelandii* nitrogenase (Av2)[25]. When similar experiments were attempted with Av1, the clusters were not susceptible to chelation by EDTA (N, N, N', N'-ethylenediaminotetraactate) or 2,2'-dipyridyl as monitored by changes in the UV-Vis absorption spectra. Likewise, thiol modifying reagents such as 2 mM iodoacetic acid (IAA), iodoacetamide, or p-mercuribenzoate did not react with the Av1 metal clusters or their ligands. In contrast, IAA reacts rapidly and specifically with the cysteinyl ligands of Av2 where the 4Fe:4S cluster is located near the protein surface[24]. The failure of these chelators and alklyating reagents to attack the Av1 clusters is consistent with the interpretation from the crystal structure which shows the FeMoco and the P-cluster to be buried >10 Å below the surface.

Two Cysteines of the α-Subunit of Av1 are Exposed in desFeMoco-Av1. Paustian et al.[20] have isolated desFeMoco-Av1 from a *nifB* mutant strain (UW45) and found that this FeMoco deficient form

($\alpha_2\beta_2\gamma_2$) retains P-clusters and can be reconstituted with FeMoco alone. This implies that the cofactor binding region has undergone a conformational change permitting the insertion. Using this desFeMoco-Av1 form, we investigated the access to the cofactor binding region by chemical modification. Cysteines susceptible to modification by IAA in desFeMoco-Av1, but not in Av1, indicate regions that are altered by the FeMoco binding.

In parallel experiments, desFeMoco-Av1 and Av1 were incubated with 2 mM [^{14}C]IAA. Samples were removed at various times of reaction, and the pattern of IAA reaction with the cysteinyl residues was determined from peptide maps. In Figure 2A is shown the cysteinyl peptide map for Av1 after a 60 minute

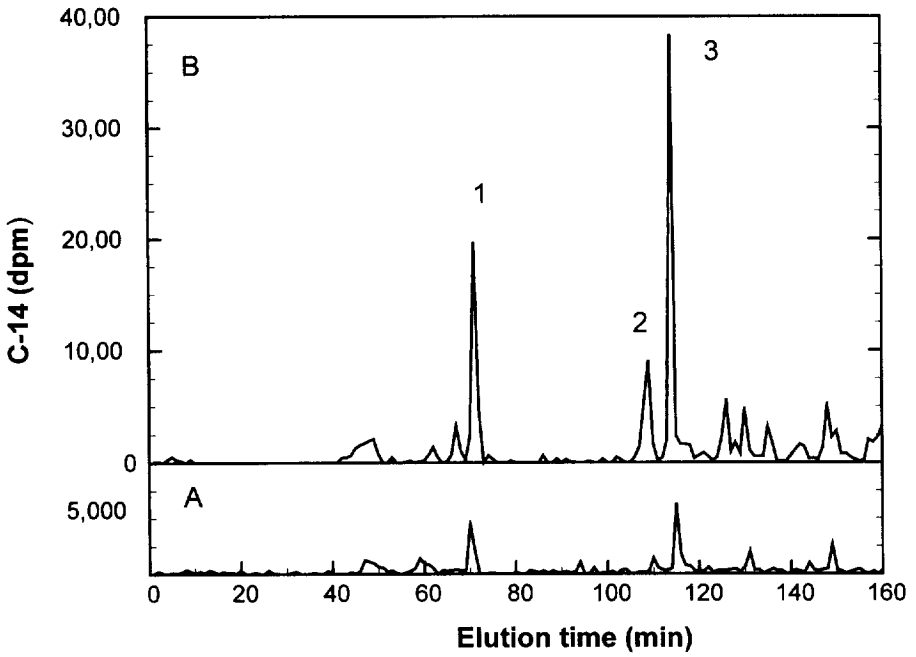


Figure 2. Peptide map of trypsin digested Av1 and des-FeMoco-Av1 exposed to [^{14}C]IAA for 60 minutes. Panel A: Peptide map of Av1. Panel B: Peptide map of desFeMoco-Av1

incubation with [^{14}C]IAA. Although only 0.6 of the 17 cysteines in the α/β pair of subunits were radiolabeled, two significant peaks of radioactivity (peaks 1 and 3) were observed. However, for these two peptides, the mole fraction labeled was only 0.13 and 0.16 respectively, emphasizing the overall low reactivity of cysteines in native Av1. (It should be noted that all 17 cysteinyl residues can be fully labeled and identified by peptide mapping if the protein is denatured before exhaustive carboxymethlyation[26] data not shown). The low level radiolabeling is consistent with the spectroscopic experiments, which showed that the metallocenters of Av1 were unaffected by treatment with IAA.

Figure 2B shows the cysteinyl peptide map for desFeMoco-Av1 after a 60 minute incubation with [^{14}C]IAA. There was only a modest increase in the total radioactivity incorporated (1.7/17) compared to the holoprotein (Av1 containing a full complement of metal clusters). Yet, essentially all of the radioactivity incorporated was found in only three peaks, indicating that selected cysteinyl residues are reactive in the absence of FeMoco. The peptides in peaks 1, 2 and 3 were identified by automated Edman degradation. The sequence of the cysteinyl peptide in both peak 2 and peak 3 was determined to be the peptide, Leu-Asn-Leu-Val-His-Cys^{275}-Tyr-Arg , of the α-subunit of Av1$^{26\text{-}27}$ with the radioactivity exclusively in αCys-275. (The splitting of the peptide into peaks 2 and 3 appears to arise because of partial oxidation of the S-carboxymethyl cysteine.) Peak 1 was identified as the peptide, Cys^{45}-Ile-Ile-Ser-Asn-Lys, also from the α subunit$^{26\text{-}27}$.

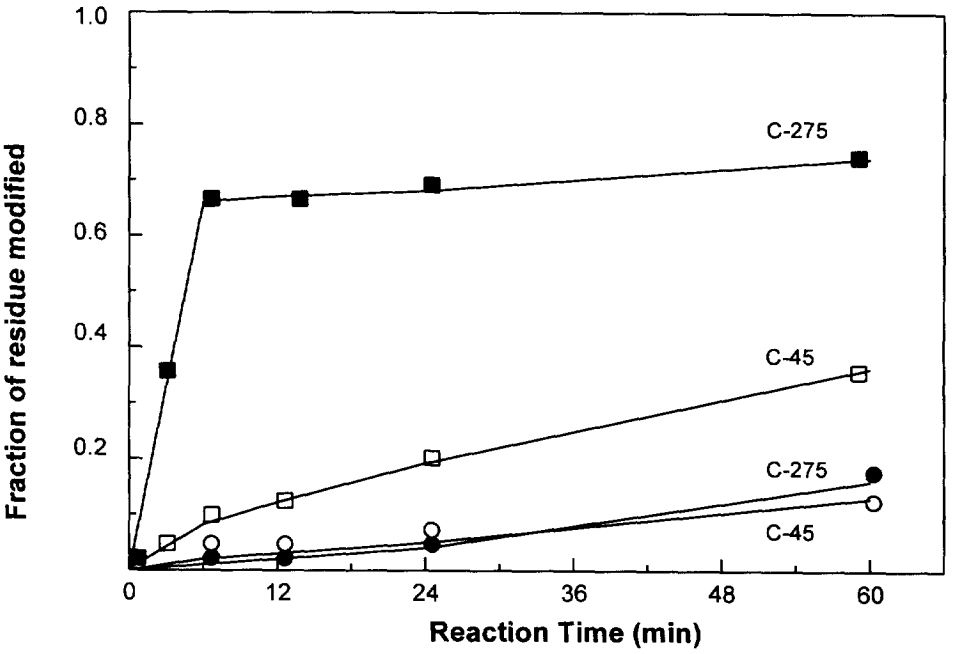


Figure 3. Time of reaction for alkylation of specific cysteines in holo-Av1 (○ , ●) and desFeMoco-Av1 (□ , ■).

These results indicate that αCys-275 and αCys-45 are exposed to chemical modification in the absence of cofactor. Equally important, *only* these two of the seventeen cysteinyl residues have become reactive,

suggesting that the overall protein structure is intact. Even the limited degree of alkylation in holo-Av1 was selective towards these two residues and can be attributed to a small amount of des-FeMoco-Av1 found in most preparations of the holo-Av1. Although both αCys-45 and αCys-275 are exposed in the absence of FeMoco, the rate and extent of their reactivity differ greatly (see Figure 3). αCys-275 was already 65 % modified by six minutes, 90 % of the ultimate level of incorporation obtained at 60 minutes. In contrast, αCys-45 was only 8 % modified at six minutes and even after 60 minutes, the modification was only 50% of that for αCys-275. Thus, αCys-275 is hyper-reactive relative to αCys-45, and, indeed, is hyper-relative compared to free cysteine or the exposed cysteinyl ligands in apo-Av2[25].

Reversible Inactivation of desFeMoco-Av1. Paustian et al.[20] observed that desFeMoco-Av1 exposed to oxygen lost the ability to be reconstituted by FeMoco. Unlike native Av1 that has been inactivated by oxygen, oxygen inactivated desFeMoco-Av1 can be partially restored by incubation with DTT (dithiothreitol). In addition, some preparations of desFeMoco-Av1 only had low levels of activity upon reconstitution but had normal levels if the protein were incubated with DTT prior to the addition of FeMoco. Both studies suggest desFeMoco-Av1 might have sulfhydryls that are subject to reversible modification. Because both αCys-45 and αCys-275 are chemically reactive in desFeMoco-Av1, they were considered prime candidates for the site of reversible modified by oxidation. As shown in Table 1, in the reversibly inactivated state of desFeMoco-Av1, neither αCys-45 nor αCys-275 is alkylated by IAA; yet, these residues are readily modified by IAA after the protein is incubation with DTT[28]. The regain of activity upon reconstitution follows the same pattern as the regain in ability to alkylate the cysteines.

Source for desFeMoco-Av1	UW 45	UW 45	DJ530	DJ530
DTT incubation	yes	no	yes	no
Activity[a]	45.3	6.7	62.3	9.3
% αCys-45 modified	29	3	na[b]	na
% αCys-275 modified	78	6	83	5.

Table 1. Activation of des-FeMoco-Av1 by incubation with DTT. Des-FeMoco-Av1 from UW45 (*nifB*) and DJ530 (*nifB, nifD:C45S*) was divided into two portions. One portion of each was incubated with 8 mM DTT for ten minutes at 30° C. Both portions were reconstituted with FeMoco and assayed by acetylene reduction as described in experimental procedures. In a separate experiment, the proteins were divided into two portions, one portion was incubated with 1 mM DTT for one hour at 30° C. Both portions of the protein were then allowed to react with 2 mM [^{14}C]IAA for 15 minutes. The fraction of the cysteines containing radiolabel were determined as described in Figure 2.

[a]nmol C_2H_4/minute. [b]not applicable.

To determine which cysteine was the likely site of reversible modification, desFeMoco-Av1 with αCys-45 changed to serine was isolated from mutant strain DJ530 (*nifB, nifD:C45S*). As for wild type desFeMoco-Av1, oxygen inactivated αC45S-desFeMoco-Av1 could be fully restored for FeMoco insertion and for IAA reactivity by incubating with DTT (See Table 1). If αC45S-desFeMoco-Av1 was reacted with [^{14}C]IAA first, subsequent incubation with DTT and FeMoco failed to restore activity. The peptide map of this protein showed that radiolabel was incorporated only into αCys-275, αCys-45 being absent (data, not shown). Together, these results indicate that of all cysteines in Av1, αCys-275 alone is necessary and sufficient for incorporation of FeMoco into desFeMoco-Av1 with concomitant formation of catalytically active enzyme.

Although intra-subunit disulfide formation might be a mechanism for reversible inactivation, as shown in Table 1, αC45S-desFeMoco-Av1 from DJ530 exhibited the same DTT reversible inactivation, as wild-type protein. Thus, disulfide formation between αCys-45 and αCys-275 cannot account for the reversible inactivation. Likewise disulfides between αCys-275 or αCys-45 on different desFeMoco-Av1 molecules are unlikely, because no increase in molecular weight was observed for the inactive material.

One reasonable possibility to explain the reversible blocking of the two cysteines is that oxidative decay products of dithionite, present in all buffers used for nitrogenase proteins, are reacting with the exposed cysteinyl residues. Dithionite undergoes a variety of chemical transformations with the following sequence of reactions, as one example of how the reversible modification might occur.

Thiosulfate is generated by the decay of dithionite[29]:

$$2\,S_2O_4^{2-} + H_2O \rightarrow S_2O_3^{2-} + 2\,HSO_3^-$$

In the presence of oxygen, thiosulfate can form tetrathionate[29]:

$$2\,S_2O_3^{2-} + O_2 + 2\,H_2O \rightarrow S_4O_6^{2-} + 2\,H_2O_2$$

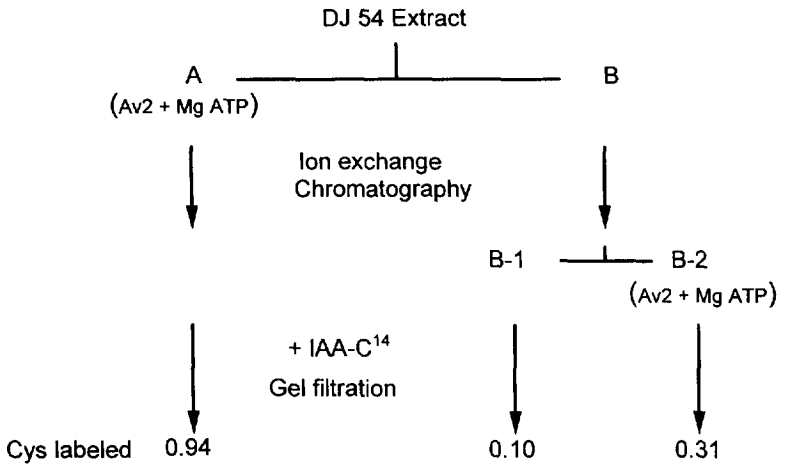
Tetrathionate is known to react with protein thiols to give the S-sulfenylsulfonate derivative[30]:

$$\text{Protein-S}^- + S_4O_6^{2-} \rightarrow \text{Protein-S-S-SO}_3^- + S_2O_3^{2-}$$

The S-sulfenylsulfonate group can be can be removed by exchange with excess thiol, such as, DTT, regenerating the free cysteine[30]. The observations made here with the hyper-reactive αCys-275 strongly suggest caution must be exercised when reconstituting Fe:S clusters in proteins where dithionite has been used as a reductant or oxygen scavenger.

DesFeMoco-Av1 Retains the Characteristic Cross-linking Reaction with Av2. Previously it has been shown that Av2 is involved in the synthesis and insertion of FeMoco[12,20-23]. This raises the question as to whether Av2 can bind to desFeMoco-Av1. To detect this interaction, the cross-linking reaction characterized by Willing et al.[31] was used. The water soluble carbodiimide EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, mediates a highly specific cross-linking reaction between Av1 and Av2. DesFeMoco-Av1 or reconstituted Av1 was incubated with Av2 and 12.5 mM EDC for fifteen minutes. The reaction mixtures were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis to determine the degree of chemical cross-linking. The results indicated that desFeMoco-Av1 and native Av1 were equally well cross-linked to Av2. Similar results have been reported by Gavini, et al.[22] using the form of desFeMoco-Av1 isolated from DJ54 (Δ*nifH*) which has the subunit composition $\alpha_2\beta_2$.

γ-subunit stabilizes an open conformation of desFeMoco-Av1. Homer, et al.[23] have shown that the activation of desFeMoco-Av1 by Av2 and MgATP in crude extracts correlates with the conversion of the $\alpha_2\beta_2$ form to the $\alpha_2\beta_2\gamma_2$ form which is then amenable to cofactor insertion. The selectivity of the cysteinyl labeling pattern observed above provides an experimental approach to investigate how γ might alter the desFeMoco-Av1 structure to facilitate the cofactor binding. The experimental protocol and results are shown in Scheme 1. An extract of DJ54 (containing desFeMoco-Av1 in the $\alpha_2\beta_2$ form) was divided in two: one portion (A) was incubated with MgATP and Av2 (the conditions necessary to incorporate γ into desFeMoco-Av1) followed by anaerobic ion exchange chromatography; the other portion (B) was directly subjected to the ion exchange chromatography. The desFeMoco-Av1 isolated in (B) was split with one portion (B-2) incubated with Av2 and MgATP. All three samples (A), (B-1), and (B-2) were incubated with [^{14}C]IAA, desalted, and the number of cysteines modified per α/β subunit pair determined. The results, shown at the end of the flow diagram, support the hypothesis that one role of Av2 and MgATP is to alter the desFeMoco-Av1, presumably by inducing tight binding of γ. This altered state has the property that one or more cysteinyl residues are uncovered and, as defined by the exposure of the thiol, is formed only in crude exacts. It is reasonable to conclude that binding of γ by desFeMoco-Av1 stabilizes an "open" form with αCys-275 exposed for binding of cofactor.



Scheme 1. Labeling of cysteines in desFeMoco-Av1 before and after incubation with Av2 and MgATP. All steps in the scheme were carried out in the presence of DTT to prevent dithionite related modification of cysteinyl residues (see text).

DISCUSSION

The metalloclusters and cysteinyl residues of Av1 were found to be unreactive to a variety of chelators and alkylating reagents, consistent with the x-ray structure, which shows the protein effectively surrounding the metal centers. In contrast, αCys-275 and αCys-45 in desFeMoco-Av1 ($\alpha_2\beta_2\gamma_2$ form) were readily alkylated by IAA, with the modification rate of αCys-275 characterized as hyper-reactive relative to other protein cysteines. Of the seventeen cysteinyl residues of an α/β-subunit pair, only two are exposed to alkylation while the P-cluster remains sequestered and unreactive to these thiol reagents. Most importantly, desFeMoco-Av1 retains the essential interactions for Av2 binding as shown by chemical cross-linking. This implies that much of the three dimensional structure of holo Av1 is already present in desFeMoco-Av1.

The absence of cysteine reactivity with IAA or iodoacetamide is consistent with the crystal structure of Av1 where the cysteines are buried; only αCys-45 and αCys-342 are near the surface with their sulfur atoms partially exposed to solvent. However, thiol reactivity as a measure of "exposure" should be interpreted with caution. For example, three cysteines, αCys-45, αCys-342, and αCys-471 are modified by ethylmercurithiosalicylate in the preparation of derivatives for the solution of the crystal structure, yet none of these are reactive to IAA. In desFeMoco-Av1, only one of these, αCys-45, is slowly modified. Therefore, one difference between Av1 and desFeMoco-Av1 must involve at least minimal conformational changes around αCys-45 which allows alkylation at this site.

The high rate of modification of αCys-275 in desFeMoco-Av1 was surprising in light of the x-ray crystal structure of Av1 where the FeMoco and αCys-275 are buried more than ten angstroms below the protein surface. αCys-275 is inaccessible and unreactive to IAA, yet the cofactor must be approached by the diverse substrates that are reduced by nitrogenase. For the high rate of alkylation of αCys-275 in desFeMoco-Av1, the region normally occupied by the cofactor must be considerably exposed. *A priori* arguments would predict an open cleft if the insertion of the cofactor is to be effected. The alkylation rate for αCys-275 suggests not only an open environment but also indicates that the cysteine has enhanced nucleophilicity. The latter presumably is a consequence of specific interactions with a basic side chain in the region which implies an organized cofactor binding region[32,33].

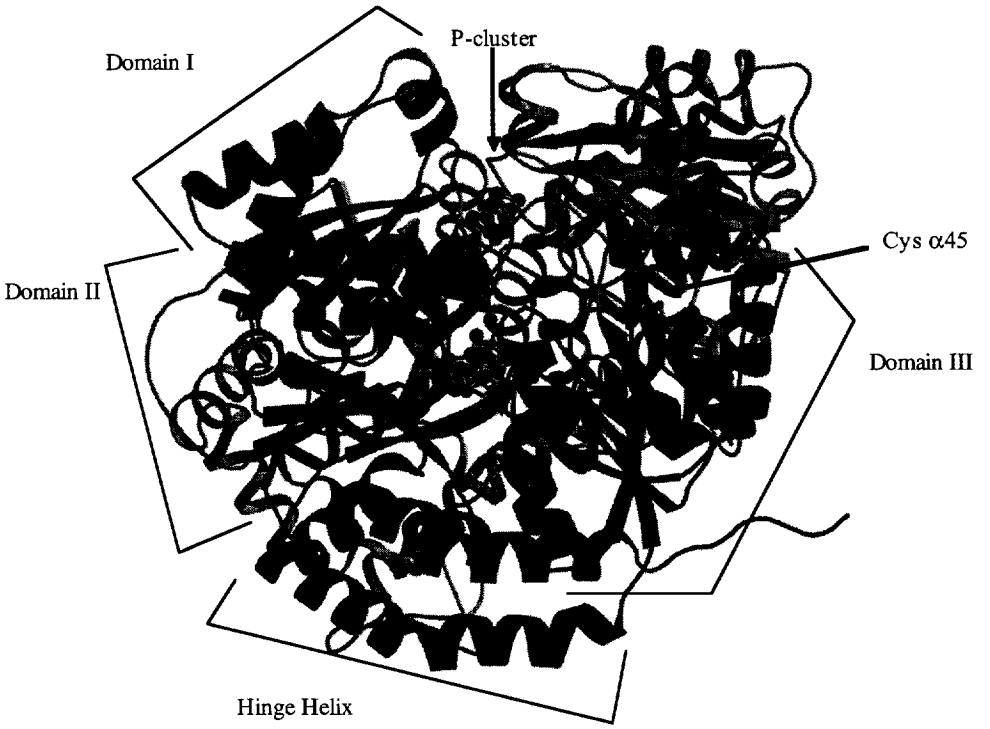


Figure 4. Ribbons diagram of the polypeptide fold of Av1 dimer (half of the native tetramer) with space filling models of the P-cluster and the FeMoco[37]. The view is into the channel at intersection of the three domains of the α-subunit (shades of gray) with the FeMoco at the bottom of the channel. The β-subunit is behind (white). Prepared with the program MOLSCRIPT[10].

To understand how the cofactor binding site might become exposed with minimal alteration in the $\alpha_2\beta_2$ desFeMoco-Av1 protein, we have examined the structure of Av1. Kim and Rees[5-6] described the α subunit of Av1 in terms of three domains and an inter-domain segment connecting domains II and III (Figure 4). At the intersection of the three domains, a channel is formed from the surface to the cofactor. This channel is filled by the van der Waals contacts between side chains and although closed, is a potential path for substrate access to the cofactor. The intersection is composed of residues, αHis-195, αSer-192, αArg-277, and αPhe-381, that form a ring immediately above the cofactor with a second ring of residues, αHis-196, αTyr-281 and αHis-383, at the protein surface. All seven of these channel forming residues are invariant among the known α-subunit sequences[34-35] while none are conserved in NifE sequences, a subunit of the proposed FeMoco assembly protein having sequence homology to the α-subunit of the MoFe-protein[36].

A number of conformational changes can be envisioned by which the inter-domain surface could be opened. What ever the change, binding of γ appears to stabilize it. Based upon our results and inspection of the crystal structure, it is tempting to speculate that the opening might be a domain rearrangement. Domain I contains the ligands to the P-cluster, many contacts with the β-subunit, and much of the Fe-protein binding groove[5,38] and these properties are retained to a large degree in desFeMoco-Av1 Thus, we would propose domain I is likely to remain essentially in the same orientation as in the holo-protein. In contrast, domain III is a more inviting region to suggest for conformational change. For example, domain III which is composed of the carboxy terminal ~130 residues and the amino terminal ~50 residues including αCys-45 has few contacts with the β-subunit and is exposed on the outside of the larger protein structure. Because αCys-45 becomes reactive to IAA in desFeMoco-Av1, it indicates some structural changes in domain III.

To provide access to the cofactor binding site, only a small rotation between domain III and the remainder of the molecule could create a sizable separation at the intersection of the domains. Again there are numerous ways to envision this occurring but one particularly noticeable structure in this regard is the long, 27 residue α-helix (residues 318-345) connecting domains II and III. Small changes along the helix or in the turns at either end of the helix could induce relatively larger changes in domain III. There is ample evidence for domain movement of this type found in other proteins[39]. In such a movement, only the carboxyl terminal segment of domain III is likely to move, leaving the amino terminal segment and potentially αCys-45 more exposed. It should be noted that the region from ~45-55 in the α-subunit was disordered in the original crystal structure[5-6]; only in the recently solved structure of the complex of Av1-Av2 was this loop able to be modeled[37]. This suggests this loop may be flexible and takes different structures in different forms

of the protein. An additional appealing feature of the domain movement model is that domain III contains the second protein ligand to the cofactor, αHis-442, and the rotation of the domain would also involve movement of this residue to allow access to the site.

The results presented here and previously strongly argue that the open form of desFeMoco-Av1 is a consequence of interactions by nucleotide bound Fe-protein and is stabilized by binding of the γ-subunit. Missing from our picture is where the γ-subunit binds and how it serves as a "wedge" to maintain the open form of desFeMoco-Av1 in absence of Fe-protein. The chemical crosslinking studies show that the γ-subunit does not interfere with Av2 association with desFeMoco-Av1 and binds at a noncompeting site. Likewise, thiol labeling in the presence of γ-subunit suggests it does not bind in these regions either.

METHODS

Construction of mutants Mutant strains of *A. vinelandii,* listed in Table 1, were generated using genomic recombination of template-directed, site-specific mutagenized M13 plasmid harboring the individual nitrogenase genes[40-41]. UW45, generated by random mutagenesis, is *nifB*[42-43]. DJ54 has a deletion in the *nif H*[12].

Anaerobic techniques and protein purification Reagents, buffers and septum-sealed vials were made anaerobic using a Schlenk manifold and argon purified by passage over BASF catalyst. Transfer of solutions was performed with argon flushed gas tight syringes or cannulae. Nitrogenase components from *A. vinelandii*, Av1 and Av2, were purified and characterized as described previously[44] using a modified programmable FPLC system. DesFeMoco-Av1 from the *A. vinelandii* mutant strains was purified initially as described by Paustian et al.[20] and subsequently with the following modifications. Approximately 80 g of freshly harvested cells were disrupted by osmotic shock. After centrifugation, the crude extract was applied to a column (5 x 20 cm) of DEAE Sepharose CL-6B. The following buffers were used elute the proteins: Buffer A; 50 mM Tris-HCl, pH 7.75, 20 % glycerol, 2 mM $Na_2S_2O_4$; Buffer B; 1.0 M NaCl, in Buffer A. The column was washed with 500 ml of 10 % buffer B in buffer A, and the proteins were eluted by a linear gradient of 10 % to 30 % buffer B. DesFeMoco-Av1 eluted between 10 and 13 milliSiemens (mS) and Av2 eluted between 16 and 18 mS. DesFeMoco-Av1 was concentrated by absorption and elution from a small column of DEAE Sepharose CL-6B. The concentrated protein was further purified by gel filtration on a 2.5 x 95 cm column of Sephacryl S-300 equilibrated in 50 mM HEPES-NaOH, pH 8.0, 50 mM NaCl, 20 % glycerol, 2 mM $Na_2S_2O_4$. The purified protein was stored at -80°C.

Activity assays Activity of the nitrogenase protein components were determined by acetylene reduction as described by Deits and Howard[46]. FeMoco was prepared by an abbreviated version of the procedure of Shah and Brill[11]. Reconstitution and assay of desFeMoco-Av1 was a modification of the method described by Shah[46]. An aliquot of desFeMoco-Av1 (0.2 to 1.0 mg) was added to a vial containing 0.3 ml of 50 mM Tris-HCl, pH 8.0, 15 mM sodium dithionite, 10 mM DTT and incubated for 10 minutes at 30°C. Av2 (0.1 mg), MgATP (5 mM final concentration) and FeMoco extract were added (final volume 1.0 ml) and incubated for 10 minutes. Additional Av2, an ATP regeneration mixture (10 mM creatine phosphate, 10 units of creatine kinase) and 1.0 ml of acetylene were added followed by incubated for 15 minutes. The assay was quenched with glacial acetic acid and the quantity of acetylene reduced to ethylene was measured by a flame ionization detector attached to a gas chromatograph.

Spectrophotometric experiments Anaerobic cuvettes were sealed with double rubber septa and subjected to repeated cycles of vacuum/argon. Buffers containing dithionite were injected into the cuvettes and allowed to stand for at least 15 minutes before adding the protein solution. Changes in absorbance resulting from destruction of the metal centers were monitored at 412 nm. Changes in absorbance due to formation of the $Fe^{2+}(\alpha,$a-dipyridyl$)_3$ complex were monitored at 520 nm.

Labeling and identification of cysteinyl peptides Time course of labeling of Av1 and desFeMoco-Av1: [^{14}C]IAA (final concentration: 2 mM) was added to 30 nmol of desFeMoco-Av1 or Av1 in 6 ml, 25 mM 3-[N-morpholino]propane-sulfonic acid (MOPS), pH 7.4, 20 % glycerol, 200 mM NaCl and 2 mM sodium dithionite. At various times of reaction, the protein was isolated by rapid gel filtration on a 2.5 x 20 cm column of Sephadex G-25 equilibrated in 50 mM Tris-HCl, pH 8.0, 2 mM sodium dithionite. The protein samples were exhaustively reduced and carboxymethylated with unlabelled IAA[26].

The protein samples were digested with 3% (w/w) tosylphenylalanyl-chloroketone treated trypsin at 30°C for 8 hours in 50 mM Tris-HCl, pH 8.0. The peptides were separated by reverse phase chromatography on a Vydac C-4 column using a gradient of acetonitrile in 0.1% trifluoroacetic acid. The peaks corresponding to peptides containing αCys-275 were further purified (data not shown) on a Vydac C-18 column using a gradient of isopropanol in 0.135 % trifluoroacetic acid.

Specific radioactivity of the Iodo[2-^{14}C]acetic acid was determined from Av1 which was exhaustively reduced and carboxymethlyated with the radioactive IAA. After acid hydrolysis of the protein, a portion was subjected to liquid scintillation counting and another to amino acid analysis. The specific radioactivity was determined from the recovery of S-carboxymethyl-cysteine. Amino acid sequences of cysteinyl peptides were determined by repetitive Edman degradation with an Applied Biosystems 470A gas phase sequencer.

This work was supported by NSF grants MCB-9513512 (JBH), MCB-9317059 (GR), MCB-9630127 (DD) and DMB 91-18689.

REFERENCES AND NOTES

1. Howard, J.B.; Rees, D.C. *Ann. Rev. Biochem.* **1994**, 63, 235-264.
2. Peters, J.W.; Fisher, K.; Dean, D.R. *Annu. Rev. Microbiol.* **1995**, 49, 335-374.
3. Burgess, B.K.; Lowe, D.J. *Chem. Rev* **1996**, 2983-3012.
4. Howard, J.B.; Rees, D.C. *Chem. Rev* **1996,** 2965-2982.
5. Kim, J.; Rees, D.C. *Science* **1992,** 257, 1677-1682.
6. Kim, J. ; Rees, D.C. *Nature* **1992,** 360, 553-560.
7. Chan, M.K.; Kim, J.; Rees, D.C. *Science* **1993**, *260*, 792-794.
8. Bolin, J.T.; Campobasso, N.; Muchmore, S.W.; T.V., M.; Mortenson, L.E. in *Molybdenum Enzymes, Cofactors and Model Systems. ACS Symposium Series No. 535,* Stiefel, E.I.; Coucouvanis, D.; Newton, W.E., Eds., American Chemical Society, Washington, D.C., **1993**, 186-195.
9. Peters, J.P., Stowell, M.H.B., Soltis, S.M.; Finnegan, M. G.; Johnson, M. L.; Rees, D.C. *Biochemistry* **1996**, 36, 1181-1187.
10. Kraulis, P.J. *J. Appl. Cryst.* **1991**, 24, 946-950.
11. Shah, V.K.; Brill, W.J. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 3249-3253.
12. Robinson, A.C.; Dean, D.R; Burgess, B.K. *J. Biol. Chem.* **1987**, 262, 14327-14332.
13. Robinson, A.C.; Chun, T.W.; Li, J.-G.; Burgess, B.K.. *J. Biol. Chem.* **1989**, 264, 10088-10095.
14. Paustian, T.D.; Shah, V.K.;Roberts, G.P. *Proc. Natl. Acad. Sci. USA* **1989**, 86, 6082-6086.
15. Hoover, T.R.; Imperial, J.; Ludden, P.W.; Shah, V.K. *Biochemistry* **1989**, 28, 2768-2771.
16. Allen, R.M.; Chatterjee, R.; Madden, M.S.; Ludden, P.W.; Shah, V.K. *Crit. Rev. Biotech.* **1994**, *14*, 225-249.
17. Allen, R. M.; Chatterjee, R.; Ludden, P. W.; Shah, V.K; *J. Biol. Chem.* **1995,** 270, 26890-26896.

18 Shah, V.K.; Allen, J. R.; Spangler, N. J.; Ludden, P. W. *J. Biol. Chem.* **1994**, 269, 1154-1158.
19. Roll, J.T., Shah, V.K.; Dean, D.; Roberts, G.P.*J. Biol. Chem.* **1995**, 270, 4432-4437.
20. Paustian, T.D.; Shah, V.K.; Roberts, G.P *Biochemistry* **1990**, 29, 3515-3522.
21. Allen, R.M.; Homer, M.J.; Chatterjee, R.; Ludden, P.W.; Roberts, G.P.; ShahV.K. *J. Biol. Chem.* **1993**, 268, 23670-23674.
22. Gavini, N.; Ma, L.; Watt, G.; Burgess, B.K. *Biochemistry* **1994**, *33*, 11842-11851.
23. Homer, M.J.; Dean, D; Roberts, G.P. *J. Biol. Chem.***1995**, 270, 24745-24752.
24. Plank, D.W.; Howard, J.B. *J. Biol. Chem.***1988**, 263, 8184-8189.
25. Hausinger, R.P.; Howard, J.B. *J. Biol. Chem.***1983**, 258, 13486-13492.
26. Lundell, D.J;. Howard, J.B. *J. Biol. Chem.***1981**, 256, 6385-6391.
27. Brigle, K.E.; Newton, W.E.; Dean, D.R. *Gene* **1985**, 37, 37-44.
28. Although DTT or 2-mercaptoethanol may be present in excess over IAA, at pH 8, the protein cysteines react at least an order of magnitude faster than these exogeneous thiols with IAA. Crestfield, A; Moore, S.; Stein, W. *J. Biol. Chem.* **1963**, 238, 2684-2691.
29. Cotton, F.A. and Wilkinson, G. *Advanced Inorganic Chemistry: A Comprehensive Text*, 4th ed. **1980** John Wiley & Sons. New York.
30. Glazer, A.N.;DeLange, R.J.; Sigman, D.S. *Chemical Modification of Proteins* **1975** American Elsevier Publishing Co., Inc. New York.
31. Willing, A.H.; Georgiadis, M.M.; Rees, D.C.; Howard, J.B. *J. Biol. Chem.* **1989**, 264, 8499-8503
32. Snyder, G.H.; Cennerazzo, M.J.; Karalis, A.J.; Field, D. *Biochemistry* **1981**, 20, 6509-6519.
33. Parente, A.; Merrifield, B.; Geraci, G.; D'Alessio, G. *Biochemistry* **1985**, 24, 1098-1104.
34. Ioannidis, I.; Buck, M. *Biochem. J.* **1987**, 247, 287-291.
35. Wang, S.-Z.; Chen, J.-S.; Johnson, J.L. *Biochemistry* **1988**, 27, 2800-2810.
36. Brigle, K.E.; Weiss, M.C.; Newton, W.E. Dean, D.R. *J. Bacteriol.* **1987**, 169, 1547-1553.
37. Schindelin, H.; Kisker, C.; Schlessman, J. L.; Howard, J. B.; Rees, D. C. *Nature* **In Press.**
38. Howard, J.B. in *Molybdenum Enzymes, Cofactors and Model Systems. ACS Symposium Series No. 535,* Stiefel, E.I.; Coucouvanis, D.; Newton, W.E., Eds., American Chemical Society, Washington, D.C., **1993**, 271-289.
39. Gerstein, M.; Lesk, A.M.; Chothia, C. *Biochemistry* **1994**, 33, 6739-6749.
40. Brigle, K.E.; Setterquist, R.A.; Dean, D.R.; Cantwell, J.S.; Weiss, M.C; Newton, W.E. *Proc. Natl. Acad. Sci. USA* **1987**, 84, 7066-7069.
41. Howard, J.B.; Davis, R.; Moldenhauer, B.; Cash, V.L; Dean, D *J. Biol. Chem* **1989**, 264, 11270-11274.
42. Shah, V.K.; Davis, L.C.; Gordon, J.K.; Orme-Johnson, W.H.; Brill, W.J. *Biochim. Biophys. Acta* **1973**, 292, 246-255.
43. Joerger, R.D.; Bishop, P.E.. *J. Bacteriol.* **1988**, 170, 1475-1487.
44. Wolle, D.; Kim, C.; Dean, D.; Howard, J.B.. *J. Biol. Chem.***1992**, 267, 3667-3673.
45. Deits, T. L.; Howard, J. B. *J. Biol. Chem.* **1990**, 265, 3859-3867.
46. Shah, V.K.; Imperial, J.; Ugalde, R.A.; Ludden, P.W.; Brill, W.J. *Proc. Natl. Acad. Sci. USA* **1986**, 83, 1636-1640.

(*Received* 10 *April* 1997; *accepted* 12 *June* 1997)