The biosynthesis of the iron-molybdenum cofactor (FeMo-co) of nitrogenase was investigated using the purified \textit{in vitro} FeMo-co synthesis system and 99Mo. The purified system involves the addition of all components that are known to be required for FeMo-co synthesis in their purified forms. Here, we report the accumulation of a 99Mo-containing FeMo-co precursor on NifNE. Apart from NifNE, NifH and NifX also accumulate 99Mo label. We present evidence that suggests NifH may serve as the entry point for molybdenum incorporation into the FeMo-co biosynthetic pathway. We also present evidence suggesting a role for NifX in specifying the organic acid moiety of FeMo-co.

Dinitrogenase (NifKD, MoFe-protein) and dinitrogenase reductase (NifH, Fe-protein) comprise the two-component complex metalloenzyme nitrogenase (1). Dinitrogenase is an \(\alpha_2\beta_2\) heterotetramer of the \(nifK\) and \(D\) gene products, while dinitrogenase reductase is an \(\alpha_2\) homodimer of the \(nifH\) gene product. The iron-molybdenum cofactor (FeMo-co) is the site of substrate reduction (2–4) and resides within the \(\alpha\) subunits of dinitrogenase (5). FeMo-co consists of iron, sulfur, and molybdenum atoms in the ratio 7:9:1, and a molecule of homocitrate that serves as a non-protein ligand to the molybdenum atom (6).

Genetic and biochemical studies in \textit{Klebsiella pneumoniae} and \textit{Azotobacter vinelandii} have revealed that the products of \(nifQ\), \(V\), \(B\), \(H\), \(X\), \(N\), and \(E\) are involved in the biosynthesis of FeMo-co (7–11). However, the exact roles played by many of these \(nif\) gene products remain uncertain. A list of the \(nif\) gene products involved in FeMo-co biosynthesis and their known/postulated roles in this process is given in Table I. Early on, the studies conducted by Imperial et al. (12) have made clear that the structural genes encoding dinitrogenase, \(nifK\) and \(D\), are not required for the biosynthesis of the cofactor per se. This suggested that FeMo-co is synthesized separately and then inserted into cofactor sites in NifKD.

An \textit{in vitro} FeMo-co biosynthesis system has been established, which involves mixing extracts from two \textit{A. vinelandii} strains with complementary defects in the synthesis of FeMo-co; alternatively, the addition of the missing component can be added to complement a particular mutant extract (13). This assay system requires at least homocitrate, molybdate (as a source of molybdenum), MgATP, reductant (sodium dithionite), NifNE, NifH, NifB-cofactor (NifB-co), and NifX.

There is limited information regarding the iron, sulfur, and molybdenum donors to FeMo-co. The metabolic product of NifB, termed NifB-co, has been shown to be comprised only of iron and sulfur (14). Studies by Allen et al. (15) with \(\text{Fe}\) and \(\text{S}\)-labeled NifB-co have conclusively shown that NifB-co functions as a specific iron and sulfur donor to FeMo-co. However, whether NifB-co is the sole iron and sulfur donor to FeMo-co or if additional components are required for this purpose still needs to be determined.

The \textit{in vivo} accumulation of molybdenum in \textit{A. vinelandii} and \textit{K. pneumoniae} was studied by Pienkos and Brill (16). They reported the accumulation of molybdenum on a non-\(nif\) protein termed Mo-storage protein (Mo-sto) in \textit{A. vinelandii}. The 99Mo incorporation study in a \(nifDK\) mutant of \textit{K. pneumoniae}, by Ugalde et al. (17), revealed that the radiolabel accumulated on a 65-kDa protein. The \textit{in vitro} FeMo-co biosynthesis system was utilized by Hoover et al. (4) with various homologs of homocitrate to determine which of the organic acids were able to support 99Mo incorporation into NifKD. Allen et al. (18) have used 99Mo and the \textit{in vitro} biosynthesis system involving various mutant strains of \textit{A. vinelandii} and have observed the accumulation of 99Mo on the non-\(nif\) chaperone-protein, gamma.

In the present study, we have used 99Mo and the purified \textit{in vitro} FeMo-co biosynthesis system to investigate the pattern of incorporation of the radiolabel. We report the accumulation of a 99Mo-containing FeMo-co precursor on NifNE, NifH, and NifX. We present evidence suggesting that NifH/NifX complex serves as the entry point for molybdenum into the FeMo-co biosynthetic pathway and that NifX may play a role in specifying the organic acid moiety of FeMo-co.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Materials—}Leupeptin, phenylmethylsulfonyl fluoride (PMSF), phosphocreatine, creatine phosphokinase, homocitrate lactone, d,L-isocitric acid, n-malic acid, 2-ketogluarate, tricarboxylic acid, and ATP (as disodium salt) were from Sigma Chemical Co. 1,2,4-Butane tricarboxylic acid was from Chem Service (West Chester, PA). Citric acid was obtained from Aldrich Chem Co. Tris base, glycerol, and glycine were from Fisher Scientific Co. Sodium dithionite (DTH) was from Fluka. Acrylamide/bisacrylamide solutions (37.5%:1%), and SDS-PAGE equipment was from Bio-Rad. Carrier-free 99Mo was obtained from the Department of BioMedical Physics, University of Wisconsin-Madison.

Buffers—25 mM Tris-HCl (pH 7.4) was used throughout unless otherwise noted. All buffers were sparged with nitrogen for at least 20 min.
prior to alternating cycles of evacuation and flushing with argon on a gassing manifold. DTH was added to a final concentration of 1 mM. Buffers used for protein purification contained 0.5 μg/ml leupeptin and 0.2 mM PMSF.

**In vitro FeMo-co Synthesis**—The in vitro FeMo-co synthesis reactions were performed in 9-ml serum vials sealed with serum stoppers. The vials were repeatedly evacuated and flushed with argon gas and rinsed with 0.3 ml of anaerobic buffer. The complete purified in vitro FeMo-co synthesis reaction mixture contained the following components: 0.1 ml of anaerobic buffer, 0.2 ml of unlabeled sodium molydate, 100 nmol of homocitrate that had been treated with base to cleave the lactone (pH 8.0), 0.2 ml of an ATP-regenerating system (containing 3.6 mM ATP, 6.3 mM MgCl₂, 51 mM creatine phosphate, 20 units/ml creatine phosphokinase, and 6.3 mM DTH in anaerobic buffer), 10 μl of a solution containing NiF-co (0.4 nmol of iron/μl), 7.5–30 μg of purified NiFE, 25 μg of purified NiFH, 15 μg of purified NiFX, 55 μg of purified apoapodinitrogenase (α₂βγγ), and 200 μg of 99Mo (1.5 million cpm, prepared in anoxic Tris-HCl buffer containing 1.7 mM DTH). The total volume of each reaction mixture was 510 μl. The reactions were incubated at 30°C for 35 min to allow FeMo-co synthesis and the insertion of the newly formed FeMo-co into apodinitrogenase. Reactions that were used to monitor only FeMo-co biosynthesis contained all the components listed above except for apodinitrogenase. The total volume for such reaction mixtures was 490 μl.

Reactions containing various organic acids in place of homocitrate were performed as described by Imperial et al. (19). The final concentrations of the various homocitrate analogs in the in vitro FeMo-co synthesis assay were 8 mM for citrate and malate, 1.6 mM for isocitrate and 2-ketoglutarate, and 16 mM for tricarballylate and for 1,2,4-butane tricarboxylate. Solutions of the various homocitrate analogs were prepared in 0.2 mM PMSF.

**TABLE I**

| nif or non-nif gene | Proposed function in FeMo-co biosynthesis | References |
|---------------------|------------------------------------------|------------|
| nifQ                | Mo processing                            | 11         |
| nifY                | Homocitrate synthesis                    | 4, 27, 28  |
| nifB                | NiF-co; Fe and S to apodinitrogenase      | 14, 15     |
| nifN                | Along with NiF is proposed to function as a scaffold on which FeMo-co is synthesized | 23, 24, 44 |
| nifE                | Along with NiFN is proposed to function as a scaffold on which FeMo-co is synthesized | 23, 24, 44 |
| nifH                | not well understood                      | 20, 37–42  |
| nifX                | not well understood                      | 10         |
| nafY                | Chaperone; binds FeMo-co specifically    | 22, 30     |

**RESULTS**

**Incorporation of ⁹⁹Mo into Components of the In Vitro FeMo-co Synthesis Reaction Mixture**—Earlier studies have indicated that the products of nifB, H, N, E, and X are required for the in vitro FeMo-co synthesis reaction (10, 13, 14, 26). Apart from the above-mentioned gene products, NiFY and nifQ are also required during FeMo-co biosynthesis in vivo for the production of homocitrate and for a proposed molybdenum processing step, respectively (11, 27, 28). Typically, the in vitro FeMo-co synthesis reaction involves mixing extracts from different mutants defective in the synthesis of the cofactor (13). In contrast, the purified in vitro FeMo-co synthesis system involves the addition of all components known to be required for cofactor synthesis, in their purified forms (10). The purified system yields lower FeMo-co synthesis activity in comparison to a system employing crude cell extracts, thus suggesting the requirement of other component(s) essential for cofactor synthesis (10). Apodinitrogenase, NiFNE, NiF-co, NiFH, and NiFX were purified as described under “Experimental Procedures.” The incorporation of ⁹⁹Mo into components of the in vitro FeMo-co synthesis reaction was monitored by separating the proteins in the reaction mixture on anoxic native gels and by phosphorimage analyses of these gels (Fig. 1). Assignment of the various bands on the gel was accomplished by immunoblotting for the relevant proteins on similar gels. When all components known to be required for FeMo-co synthesis, namely NiFNE, NiFH, NiFX, NiF-co, MgATP, and homocitrate are present (lane 1), incorporation of ⁹⁹Mo into three distinct bands can be observed. In a minus-homocitrate reaction, as shown in lane 2, there is higher labeling of NiFH as well as incorporation of label into a slower-migrating band labeled NiFNE. An important feature to be noted here is that the total amount of radiolabel incorporated into the various proteins is higher in lane 2 versus lane 1 of Fig. 1. This result was observed consistently, and one possible explanation for this phenomenon is the inability of the proteins in the complete reaction mixture (lane 1) to bind finished FeMo-co. FeMo-co, being unstable in aqueous solutions, disintegrates, leading to a lower accumulation of the total radiolabel in a “complete” reaction mixture. However, when one of the components necessary for FeMo-co synthesis is missing from the reaction mixture, the cofactor biosynthetic pathway is blocked at a particular step. In this scenario, FeMo-co intermediates are still bound to various proteins, and thus the total amount of radiolabel in reactions missing an

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Rubio et al., unpublished results.
essential component for cofactor biosynthesis is higher than the reaction containing all the necessary components.

The minus-MgATP (lane 3), minus-NifH (lane 4), minus-NiFe (lane 6), and minus-NifB-co (lane 7) reactions do not show incorporation of radiolabel into any distinct bands. This suggests that the incorporation of $^{99}$Mo into NiFe, NiH, and NiF is dependent on the presence of MgATP, NiFe, NiF-co and NiH. The minus-NiF reaction (lane 5), however, showed the incorporation of $^{99}$Mo into NiH and to a lesser extent into NiFe, indicating that NiF likely plays a role in the latter part of the cofactor biosynthetic pathway.

**Incorporation of $^{99}$Mo-containing FeMo-co Precursor into NiFe**—NiFe is believed to play a catalytic role in the early part of the FeMo-co biosynthetic pathway (23). Fig. 1 shows that $^{99}$Mo incorporation into NiFe is dependent on the presence of MgATP (lane 3), on NiH (lane 4), and on NiF-co (lane 7). A homocitrate-deficient reaction (lane 2) showed a higher level of incorporation of $^{99}$Mo into NiFe, suggesting that FeMo-co biosynthesis may be blocked at the point where homocitrate is added to the FeMo-co precursor. The addition of homocitrate (lane 1) allowed cofactor biosynthesis to proceed beyond this point, and consequently a lower level of labeling of NiFe was observed. The data presented here suggest that the precursor of FeMo-co on NiFe is homocitrate-deficient. Because the incorporation of $^{99}$Mo is dependent on the presence of MgATP, NiF-co, and NiH and because NiFe alone in the presence of MgATP did not show any incorporation of $^{99}$Mo (data not shown), we conclude that the radiolabel on NiFe is not adventitious. Quantitation of the $^{99}$Mo label on NiFe was performed as described under “Experimental Procedures” and revealed 0.6 nmol of molybdenum per nmol NiFe.

In the above reactions excess NiFe (30 μg) was used to observe the incorporation of $^{99}$Mo label into NiFe. When a catalytic quantity of NiFe (7.5 μg) was used, $^{99}$Mo accumulation on NiFe was greatly diminished; but this level of NiFe was sufficient for the radiolabel accumulation on NiH and NiF, as can be observed in Figs. 2 and 3. Prior to this study, $^{99}$Mo-labeling of NiFe has not been observed. Previous studies (17, 18) have involved the use of crude cell extracts, and two possible reasons for not observing the incorporation of $^{99}$Mo into NiFe could be the interference from other proteins present in the cell extracts, or that the synthesis of FeMo-co is not adventitious. Quantitation of the $^{99}$Mo label on NiFe was performed as described under “Experimental Procedures” and revealed 0.6 nmol of molybdenum per nmol NiFe.
that significant incorporation of the heterometal into NiFH occurred only when components known to be required for FeMo-co biosynthesis were present in the reaction mixture. For example, when MgATP, NiFNE, or NiFB-co (Fig. 1, lanes 3, 6, and 7) were not included in the reaction mixture, no incorporation of $^{99}$Mo into NiFH could be observed. However, a minimal level of radiolabel was incorporated into NiFH when a high concentration of NiFH (>5 nmol) was incubated with $^{99}$Mo and MgATP (Fig. 2, lane 5). This low level of nonspecific binding to molybdenum possibly occurs at the $PO_4^-$-binding site in NiFH. It should be noted here that Georgiadis et al. (29) have reported the presence of a molybdenum atom in the structure of NiFH. In a minus-homocitrate reaction mixture, a higher level of incorporation was consistently observed (lane 2 in Figs. 1 and 2), suggesting that the FeMo-co precursor on NiFH lacked homocitrate. Quantitation of radiolabel on NiFH performed as described under “Experimental Procedures” indicated 0.54 nmol of molybdenum per nmol NiFH. Upon the addition of the non-nif chaperone protein NafY (gamma) to the reaction mixture (Fig. 2, lane 3), the label is seen on a slightly faster migrating species than NiFH that is labeled NaFY, as judged by the migrating pattern of NaFY-FeMo-co species on similar gels as determined by immunoblotting. This demonstrates a transfer of finish FeMo-co from NiFH and NiFX to NaFY. Homer et al. (30) have shown that NaFY specifically binds FeMo-co, by in vitro as well as in vivo studies. When purified apodinitrogenase ($\alpha_2\beta_2\gamma_2$) is added to the reaction mixture (lane 4), the $^{99}$Mo label can be observed at the dinitrogenase position with a concomitant decrease in the amount of label associated with NiFH and with NiFX, indicating that the completed FeMo-co has been inserted into apodinitrogenase.

**Incorporation of $^{99}$Mo-containing FeMo-co Precursor into NiFX—**Using the purified in vitro FeMo-co synthesis system by Shah et al. (10), NiFX has been shown to be required for FeMo-co synthesis. When all components known to be required for FeMo-co synthesis are present in the reaction mixture including NiFH, NiFX, and catalytic amounts of NiFNE (7.5 $\mu$g), $^{99}$Mo-radiolabel is observed on NiFX, apart from NiFNE and NiFH (Fig. 1). This incorporation of $^{99}$Mo into NiFX is dependent on the presence of MgATP, NiFH, NiFNE, and NiFB-co (Fig. 1, lanes 3, 4, and 6, 7). There was no incorporation of the radiolabel when NiFX was incubated by itself with $^{99}$Mo in the presence or absence of MgATP (data not shown). However, a minus-NiFX reaction could support the incorporation into NiFNE as well as NiFH (Fig. 1, lane 5), suggesting that NiFX plays a role in the latter part of the FeMo-co biosynthetic pathway.

The effect of various organic acids on the incorporation of $^{99}$Mo into the components of the purified in vitro FeMo-co biosynthesis system was studied by including various organic acids in the reaction mixture. The reaction mixtures included NiFH, NiFX, and catalytic amounts of NiFNE (7.5 $\mu$g) and were performed as described in the “Experimental Procedures” section. The results of this study, shown in Fig. 3, indicate that there is incorporation of $^{99}$Mo into NiFH as well as NiFX. However, while the incorporation of the radiolabel into NiFH occurred no matter what organic acid was present in the reaction mixture, the incorporation into NiFX was dependent on the presence of certain organic acids. Only homocitrate, isocitrate, malate, citrate, and 2-ketoglutarate could support the incorporation of $^{99}$Mo into NiFX (Fig. 3, lanes 1, 3, 4, and 7, 8). The organic acids 1,2,4-butanetricarboxylate and tricarballylate (lanes 5 and 6) did not support the synthesis of FeMo-co and the incorporation of radiolabel into NiFX. Previous studies by Hoover et al. (4) and by Imperial et al. (19) have shown that both organic acids 1,2,4-butanetricarboxylate and tricarballylate did not support the incorporation of $^{99}$Mo into dinitrogenase when homocitrate was replaced with these organic acids. Both 1,2,4-butanetricarboxylate and tricarballylate have also been involved in the biosynthesis of FeMo-co. The salient features of incorporation of $^{99}$Mo into dinitrogenase were performed as described under “Experimental Procedures.” Lane 1, reaction including all components required for the biosynthesis of FeMo-co and apodinitrogenase ($\alpha_2\beta_2\gamma_2$) was shown in Fig. 2, lane 1, minus-NifB-co reaction; lane 3, minus-NiFH reaction; lane 4, minus-NiFX reaction; lane 5, minus-MgATP reaction. The table below the figure indicates the presence (+) or absence (−) of the particular component. The position of dinitrogenase is indicated. Specific activities (nmol C$_2$H$_4$ per min per mg apodinitrogenase) are reported; n.d., not determined.

![Phosphorimager analyses of anoxic native gel illustrating the NifX-dependent incorporation of $^{99}$Mo into apodinitrogenase](image)

**Fig. 4.** Phosphorimager analyses of anoxic native gel illustrating the NifX-dependent incorporation of $^{99}$Mo into apodinitrogenase. The in vitro FeMo-co synthesis reactions containing all the components required for the synthesis of FeMo-co including apodinitrogenase were performed as described under “Experimental Procedures.” Lane 1, reaction including all components required for the biosynthesis of FeMo-co and apodinitrogenase ($\alpha_2\beta_2\gamma_2$); lane 2, minus-NifB-co reaction; lane 3, minus-NiFH reaction; lane 4, minus-NiFX reaction; lane 5, minus-MgATP reaction. The table below the figure indicates the presence (+) or absence (−) of the particular component. The position of dinitrogenase is indicated. Specific activities (nmol C$_2$H$_4$ per min per mg apodinitrogenase) are reported; n.d., not determined.

Based on the above results, it seemed likely that NiFX played a role in the latter part of the cofactor biosynthetic pathway. To further analyze the role of NiFX, we monitored the incorporation of $^{99}$Mo label into dinitrogenase in reactions including and excluding NiFX. The in vitro FeMo-co biosynthesis assays were performed as described under “Experimental Procedures.” The complete reaction (Fig. 4, lane 1) shows the incorporation of $^{99}$Mo into dinitrogenase. A specific activity of 134.5 nmol per min per mg of protein was obtained and this suggested the formation of FeMo-co and its insertion into apodinitrogenase ($\alpha_2\beta_2\gamma_2$). In the absence of NiFB-co, NiFH, or MgATP there was no incorporation of the radiolabel into dinitrogenase. However, a minus-NiFX reaction (lane 4) showed a much lower level of label incorporated into dinitrogenase. This reaction also showed a lower specific activity (29.8 nmol) than the reaction containing NiFX. Quantitation of the radiolabel on dinitrogenase revealed −4.5 times lower level of label on the dinitrogenase band in the minus-NiFX reaction when compared with the reaction that contained NiFX. These data suggest a role for NiFX in the insertion process of the cofactor into apodinitrogenase; however, this role might not be absolutely required.

**DISCUSSION**

We have studied the accumulation of $^{99}$Mo into proteins involved in the biosynthesis of FeMo-co. The salient features of
the present study are: 1) NifNE, NifH, and NifX accumulate 9$^{99}$Mo-FeMo-co precursors; 2) transfer of 9$^{99}$Mo label from NifH and NifX to NafY or apodinitrogenase, indicating the presence of completed FeMo-co; 3) involvement of NifX in specifying organic acid moiety of FeMo-co; and 4) requirement of NifNE and NifH for the incorporation of molybdenum into the FeMo-co biosynthetic pathway.

Based on the results presented in this study and from previous studies with $^{55}$Fe-NifB-co (20), the following hypothesis for the biosynthesis of FeMo-co is consistent with our results. NifNE binds NifB-co; then NifH binds the NifNE-NifB-co complex. Our previous results suggest that NifH and NifNE form a complex (42), and results presented here suggest that only when both NifH and NifNE are present is either protein capable of binding molybdenum. Hence, we propose that the addition of molybdenum to NifB-co occurs within the NifNE-NifB-co-NifH complex to yield a Mo-containing FeMo-co precursor. The binding of NifB-co to NifNE is one of the early steps in FeMo-co biosynthesis. This event has been observed as a change in the mobility of NifNE on anoxic native gels by Roll et al. (23) and by visualizing $^{55}$Fe label on NifNE when radiolabeled NifB-co was used by Allen et al. (15) and Rangaraj et al. (20). Because the binding of NifB-co to NifNE does not require the presence of any additional factors such as MgATP or NifH, this step has been suggested as one of the first steps in the cofactor biosynthetic pathway. It is not yet known if any change is brought in about NifB-co upon its binding to NifNE. The role of NifNE in FeMo-co biosynthesis is thought to be that of a scaffolding protein upon which FeMo-co is synthesized (44). This is supported by the facts that NifNE and dinitrogenase share high sequence similarity and that apodinitrogenase is not required for FeMo-co synthesis. In A. vinelandii, NifHNE is absolutely required for the biosynthesis of FeMo-co as nifN or E mutants show a definite nif phenotype. However, a recent report by Siemann et al. (31) reveals that in Rhodobacter capsulatus NifNE is not essential for the biosynthesis of FeMo-co, though its presence greatly enhances the content of intact FeMo-co-containing NifDK.

NifB-co has been shown to function as a specific iron and sulfur donor to FeMo-co. It is not known whether all of the iron and sulfur of FeMo-co are donated solely by NifB-co, but it seems likely that a major portion of the iron and sulfur in FeMo-co is derived from NifB-co. Though the structure of NifB-co is yet to be solved, it is known that NifB-co contains iron and sulfur, but not molybdenum (14). NifU and NifS are believed to be involved in mobilizing iron and sulfur (32, 33), respectively, into NifB for the synthesis of NifB-co because NifB-co levels in K. pneumoniae mutants lacking nifS and nifU are greatly decreased.3

The next step in FeMo-co biosynthesis involves NifH. NifH has been shown to be a multifunctional “moonlighting” protein (34) playing diverse roles in the nitrogenase enzyme system: 1) substrate reduction wherein it functions as the obligate electron donor to dinitrogenase (35); 2) the maturation of apodinitrogenase wherein its presence is required for associating NafY with the NafY-deficient apodinitrogenase (36); and 3) the biosynthesis of FeMo-co (8, 26, 37). Its role in substrate reduction is best understood, while the specific role(s) of NifH in the latter two processes is obscure. The facts that several site-specifically altered forms of NifH showing altered MgATP reactivity function in cofactor biosynthesis (38–40) and that the 4Fe-4S cluster of the protein is not required for its function in this process (41) argue for a role that is very different from the NifH role in substrate reduction. We have shown in the present study that a $^{99}$Mo-labeled FeMo-co precursor accumulates on NifH and that this accumulation of radiolabel is dependent upon the presence of NifB-co and NifNE as well as MgATP. Because the presence of both NifNE and NifH is required to observe any accumulation of $^{99}$Mo, we propose that molybdenum is added to the FeMo-co precursor bound by the NifNE-NifH complex. This hypothesis is further strengthened by the study of L127A NifH (a site-specifically altered form of NifH) and NifNE that suggested an interaction of NifH with only the NifB-co associated form of NifNE (42). Studies with $^{55}$Fe-NifB-co also show accumulation of a FeMo-co precursor on NifH (20).

In a minus-NifH reaction there is no accumulation of molybdenum on any protein, suggesting that NifH is necessary for the entry of molybdenum into the FeMo-co biosynthetic pathway. Data presented in this study suggest that the action of NifH occurs prior to the addition of homocitrate, as a minus-homocitrate reaction showed a higher level of NifH labeling than a reaction wherein homocitrate was present. We have presented evidence for the involvement of NifH along with NifNE in the addition of heterometal, molybdenum, to the cofactor precursor. In this context, it seems likely that VnfH and AnfH, NifH counterparts in the vnf and the anf systems, serve in a similar manner and specify V and Fe respectively, during FeV-co and FeFe-co biosyntheses.

The next step in FeMo-co biosynthesis involves the action of NifD. The presence of NifD is not necessary for the accumulation of $^{99}$Mo label on NifH. This suggests that NifX plays a role in the latter part of the FeMo-co biosynthetic pathway. In a previous study we have shown that NifX is capable of binding NifB-co and that a $^{55}$Fe-labeled FeMo-co precursor accumulated on NifX (20). Recent data suggest that NifB-co can be transferred to NifNE. However, whether this transfer is physiologically relevant remains to be determined. In the present study, we have shown the homocitrate-dependent labeling of NifX, suggesting a role for NifX in specifying the organic acid moiety in FeMo-co. Also, an enhancement in incorporation of the $^{99}$Mo-radiolabel into apodinitrogenase in the presence of NifX that also correlates with C2H2 reduction activity has been observed. This suggests a role for NifD in the cofactor insertion process. In this context, VnfX, the NifX homolog in the vanadium nitrogenase system, has also been shown to accumulate a FeV-co precursor, and Rüttimann et al. (43) have shown the homocitrate-dependent transfer of the VnfX-bound FeV-co precursor to apodinitrogenase.

The non-nif protein NafY (aka gamma) has been postulated to function as a chaperone-insertase in the biosynthesis of NifDK. Evidence for the transfer of $^{99}$Mo label from NifH and NifX to NafY is seen in Fig. 2, suggesting the completion of FeMo-co synthesis. Homer et al. (30) have shown that NafY binds FeMo-co specifically and have postulated a role for NafY in the cofactor insertion process. However, Rubio et al. (22) have shown that nafY mutants exhibit a nif phenotype only under stress conditions. One interpretation of their data is that NafY is required for the stability of apodinitrogenase in an open conformation that is favorable to the insertion of FeMo-co.

In vivo, FeMo-co biosynthesis might not occur in sequential steps on uncomplicated proteins. A FeMo-co biosynthetic complex comprised of NifNE, NifX, and NifH might possibly exist, to which NifB-co, Mo, and homocitrate are supplied by NifB, NifQ, and NifY, respectively. The FeMo-co produced in the complex might be bound by NafY, which delivers the cofactor to apodinitrogenase. Results presented in this study and other studies were obtained with the use of either purified proteins or the use of cell-free extracts. We believe that the FeMo-co biosynthetic complex in these cases has been teased apart so that

3 P. Rangaraj and P. W. Ludden, unpublished data.
FeMo-co biosynthesis can be studied as several individual steps rather than as one concerted, continuous event.

Though the sequence of steps in the FeMo-co biosynthetic pathway is better understood at present than a few years ago, the exact roles played by NiFeH and NiFeX in this process remain to be explored. Questions concerning the role of MgATP and whether nucleotide hydrolysis is required for FeMo-co synthesis also remain to be addressed. At some stage reductant is required during the biosynthesis of FeMo-co, presumably for the reduction of Mo(VI) to Mo(IV), the formal oxidation state required during the biosynthesis of FeMo-co (46). The exact step at which reductant is required is also remain to be addressed. Whether nucleotide hydrolysis is required for FeMo-co synthesis can be studied as several individual steps rather than as one concerted, continuous event.

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