Homocitrate Is a Component of the Iron-Molybdenum Cofactor of Nitrogenase†

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ABSTRACT: When apodinitrogenase (lacking FeMo-co) was activated with FeMo-co synthesized in vitro in the presence of ^{3}H-labeled homocitrate, label was incorporated into dinitrogenase. The physical association of the label with FeMo-co was demonstrated by reisolation and purification of the cofactor from dinitrogenase. The presence of homocitrate in FeMo-co was established by NMR analysis of the organic acid extracted from dinitrogenase. Quantitation of homocitrate in dinitrogenase showed it to be present at a 1:1 ratio with molybdenum.

Nitrogenase catalyzes the ATP[1]-dependent reduction of N_2 to ammonium (Bulen & LeComte, 1966) and consists of two proteins: dinitrogenase (MoFe protein or component I) and dinitrogenase reductase (Fe protein or component II) (Bulen & LeComte, 1966; Hageman & Burris, 1978). Substrate reduction occurs on dinitrogenase and is associated with a unique prosthetic group, the iron-molybdenum cofactor (FeMo-co) (Shah et al., 1977; Rawlings et al., 1978; Hawkes et al., 1984). FeMo-co has an approximate composition of $MoFe_{6-8}S_{6-9}$ (Shah et al., 1977; Nelson et al., 1983; Yang et al., 1982). Biochemical and genetic studies of Nif$^-$ (nitrogen fixation) mutants of *Klebsiella pneumoniae* indicate that the *nifB*, *nifQ*, *nifN*, *nifE*, *nifV*, and *nifH* gene products are required for FeMo-co biosynthesis (Shah et al., 1977, 1988; Hawkes et al., 1984; Roberts et al., 1978; Imperial et al., 1984; Filler et al., 1986; Robinson et al., 1987; McLean & Dixon, 1981; McLean et al., 1983). An in vitro system for the synthesis of FeMo-co has been described that requires molybdate, ATP, at least the gene products of *nifB*, *nifN*, and *nifE* (Shah et al., 1986), dinitrogenase reductase (or the *nifH* gene product) (Robinson et al., 1987; Shah et al., 1988), and (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid (homocitric acid) (Hoover et al., 1987). The synthesis and accumulation of homocitrate in *K. pneumoniae* is correlated with the presence of a functional *nifV* gene (Hoover et al., 1986, 1987). We have followed the fate of ^{3}H-labeled homocitrate in the in vitro FeMo-co synthesis system and report here that homocitrate is a component of FeMo-co.

Materials and Methods

A 25-mg sample of homocitric acid lactone (Sigma Chemical Co.) was furnished to Amersham Corp. for tritium labeling. The ^{3}H-labeled homocitric acid lactone was purified by reverse-phase C18 HPLC (Hoover et al., 1987). The specific activity of the purified, ^{3}H-labeled homocitric acid lactone was estimated to be 20 mCi·mmol^{-1}. The lactone was converted to the free acid by the addition of NaOH. The *nifB* gene product (designated NIFB) from *K. pneumoniae* strain UN1100 (*nifE4420*) (MacNeil et al., 1978) was purified for use in these experiments by elution from the membranes with 1% *N*-laurylsarcosine (Shah et al., 1988) in 100 mM Tris-HCl buffer, pH 7.4. *Azotobacter vinelandii* extracts were prepared by osmotic shock (Shah et al., 1972), and endogenous homocitrate was removed from these extracts by anaerobic AG1-X8 (Bio-Rad) anion-exchange chromatography.

Incorporation of [^{3}H]Homocitrate into FeMo-co. The in vitro FeMo-co synthesis reaction mixture contained 30 mL of *K. pneumoniae* membrane extract (about 300 mg of protein), 60 mL of *A. vinelandii* strain UW45 (*nifB*$^-$) extract (about 1 g of protein), and 1.8 mL of a 5 mM solution of purified ^{3}H-labeled homocitrate as described (Shah et al., 1988; Hoover et al., 1988). In a control reaction mixture, the *K. pneumoniae* membrane extract was replaced with 1% *N*-laurylsarcosine in 100 mM Tris-HCl buffer. The reaction mixtures were incubated for 45 min at 30 °C. Samples from the reaction mixtures were assayed for acetylene reduction following a 45-min incubation period at 30 °C (Shah et al., 1972). To both the experimental and control reaction mixtures was added 30 mg of purified *A. vinelandii* dinitrogenase (specific activity 1700 nmol of ethylene formed·min^{-1}·mg^{-1}) as a carrier.

Purification of Labeled Dinitrogenase. Dinitrogenase from the reaction mixtures was then purified by a modification of the method described by Shah and Brill (1973). Following anaerobic DEAE-cellulose chromatography, the dinitrogenase preparations were applied to a 90 × 2.5 cm Sephacryl S-200 column that was equilibrated and developed with 50 mM NaCl and 2.5 mM sodium citrate in 50 mM Tris-HCl buffer, pH 7.4. Fractions containing dinitrogenase were applied to anaerobic DEAE-cellulose columns equilibrated with 0.15 M NaCl in 25 mM Tris-HCl buffer, pH 7.4. The columns were washed with 0.15 M NaCl, and dinitrogenase was eluted with 0.25 M NaCl in 25 mM Tris-HCl, pH 7.4. Fractions containing dinitrogenase were heat-treated (Shah & Brill, 1973) and applied to the Q-Sepharose columns equilibrated with 0.2 M NaCl in 25 mM Tris-HCl buffer, pH 7.4. The columns were washed with 0.2 M NaCl, and dinitrogenase was eluted with 0.35 M NaCl in 25 mM Tris-HCl buffer, pH 7.4. All buffers contained 1.7 mM sodium dithionite, and all manipulations were done anaerobically.

Dinitrogenase specific activity was determined by acetylene reduction assays in the presence of excess dinitrogenase re-

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[1] Abbreviations: FeMo-co, iron-molybdenum cofactor of nitrogenase; homocitric acid, 2-hydroxy-1,2,4-butanetricarboxylic acid; ATP, adenosine 5′-triphosphate; BCA, bicinchoninic acid.

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Table I: Incorporation of [^{3}H]Homocitrate into FeMo-co

step	experimental[a] dinitrogenase[c] (units × 10^{-3})	% recovery	^{3}H dpm	dpm/mg of dinitrogenase	control[b] dinitrogenase[c] (units × 10^{-3})	% recovery	^{3}H dpm
crude preparation[d]	52.6 (30.9)	100	4.2×10^8	1.3×10^7	51.1 (30)	100	4.2×10^8
DEAE-cellulose	48.0 (28.2)	91.3	ND		45.6 (26.8)	89.3	ND
Sephacryl S-200	46.5 (27.3)	88.3	2.9×10^5	1.1×10^4	41.3 (24.3)	81.0	0
DEAE-cellulose	45.7 (26.9)	87.1	3.0×10^5	1.1×10^4	40.5 (23.8)	79.3	0
heat treatment	39.0 (22.9)	74.1	ND		38.3 (22.5)	75.0	0
Q-Sepharose	39.1 (23.0)	74.4	2.2×10^5	9.6×10^3			

[a] Complete in vitro FeMo-co synthesis system. [b] NIFB omitted from in vitro FeMo-co synthesis system. [c] Unit is defined as nanomoles of ethylene formed per minute; estimated milligrams of dinitrogenase (in parentheses) from C_2H_2 reduction assays using a specific activity of 1700 nanomoles of ethylene formed per minute per milligram; approximately 0.9 mg of apodinitrogenase was activated in the complete in vitro FeMo-co synthesis. [d] Added 30 mg of dinitrogenase (specific activity 1700 nanomoles of ethylene formed per minute per milligram) as carrier to both samples. ND, not determined.

ductase (Shah & Brill, 1973). Protein concentrations were determined by the BCA method (Smith, P. K., et al., 1985) using bovine serum albumin as standard. Samples were counted for tritium with a Tri-Carb 4430 (Packard Instruments, Inc.) scintillation counter. Efficiency of counting was determined from a quench curve for tritium prepared with [^{3}H]toluene.

Isolation and Purification of FeMo-co from ^{3}H-Labeled Dinitrogenase. FeMo-co was extracted from purified dinitrogenase samples in *N*-methylformamide and subjected to anaerobic Sephadex G-100 gel filtration chromatography in *N*-methylformamide (Shah & Brill, 1977). Fractions from this column were collected anaerobically and assayed for FeMo-co activity with extracts of *A. vinelandii* strain UW45 (Shah & Brill, 1977), and the level of tritium was determined by liquid scintillation.

Isolation and Purification of Homocitrate from Dinitrogenase. Approximately 30 mL of dinitrogenase (980 mg of protein, specific activity 1700 mmol of ethylene formed·min^{-1}·mg^{-1}) along with a small amount of purified ^{3}H-labeled dinitrogenase (labeled in the in vitro FeMo-co synthesis system with [^{3}H]homocitrate) was added dropwise to 600 mL of aerobic acetone containing 4.5 mL of 4 N HCl at 4 °C with continuous stirring over a 15-min period. The mixture was stirred for an additional 15 min, precipitated protein was removed by filtration, and the filtrate was evaporated to dryness with a rotary evaporator under vacuum at 40–50 °C. The residue was dissolved in 4 mL of distilled water, the pH was adjusted to 8.0 with NaOH, and the resultant mixture was chromatographed on an AG1-X8 formate column (0.75 × 26 cm). The column was eluted with a 200-mL linear gradient of 0–6 N formic acid (Tucci & Ceci, 1972). Five-milliliter fractions were collected, and an aliquot of each fraction was assayed for the presence of tritium. The fractions containing tritium were pooled, evaporated to dryness, and dissolved in distilled water, and the pH was adjusted to 8. This preparation was used in the in vitro FeMo-co synthesis assay and the level of homocitrate quantified by comparing the activity with authentic homocitrate (Sigma Chemical Co.).

Small-Scale Extraction of Homocitrate from Dinitrogenase. The acidified acetone extraction procedure described above was carried out with 10 mg of dinitrogenase and 6 mL of acidified acetone in a 15-mL Corex centrifuge tube. Following this treatment, the contents were centrifuged at 10000*g* for 10 min, and the supernatant was transferred to another centrifuge tube and evaporated to dryness with a Speed-Vac concentrator (Savant). The residue was dissolved in 0.5 mL of distilled water and transferred to a microfuge tube, the pH was adjusted to 8 with NaOH, and the resultant mixture was frozen overnight at −20 °C. The sample was thawed and centrifuged at 15000*g* for 5 min to remove the brown precipitates that inhibit in vitro FeMo-co synthesis. Aliquots of the supernatant were used in the in vitro FeMo-co synthesis system.

Results

Tritium-labeled homocitrate was used in the in vitro FeMo-co synthesis system to determine whether it was incorporated into FeMo-co. Following the in vitro FeMo-co synthesis reaction, the dinitrogenase that was activated (along with dinitrogenase added as carrier) was purified (Table I). A control reaction mixture, in which the source of the *nifB* gene product (designated NIFB) was omitted, was treated in the same manner. Following gel filtration chromatography, no ^{3}H was associated with the control preparation, whereas ^{3}H was observed in the experimental preparation. Further, over the last three steps of the purification protocol, the ratio of ^{3}H:dinitrogenase activity remained constant in the experimental preparation. Approximately 7.6 nmol of FeMo-co was synthesized in the experimental reaction mixture, assuming a specific activity of 275 nmol of acetylene reduced·min^{-1}·ng-atom of Mo (Hawkes et al., 1984; Shah, 1986). At a homocitrate:Mo ratio of 1, the expected level of ^{3}H would be 2.5×10^5 dpm for the purified dinitrogenase sample (taking into consideration the percent recovery of dinitrogenase during purification). In two separate experiments, purified dinitrogenase preparations contained 79–88% of the expected amount of ^{3}H. These data indicate that homocitrate (or a portion of the molecule) is associated with dinitrogenase only under conditions in which FeMo-co synthesis occurs. Further, our control experiment demonstrates that [^{3}H]homocitrate does not bind nonspecifically to apodinitrogenase, to other proteins in UW45 extract, or to the dinitrogenase added as carrier.

FeMo-co was extracted from the purified dinitrogenase of the experimental preparation. The procedure for FeMo-co extraction (Shah & Brill, 1977) includes the following: unfolding dinitrogenase with a brief treatment with citric acid; neutralization to the isoelectric point of the protein, which results in its precipitation; washing the protein pellet with *N*,*N*-dimethylformamide; and finally extraction of FeMo-co from the protein pellet with a basic solution of *N*-methylformamide. The majority (>80%) of the ^{3}H associated with the purified protein was extracted into *N*-methylformamide along with the FeMo-co. Gel filtration (Shah & Brill, 1977) of the isolated FeMo-co demonstrated that the ^{3}H comigrated with FeMo-co activity (Figure 1). In a separate experiment, when a mixture of unlabeled FeMo-co and [^{3}H]homocitrate was subjected to the same Sephadex G-100 column, tritium counts eluted as a distinct peak after the FeMo-co peak. These data clearly demonstrate that homocitrate does not bind to the FeMo-co nonspecifically. These data further show that

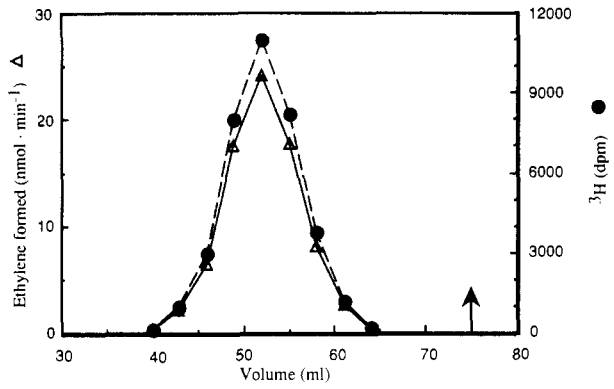

FIGURE 1: Sephadex G-100 gel filtration of ^{3}H-labeled FeMo-co. In a separate experiment, when a mixture of unlabeled FeMo-co and [^{3}H]homocitrate was subjected to the same column, tritium counts eluted as a distinct peak (marked by arrow) after the FeMo-co peak.

homocitrate does not exchange with the organic moiety in preformed FeMo-co.

Part of the ^{3}H-labeled FeMo-co isolated from in vitro activated dinitrogenase was used to activate apodinitrogenase (lacking FeMo-co) from *A. vinelandii* strain UW45. The resulting holodinitrogenase was purified by DEAE-cellulose chromatography and Sephacryl S-200 gel filtration chromatography. Greater than 80% of the ^{3}H that was present in the FeMo-co preparation copurified with the dinitrogenase activity. Thus, the ^{3}H-labeled moiety that had been used to activate apodinitrogenase and then reisolated still functions as FeMo-co. Taken together, the above data demonstrate that homocitrate (or portion of the molecule) is associated with FeMo-co, and it can be concluded that homocitrate functions as a substrate in FeMo-co biosynthesis.

Previous attempts to demonstrate the release of homocitrate (with the aid of the in vitro FeMo-co synthesis assay) from acid-hydrolyzed dinitrogenase and FeMo-co were unsuccessful (Hoover et al., 1986). Therefore, to determine if homocitrate is modified or if only a part of the molecule is incorporated into the FeMo-co, the organic component was isolated and purified from twice crystallized *A. vinelandii* dinitrogenase. A small amount of purified, ^{3}H-labeled dinitrogenase (labeled in the in vitro FeMo-co synthesis system with [^{3}H]homocitrate) was included as a tracer to follow the purification of the organic component. The organic component was extracted from 30 mL of dinitrogenase (980 mg of protein, specific activity 1700 nmol of ethylene formed·min^{-1}·mg^{-1}) and purified by the procedure described under Materials and Methods. The fractions containing tritium were pooled, evaporated to dryness, and dissolved in distilled water, and the pH was adjusted to 8 with NaOH. This preparation was used as a source of homocitrate in the in vitro FeMo-co synthesis assay and the level of homocitrate quantitated by comparison to a standard curve prepared by using authentic homocitrate. From the in vitro FeMo-co synthesis assay, the level of (*R*)-homocitrate recovery was estimated to be 6970 nmol. At a homocitrate:Mo ratio of 1, the expected level of homocitrate would be 8000 nmol in 980 mg of dinitrogenase. Thus, greater than 87% of expected homocitrate was recovered from dinitrogenase. These data also clearly demonstrate that 1 mol of homocitrate is present per molybdenum in dinitrogenase. Data presented above have already demonstrated that tritium label from homocitrate is incorporated into the FeMo-co. Taken together, these data clearly demonstrate that the organic component of FeMo-co remains homocitrate.

A fraction of homocitrate isolated and purified from dinitrogenase was subjected to C18 reverse-phase HPLC (Hoover et al., 1987). The tritium comigrated with homocitrate and was active in the in vitro FeMo-co synthesis system. Proton NMR further confirmed that this material was homocitrate (Figure 2).

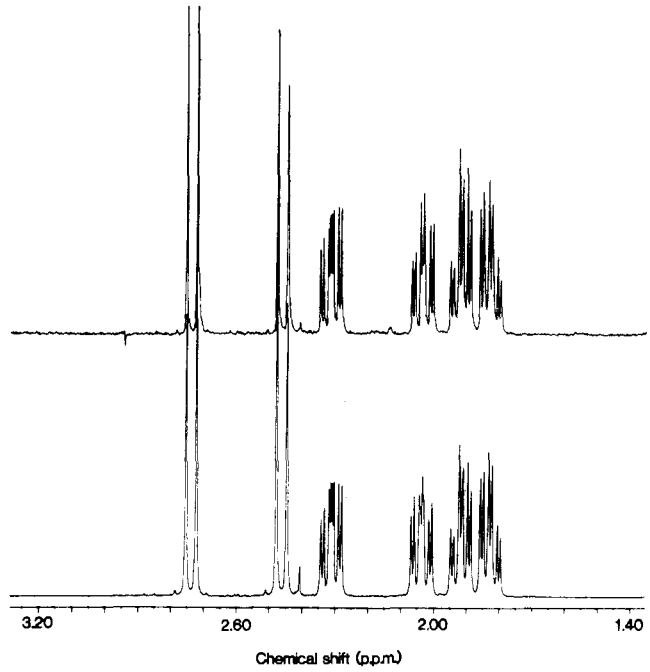

FIGURE 2: Proton NMR spectra of synthetic homocitric acid (bottom) and homocitric acid isolated from *A. vinelandii* dinitrogenase (top). Spectra were recorded as described (Hoover et al., 1987) on a Bruker AM500 spectrometer operating at 500.13-MHz ^{1}H frequency.

Discussion

Dinitrogenase from NifV$^-$ mutants effectively reduces acetylene but not N_2, and unlike the wild-type enzyme, H_2 evolution from NifV$^-$ dinitrogenase is inhibited by CO (McLean & Dixon, 1981; McLean et al., 1983). This altered substrate specificity and inhibitor susceptibility have been demonstrated to be the result of a defective FeMo-co (Hawkes et al., 1984). Strains with mutations affecting the *nifV* product fail to synthesize homocitrate (Hoover et al., 1986, 1987), and it has been suggested that, in the absence of homocitrate, NifV$^-$ mutants use citrate for FeMo-co biosynthesis (Hoover et al., 1988). The use of citrate, in place of homocitrate, in the in vitro FeMo-co synthesis system resulted in the formation of dinitrogenase with the NifV$^-$ phenotype.

Our attempts to demonstrate exchange of homocitrate into NifV$^-$ FeMo-co have been unsuccessful. Incubation of NifV$^-$ FeMo-co with a 200-fold excess of (*R*)-homocitrate at 30 °C for 1 h did not change the substrate reduction properties of the NifV$^-$ FeMo-co (data not shown). This is consistent with the data presented here, indicating that homocitrate is incorporated into the wild-type FeMo-co during its synthesis, is an integral part of the cofactor, and cannot be readily exchanged.

Within experimental error, there are no differences in the Fe:Mo ratio between wild-type and NifV$^-$ FeMo-co (Hawkes et al., 1984). With EPR (McLean et al., 1987), Mössbauer (McLean et al., 1985), Mo-extended X-ray absorption fine structure (EXAFS) (McLean et al., 1987; Eidness et al., 1986), and low-temperature magnetic circular dichroism (Smith, B. E., et al., 1985) spectroscopic techniques, no differences are observed between wild-type and NifV$^-$ dinitrogenases, indicating that the environment of the spectrally observable components of wild-type and NifV$^-$ FeMo-co are very similar. Differences between the wild-type and NifV$^-$

enzymes are observed with ^{95}Mo electron nuclear double resonance (ENDOR) spectroscopy (McLean et al., 1987). From these ENDOR experiments, it has been suggested that the molybdenum site is perturbed in the NifV$^-$ FeMo-co, possibly due to an alteration of the non-sulfur ligands at molybdenum (McLean et al., 1987). Mo K-edge X-ray absorption edge and near-edge structure data from dinitrogenase and isolated FeMo-co suggest a local environment of three S and three O (or N) ligands around the Mo (Conradson et al., 1985). Given that the only significant spectroscpic differences observed between wild-type and NifV$^-$ dinitrogenase have been with ^{95}Mo ENDOR (McLean et al., 1987), and the association of homocitrate with FeMo-co reported here, it seems reasonable that homocitrate may provide some or all of the oxygen ligands to Mo in FeMo-co. The *nifV* gene product is also required for synthesis of the vanadium-containing dinitrogenase of *Azotobacter chroococcum* (D. Evans and R. Robson, personal communication), and a similar function for homocitrate is to be expected in this system.

A possible explanation for the failure of earlier attempts (Hoover et al., 1986) to demonstrate the release of homocitrate from dinitrogenase and oxidized FeMo-co may be that the concentrations of thiomolybdates (Shah et al., 1985) and other salts present in the preparations were inhibitory to the functional assays. Our observations with the concentrated acidified acetone extract of dinitrogenase substantiated this possibility. Addition of increasing amounts of this preparation to the in vitro FeMo-co synthesis system showed progressively lower activities than expected from the amount of homocitrate in the sample. After purification the same preparation showed activities proportional to the amount added and was used for quantitation of (*R*)-homocitrate by the in vitro FeMo-co synthesis system.

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