Dinitrogenase with Altered Substrate Specificity Results from the Use of Homocitrate Analogues for in Vitro Synthesis of the Iron–Molybdenum Cofactor†

Timothy R. Hoover, Juan Imperial, Jihong Liang, Paul W. Ludden,* and Vinod K. Shah

Department of Biochemistry and Center for the Study of Nitrogen Fixation, College of Agriculture and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received November 18, 1987; Revised Manuscript Received January 12, 1988

ABSTRACT: The in vitro synthesis of the iron–molybdenum cofactor (FeMo-co) of nitrogenase requires homocitrate (2-hydroxy-1,2,4-butanetricarboxylic acid). Homocitrate is apparently synthesized by the *nifV* gene product. In the absence of homocitrate, no FeMo-co is formed in vitro, as determined from coupled C_2H_2 reduction assays and the lack of ^{99}Mo label incorporation into apodinitrogenase. Several organic acids were tested for their ability to replace homocitrate in the FeMo-co synthesis system. With appropriate homocitrate analogues, aberrant forms of FeMo-co are synthesized that exhibit altered substrate specificity and inhibitor susceptibility. Homoisocitrate (1-hydroxy-1,2,4-butanetricarboxylic acid) and 2-oxoglutarate facilitated the incorporation of ^{99}Mo into apodinitrogenase in the FeMo-co synthesis system, yielding a dinitrogenase that effectively catalyzed the reduction of protons but not C_2H_2 or N_2. Citrate also promoted the incorporation of ^{99}Mo into apodinitrogenase, and the resulting holodinitrogenase reduced protons and C_2H_2 effectively but not N_2. In addition, proton reduction from this enzyme was inhibited by CO. The properties of the homodinitrogenase formed in the presence of citrate were reminiscent of those of the *Klebsiella pneumoniae* NifV$^-$ dinitrogenase. We also observed low rates of HD formation from NifV$^-$ dinitrogenase compared to those from the wild-type enzyme. No HD formation was observed with the dinitrogenase activated in vitro in the presence of citrate. We propose that in vivo NifV$^-$ mutants utilize citrate for FeMo-co synthesis.

Nitrogenase catalyzes the reduction of N_2 to ammonium, as well as the reduction of several low molecular weight compounds with triple bonds. In the absence of any substrate, nitrogenase catalyzes the reduction of protons to H_2. The reduction by nitrogenase of all substrates, except protons, is inhibited by CO (Hwang et al., 1973; Rivera-Ortiz & Burris, 1975). In the presence of D_2 and N_2, nitrogenase catalyzes a reductant-dependent formation of HD (Hoch et al., 1960). HD formation is likewise inhibited by CO, indicating that H_2 is generated by two independent nitrogenase reactions (Burris, 1985).

Nitrogenase is composed of two proteins, dinitrogenase (component I or molybdenum–iron protein) and dinitrogenase reductase (component II or iron protein) (Bulen & LeComte, 1966; Hageman & Burris, 1978). Dinitrogenase contains an unique prosthetic group [iron–molybdenum cofactor (FeMo-co)[1]] that is comprised of Fe, Mo, and S (Shah & Brill, 1977). FeMo-co has been proposed as the site of substrate reduction (Shah et al., 1978; Hawkes et al., 1984; Smith et al., 1985). In *Klebsiella pneumoniae*, at least six *nif* (nitrogen-fixation) gene products (*nifQ*, *nifB*, *nifV*, *nifN*, *nifE*, and *nifH*) are involved in the synthesis of active FeMo-co. The *nifQ* gene product is apparently required for early steps in the processing of Mo for FeMo-co (Imperial et al., 1984). Mutants in *nifB*, *nifN*, and *nifE* produce an apodinitrogenase that can be activated in vitro with purified FeMo-co (Shah & Brill, 1977; Roberts et al., 1978; Ugalde et al., 1984). Certain strains of *K. pneumoniae* and *Azotobacter vinelandii* with mutations in *nifH* (the structural gene for dinitrogenase reductase) fail to synthesize FeMo-co (Filler et al., 1986; Robinson et al., 1987).

Dinitrogenase from mutants altered in *nifV* effectively reduces acetylene but not N_2; furthermore, H_2 evolution by the enzyme is inhibited by CO (McLean & Dixon, 1981; McLean et al., 1983). When apodinitrogenase was activated with FeMo-co extracted from dinitrogenase of a NifV$^-$ mutant, the resulting holodinitrogenase had the substrate-reducing properties of the enzyme from the NifV$^-$ mutant, indicating that the defect in NifV$^-$ mutants is in FeMo-co biosynthesis (Hawkes et al., 1984). It is of obvious interest to determine what differences between wild-type and NifV$^-$ FeMo-co result in this altered substrate specificity and inhibitor susceptibility. The dinitrogenase from NifV$^-$ mutants has been extensively studied with electron paramagnetic resonance (EPR) (Eidsness et al., 1986; McLean et al., 1987), Mössbauer (McLean et al., 1985), Mo-extended X-ray absorption fine structure (EXAFS) (McLean et al., 1987), and ^{57}Fe, ^{1}H, and ^{95}Mo electron nuclear double resonance (ENDOR) (McLean et al., 1987) spectroscopy. With these techniques, no differences were detected between the wild-type and NifV$^-$ enzymes, except for minor differences in the ENDOR spectra (McLean et al., 1987).

Recently, an in vitro system for the synthesis of FeMo-co was described (Shah et al., 1986). The assay requires molybdate, ATP, at least the *nifB*, *nifN*, and *nifE* gene products (Shah et al., 1986), dinitrogenase reductase (J. Imperial, unpublished data), and a low molecular weight factor (V factor) which is produced only in NifV$^+$ strains of *K. pneumoniae* and other diazotrophs (Hoover et al., 1986). V factor has been identified as (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid (homocitrate) (Hoover et al., 1987). The *R* isomer also

† This research was supported by the College of Agriculture and Life Sciences at the University of Wisconsin—Madison and by NIH Grant R01GM35332-01A1.

[1] Abbreviations: FeMo-co, iron–molybdenum cofactor of nitrogenase; ATP, adenosine 5′-triphosphate; homocitric acid, 2-hydroxy-1,2,4-butanetricarboxylic acid; homoisocitric acid, 1-hydroxy-1,2,4-butanetricarboxylic acid; tricarballylic acid, 1,2,3-propanetricarboxylic acid.

0006-2960/88/0427-3647$01.50/0

serves as the intermediate in lysine biosynthesis in yeasts and fungi (Thomas et al., 1966). Citrate was reported to partially substitute for homocitrate in the in vitro FeMo-co synthesis assay at concentrations 100-fold higher than those required for homocitrate (Hoover et al., 1987). We report here on the use of citrate and other analogudes of homocitrate in the in vitro FeMo-co synthesis system and the properties of the resulting holodinitrogenase formed with each of these analogues.

Materials and Methods

Reagents. Homocitric acid lactone was obtained from Sigma Chemical Co. The lactone was converted to the free acid form by preparation of solutions in dilute NaOH. Citric acid of highest purity was obtained from Aldrich Chemical Co. Homoisocitrate was purchased from Research Plus, Inc., Bayonne, NJ. Carrier-free $Na_2{}^{99}MoO_4$ was purchased from Cintichem, Inc., Tuxedo, NY. All other chemicals were of analytical grade.

Bacterial Strains and Growth Conditions. K. pneumoniae wild-type strain UN is a reisolate of strain M5a1, originally from P. W. Wilson's collection. *K. pneumoniae* mutant strains UN1100 (*nifE4420*) and UN1991 (*nifV4945*) have been described (MacNeil et al., 1978). *A. vinelandii* mutant strain CA12 which has a deletion of the structural genes for nitrogenase (Δ*nifHDK*) (Bishop et al., 1986) was kindly provided by D. R. Dean. Growth and derepression for nitrogenase of *K. pneumoniae* has been described (Shah et al., 1983; Imperial et al., 1984). CA12 was grown in a 300-L fermentor in modified Burk medium (Strandberg & Wilson, 1968) supplemented with 25 mM ammonium acetate and 0.1 mM sodium tungstate and lacking sodium molybdate. Ammonium concentrations in the culture medium were followed by assaying samples with Nessler's reagent (Strandberg & Wilson, 1968). Cells were harvested 4 h after the ammonium was depleted from the medium.

Preparation of Crude Extracts. K. pneumoniae extracts were prepared as previously described (Shah et al., 1986). *A. vinelandii* extracts were prepared by osmotic shock (Shah et al., 1972). Extracts were desalted on an anaerobic Sephadex G-25 column (Hoover et al., 1986).

In Vitro FeMo-co Synthesis Assay. The assays for the in vitro synthesis of FeMo-co were performed as described (Hoover et al., 1986), except extracts of the *A. vinelandii* mutant CA12 were used as the source of the *nifB* and *nifNE* gene products and replaced the UW45 extracts. Solutions of organic acids which were tested for their ability to replace homocitrate in the assay were neutralized with NaOH. Preparations of apodinitrogenase activated in the in vitro FeMo-co synthesis system in the presence of various organic acids were handled as follows. To 15 mL of an ATP-generating mixture (Shah et al., 1972) containing 5 mM sodium dithionite and 50 μM sodium molybdate, 15 mL of desalted UN1100 extract and 7.5 mL of desalted CA12 extract were added, along with one of the following organic acids: (1) no addition (control); (2) homocitrate (0.2 mM); (3) citrate (4 mM); (4) 2-oxoglutarate (4 mM); (5) homoisocitrate (0.8 mM). Final concentrations of each organic acid in the reaction mixture are given. The reaction mixtures were incubated at 30 °C for 45 min. Dinitrogenase from each of the mixtures was partially purified by anaerobic DEAE-cellulose column chromatography as described (Shah & Brill, 1973). Dinitrogenase from *K. pneumoniae* UN (wild type) and UN1991 ($NifV^-$) extracts was partially purified in the same manner.

Nitrogenase Assays. All assays were done with an excess of dinitrogenase reductase [6–10 mol (mol of dinitrogenase)$^{-1}$]. Assays for acetylene reduction (Shah et al., 1986) and N_2 reduction (Shah et al., 1972) by nitrogenase have been described. Proton reduction by nitrogenase was assayed by measuring the formation of H_2 with a Gow-Mac thermal conductivity gas chromatograph equipped with a Porapak R (Waters Associates) column. The assay mixture for H_2 evolution contained an ATP-generating system (Shah et al., 1972), 5 mM sodium dithionite, and partially purified dinitrogenase. Assays were done in 9-mL serum vials under an argon atmosphere and were started by the addition of purified dinitrogenase reductase from *A. vinelandii* (Shah & Brill, 1973). Where indicated, 100 μL of CO (1.1 kPa) was added to the vials prior to the start of the assays.

HD formation from nitrogenase was assayed at room temperature (24 °C) with a Varian MAT 250 isotope ratio mass spectrometer equipped with a 1.6-mL membrane-leak reaction chamber (Simpson, 1985). Dinitrogenase samples, along with an ATP-generating system (Shah et al., 1972) and 5 mM sodium dithionite, were equilibrated under an atmosphere of 10% N_2 in D_2. The mixture was placed in the membrane-leak chamber, and initial rates of HD diffusion through the membrane in the absence of dinitrogenase reductase were established. Purified dinitrogenase reductase was added to the samples to start the production of HD. Reactions were continued for 8 min. A standard curve for HD diffusion rates (no enzyme present) versus HD concentrations was established, which was used to correct the observed rates of HD formation.

Dinitrogenase was quantitated with an enzyme-linked immunosorbent assay (ELISA) with rabbit antiserum directed against *K. pneumoniae* dinitrogenase and anti-rabbit IgG–alkaline phosphatase conjugate. The UN dinitrogenase preparation was used for preparing the standard curve by assuming a specific activity of 2500 nmol of C_2H_2 reduced min^{-1} mg^{-1}.

Incorporation of ^{99}Mo into Dinitrogenase. In vitro FeMo-co synthesis reactions were carried out as described above, except the concentration of nonradioactive molybdate was lowered 100-fold and $Na_2{}^{99}MoO_4$ (0.5 μCi per assay, carrier free) was added. After incubation, portions of the reaction mixtures were subjected to polyacrylamide gel electrophoresis under native, anaerobic conditions, followed by autoradiography (Imperial et al., 1984; our unpublished data). From the autoradiograms, the identity of in vitro ^{99}Mo-labeled dinitrogenase was confirmed with (1) partially purified dinitrogenase labeled in vivo with ^{99}Mo, (2) *K. pneumoniae* extracts containing apodinitrogenase which was reconstituted by addition of Fe^{99}Mo-co labeled in vivo, (3) purified dinitrogenase (following staining), and (4) immunoblotting techniques. Extracts of *A. vinelandii* cultures repressed for nitrogenase and labeled with ^{99}Mo were used to identify the molybdenum-storage protein (Pienkos & Brill, 1981).

Results

The *A. vinelandii* mutant CA12 was used in this study for FeMo-co synthesis because it lacked the structural genes for dinitrogenase. This allowed us to use *K. pneumoniae* extracts as the source of apodinitrogenase in the in vitro FeMo-co synthesis system, making it possible to directly compare our results with the work done by other groups on the $NifV^-$ dinitrogenase from *K. pneumoniae*.

With desalted extracts of CA12 and UN1100, 0.1 mM homocitrate saturated the in vivo FeMo-co synthesis system, as measured in the coupled C_2H_2 reduction assay (Figure 1). Under these conditions, nearly 75% of the native apodinitrogenase was activated, as determined by the addition of purified FeMo-co to the UN1100 extracts (Shah & Brill, 1977). At 10-fold higher concentrations, citrate showed sig-

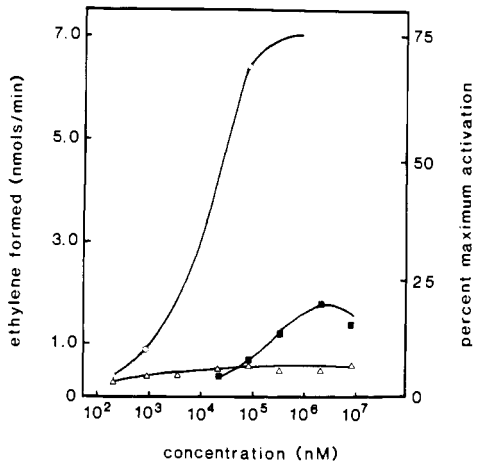

FIGURE 1: Titration of the in vitro FeMo-co synthesis mixture with homocitrate (○), citrate (■), and homoisocitrate (△). The FeMo-co synthesis mixture contained 100-μL desalted CA12 (2 mg of protein) and 200-μL UN1100 (6 mg of protein) extracts, along with 200 μL of an ATP-regenerating mixture (Shah et al., 1972) which contained 5 mM sodium dithionite and 50 μM sodium molybdate. The reaction mixtures were incubated at 30 °C for 30 min, after which 800 μL of additional ATP-regenerating mixture containing 5 mM sodium dithionite was added, and C_2H_2 reduction activities were determined (Shah et al., 1986). Concentrations of the organic acids are reported for the 30-min incubation.

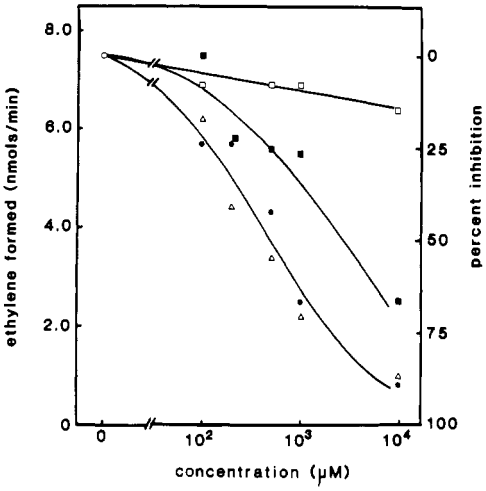

FIGURE 2: Titration of the complete in vitro FeMo-co synthesis mixture with homoisocitrate (△), 2-oxoglutarate (●), citrate (■), and tricarballylate (□). The complete FeMo-co synthesis system contained 0.1 mM homocitrate. Concentrations of the organic acids are reported for the 30-min incubation.

nificant activity in the assay. However, the levels of activation never reached the levels of activation observed with homocitrate. The FeMo-co synthesis assay mixture was titrated with a number of other organic acids. Homoisocitrate was one analogue of homocitrate which gave low, but reproducible, increases in the C_2H_2 reduction activity (Figure 1). Low increases in activity were also observed with 2-oxoglutarate, 3-oxoglutarate, 3-oxoadipate, and citramalate. No increases in activity were observed with malate, oxaloacetate, fumarate, succinate, adipate, 2-oxoadipate, tricarballylate, glutarate, L-2-hydroxyglutarate, 2-oxocaproate, glutamate, or glutamine.

Several of these organic acids were tested for their ability to inhibit in vitro FeMo-co synthesis. Organic acids which were selected were citrate, homoisocitrate, 2-oxoglutarate, and tricarballylate (Figure 2). Assay mixtures containing 0.1 mM homocitrate were titrated with each of the compounds. To distinguish between inhibition of FeMo-co synthesis and inhibition of the coupled C_2H_2 reduction assay, organic acids

Table I: Inhibition of Apodinitrogenase Activation

organic acid added	C_2H_2 reduction activity (nmol reduced min^{-1}) after in vitro synthesis	plus FeMo-co	plus FeMo-co minus CA12 extract
no addition	0.15	2.7	3.8
homocitrate (90 μM)	2.7	2.4	3.6
citrate (9 mM)	0.60	2.5	4.0
homoisocitrate (90 μM)	0.28	1.2	3.6
2-oxoglutarate (9 mM)	0.15	1.6	2.7
tricarballylate (9 mM)	0.15	2.6	3.6

were added to some assays immediately before the C_2H_2 reduction assays were started. At the highest concentration for each of the organic acids (10 mM), C_2H_2 reduction activities were inhibited by 12, 15, 24, and 26% for tricarballylate, citrate, 2-oxoglutarate, and homoisocitrate, respectively. After correction for these levels of inhibition of C_2H_2 reduction activity, homoisocitrate and 2-oxoglutarate inhibited FeMo-co synthesis by at least 65%, citrate inhibited FeMo-co synthesis by about 50%, and tricarballylate did not inhibit FeMo-co synthesis.

In order to directly determine the effects of these homocitrate analogues on FeMo-co synthesis, ^{99}Mo was used to follow incorporation of Mo (as FeMo-co) into apodinitrogenase. In the absence of homocitrate (Figure 3, lane 1), no incorporation of ^{99}Mo into apodinitrogenase was observed. Addition of homocitrate to the FeMo-co synthesis reaction mixture resulted in the incorporation of ^{99}Mo into apodinitrogenase (Figue 3, lane 2). Increasing the concentration of homocitrate in the reaction mixture resulted in increased amounts of label incorporation into apodinitrogenase (Figure 3, lane 3). Interestingly, the substitution of homoisocitrate, 2-oxoglutarate, or citrate for homocitrate in the reaction mixture also resulted in the labeling of apodinitrogenase (Figure 3, lanes 4, 6, and 7). As with homocitrate, increasing the concentrations of these analogues resulted in increased amounts of label incorporation into apodinitrogenase (Figure 3, lane 5), except for 2-oxoglutarate for which ^{99}Mo incorporation into apodinitrogenase decreased with increasing concentrations (data not shown). No incorporation of label into apodinitrogenase was observed when homocitrate was replaced with tricarballylate (Figure 3, lane 8).

If the ^{99}Mo incorporated into apodinitrogenase was the result of synthesis and insertion of products similar to FeMo-co (henceforth referred to as FeMo-co analogues), then we would expect to observe inhibition of apodinitrogenase activation by purified FeMo-co following incubation of the complete in vitro FeMo-co synthesis system with the various organic acids. In vitro FeMo-co synthesis assays were done with each of the organic acis with limiting amounts of *K. pneumoniae* UN1100 extract (1.5 mg of protein, instead of 6 mg), followed by addition of purified FeMo-co. Incubations with both homoisocitrate and 2-oxoglutarate resulted in inhibition of activation of apodinitrogenase by purified FeMo-co (Table I). With homocitrate, essentially 100% of the apodinitrogenase was activated through in vitro FeMo-co synthesis. With the citrate incubations, a low level of in vitro FeMo-co synthesis and no inhibition of apodinitrogenase activation by purified FeMo-co were observed. Incubations with tricarballylate or no added organic acid resulted in essentially no FeMo-co synthesis and no inhibition of apodinitrogenase activation by purified FeMo-co. At the concentrations of organic acids used in the assays, only 2-oxoglutarate showed any inhibition of C_2H_2 reduction activity (about 30%). Incubations in the absence

Table II: Substrate Specificity of Dinitrogenase[a]

	C_2H_2 reduction activity[b]	proton reduction activity[c]		% inhibition of H_2 evolution by CO	N_2 reduction activity[d]	rate of HD formation[e]	C_2H_2 reduction activity/N_2 reduction activity
		−CO	+CO				
Dinitrogenase from *K. pneumoniae* Wild-Type and NifV⁻ Extracts							
UN (wild-type)	2500	3500	3600	−2.8	460	240	5.4
UN1991 (NifV⁻)	2200	3200	1700	53	240	23	11.0
Dinitrogenase Activated in Vitro in the Presence of Various Organic Acids							
no added organic acid	8.6	44	38	14	<4	<6	
plus homocitrate	540	720	820	−14	94	32	5.7
plus citrate	310	270	140	48	16	<6	19
plus homoisocitrate	44	390	360	7.7	<4	<6	
plus 2-oxoglutarate	41	180	190	−5.5	<4	<6	

[a] Activities reported as nmol reduced (or formed) min^{-1} (mg of dinitrogenase)$^{-1}$. [b] Assayed 10 μg of dinitrogenase from UN and UN1991 and 10–20 μg of dinitrogenase from samples from in vitro FeMo-co synthesis. [c] Assayed 50–70 μg of dinitrogenase from UN and UN1991 and 90–200 μg of dinitrogenase from samples from in vitro FeMo-co synthesis. [d] Assayed 10–20 μg of dinitrogenase from UN and UN1991 and 40–60 μg of dinitrogenase from samples from in vitro FeMo-co synthesis. [e] Assayed 50–60 μg of dinitrogenase from UN and UN1991 and 60–100 μg of dinitrogenase from samples from in vitro FeMo-co synthesis.

of CA12 extract always resulted in higher levels of activation of apodinitrogenase by purified FeMo-co. The nature of the inhibition by CA12 extact on apodinitrogenase activation is not understood.

Finally, preparations of apodinitrogenase activated with FeMo-co analogues were tested for their abilities to reduce C_2H_2, N_2, and protons. The ability of each of these preparations to catalyze HD formation was also assayed. These activities were compared to the activities of the wild-type and the NifV⁻ enzymes (Table II). The wild-type enzyme reduced C_2H_2, N_2, and protons effectively, H_2 evolution was not inhibited by CO, and high rates of HD formation were observed. As reported previously (McLean & Dixon, 1981), the NifV⁻ enzyme reduced C_2H_2 and protons effectively, N_2 was reduced less effectively, and H_2 evolution was inhibited by CO. Low rates of HD formation catalyzed by the NifV⁻ dinitrogenase were observed.

The apodinitrogenase activated in vitro with no added organic acid showed little or no reduction of all three substrates (Table II). Apodinitrogenase activated in vitro in the presence of homocitrate, like wild-type dinitrogenase, reduced C_2H_2, N_2, and protons effectively, H_2 evolution was not inhibited by CO, and high rates of HD formation were catalyzed by the enzyme. The apodinitrogenase activated in vitro in the presence of citrate reduced C_2H_2 and protons effectively, N_2 was reduced less effectively, H_2 evolution from the enzyme was inhibited by CO, and no HD formation was observed. Apodinitrogenase activated in vitro in the presence of 2-oxoglutarate or homoisocitrate reduced C_2H_2 poorly, reduced protons well, and did not reduce N_2. With these preparations, HD formation was undetected, and H_2 evolution was not inhibited by CO.

Discussion

The observation that citrate could replace homocitrate for in vitro FeMo-co synthesis (Hoover et al., 1987) led to the examination of other di- and tricarboxylic acids in FeMo-co synthesis. Of the 17 organic acids tested, only citrate yielded a FeMo-co analogue capable of producing a dinitrogenase effective in the C_2H_2 reduction assay. Interestingly, tricarballylate, which differs from citrate in that it lacks the hydroxyl group, demonstrated no FeMo-co synthesis activity, as determined from the C_2H_2 reduction assay and ^{99}Mo incorporation data. This means that the hydroxyl group is important for either recognition or function of citrate (and presumably homocitrate also) in FeMo-co synthesis.

DL-Isocitrate was also tested for its ability to replace homocitrate in the FeMo-co synthesis system. Activities in the coupled C_2H_2 reduction assay were approximately 2-fold higher with citrate compared to isocitrate (data not shown). The inhibition of FeMo-co synthesis (in the presence of 0.1 mM homocitrate) by isocitrate was similar to the inhibition observed with citrate. Because these experiments were done with crude extracts, it is not possible to distinguish between recognition of isocitrate by the FeMo-co synthesis system and conversion of isocitrate to citrate. It does not seem likely that conversion of citrate to isocitrate is required in the FeMo-co synthesis system, as activities with citrate were substantially higher than activities with isocitrate.

In view of the strong inhibition of FeMo-co synthesis by homoisocitrate and 2-oxoglutarate, it seemed reasonable to expect that these analogues would inhibit incorporation of ^{99}Mo into apodinitrogenase. It was somewhat surprising, therefore that these analogues facilitated the incorporation of ^{99}Mo into apodinitrogenase. Incorporation of label into dinitrogenase in the presence of citrate, homoisocitrate, or 2-oxoglutarate required the complete FeMo-co synthesis system—i.e., CA12 extract, UN1100 extract, and ATP. Tricarballylate served as a useful control, as it neither inhibited FeMo-co synthesis nor facilitated the incorporation of ^{99}Mo into apodinitrogenase. These data imply that in the in vitro FeMo-co synthesis system in the presence of homocitrate analogues the products formed are FeMo-co analogues.

That these FeMo-co analogues were bound to dinitrogenase at the FeMo-co binding site was demonstrated by the inhibition of apodinitrogenase activation by purified FeMo-co following incubation of the homocitrate analogues in the complete FeMo-co synthesis system. Inhibition of apodinitrogenase activation occurred following incubation with homoisocitrate (and 2-oxoglutarate to some extent), but not with tricarballylate. The fact that we did not observe 100% inhibition of apodinitrogenase activation may be explained by low levels of FeMo-co analogue synthesis.

Dinitrogenase preparations containing these FeMo-co analogues were able to reduce some of the substrates of nitrogenase. In particular, FeMo-co analogues synthesized in the presence of homoisocitrate or 2-oxoglutarate were relatively effective at reducing protons but not C_2H_2 or N_2. Perhaps the most physiologically significant FeMo-co analogue was synthesized in the presence of citrate, as its substrate affinity and inhibitor susceptibility properties were reminiscent of the properties of the NifV⁻ FeMo-co described previously

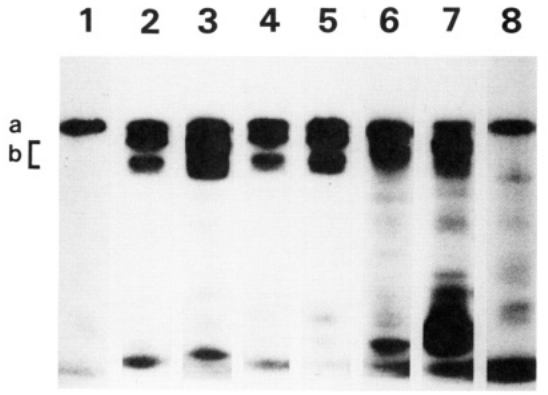

FIGURE 3: Autoradiography of in vitro FeMo-co synthesis reactions separated by anaerobic native gel electrophoresis. Reactions were carried out with $^{99}MoO_4^{2-}$ and different analogues of homocitrate. (Lane 1) No homocitrate analogue; (lane 2) homocitrate (0.03 mM); (lane 3) homocitrate (0.3 mM); (lane 4) homoisocitrate (0.15 mM); (lane 5) homoisocitrate (0.75 mM); (lane 6) 2-oxoglutarate (0.75 mM); (lane 7) citrate (7.5 mM); (lane 8) tricarballylate (7.5 mM). (a) Molybdenum-storage protein; (b) dinitrogenase. Other details are given in the text.

(McLean & Dixon, 1981; Hawkes et al., 1984) and in this study. The results reported here provide a reasonable explanation for the NifV$^-$ phenotype—i.e., citrate is used in place of homocitrate for FeMo-co biosynthesis in these mutants.

The FeMo-co formed in vitro in the presence of homocitrate was identical with wild-type FeMo-co, with regard to substrate affinities and sensitivity to CO. In the large-scale preparations, approximately 20% of the apodinitrogenase was activated in vitro in the presence of homocitrate, as indicated by the specific activity for C_2H_2 reduction. It was not possible to determine the extent of apodinitrogenase activation in vitro in the presence of the homocitrate analogues, as we do not know what specific activities to expect for dinitrogenase with these FeMo-co analogues.

HD formation with the NifV$^-$ dinitrogenase was found to proceed at rates much lower than those for the wild-type enzyme (Table II). Two models for explaining the requirement of N_2 for HD formation have been proposed (Burgess, 1985; Guth & Burris, 1983), and further studies with the NifV$^-$ enzyme may prove useful in resolving these models.

In summary, the results presented here demonstrate that there is no synthesis of FeMo-co in vitro in the absence of homocitrate, as indicated by the coupled C_2H_2 reduction, N_2 reduction, proton reduction, and HD formation assays and by the incorporation of ^{99}Mo into apodinitrogenase. Furthermore, if homocitrate is replaced in the in vitro FeMo-co synthesis assay system with a suitable analogue, such as citrate, 2-oxoglutarate, or homoisocitrate, then aberrant forms of FeMo-co are synthesized which exhibit altered substrate specificity. It seems likely that in vivo in the absence of homocitrate, as in the case of NifV$^-$ mutants, such an improperly formed FeMo-co is synthesized rather than an incompletely formed FeMo-co as suggested by others (Hawkes et al., 1984). In light of the observation that both citrate and 2-oxoglutarate are recognized as analogues for FeMo-co synthesis, it cannot be ruled out that the NifV$^-$ dinitrogenase formed in vivo contains a mixture of FeMo-co analogues.

The next questions to be addressed concern the role of homocitrate in FeMo-co synthesis. The results presented here suggest that homocitrate, or a part of homocitrate, is incorporated into FeMo-co. We have not been able to demonstrate the release of homocitrate from acid-hydrolyzed dinitrogenase or O_2-denatured FeMo-co (Hoover et al., 1986). This does not, however, preclude the possibility that part of the homocitrate molecule is incorporated into FeMo-co.

ACKNOWLEDGMENTS

We thank Claire Knocke for her excellent technical assistance and Gary Roberts and Robert Burris for helpful discussions. J.I. is on leave from the CSIC of Spain.

REFERENCES

Bishop, P. E., Premakumar, R., Dean, D. R., Jacobson, M. R., Chsnell, J. R., Rizzo, T. M., & Kopczynski, J. (1986) *Science (Washington, D.C.) 232*, 92–94.

Bulen, W. A., & LeComte, J. R. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 979–986.

Burgess, B. K. (1985) in *Nitrogen Fixation Research Progress* (Evans, H. J., Bottomley, P. J., & Newton, W. E., Eds.) pp 543–549, Martinus Nijhoff, Boston.

Burris, R. H. (1985) *Physiol. Veg. 23*, 843–846.

Eidsness, M. K., Flank, A. M., Smith, B. E., Flood, A. C., Garner, C. D., & Cramer, S. P. (1986) *J. Am. Chem. Soc. 108*, 2746–2747.

Filler, W. A., Kemp, R. M., Ng, J. C., Hawkes, T. R., Dixon, R. A., & Smith, B. E. (1986) *Eur. J. Biochem. 160*, 371–377.

Guth, J. H., & Burris, R. H. (1983) *Biochem. J. 213*, 741–749.

Hageman, R. V., & Burris, R. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2699–2702.

Hawkes, T. R., McLean, P. A., & Smith, B. E. (1984) *Biochem. J. 217*, 317–321.

Hoch, G. E., Schneider, K. C., & Burris, R. H. (1960) *Biochim. Biophys. Acta 37*, 273–279.

Hoover, T. R., Shah, V. K., Roberts, G. P., & Ludden, P. W. (1986) *J. Bacteriol. 167*, 999–1003.

Hoover, T. R., Robertson, A. D., Cerny, R. L., Hayes, R. N., Imperial J., Shah, V. K., & Ludden, P. W. (1987) *Nature (London) 329*, 855–857.

Hwang, J. C., Chen, C. H., & Burris, R. H. (1973) *Biochim. Biophys. Acta 292*, 256–270.

Imperial, J., Ugalde, R. A., Shah, V. K., & Brill, W. J. (1984) *J. Bacteriol. 158*, 187–194.

MacNeil, T., MacNeil, D., Roberts, G. P., Supiano, M. A., & Brill, W. J. (1978) *J. Bacteriol. 136*, 253–266.

McLean, P. A., & Dixon, R. A. (1981) *Nature (London) 292*, 655–656.

McLean, P. A., Smith, B. E., & Dixon, R. A. (1983) *Biochem. J. 211*, 589–597.

McLean, P. A., True, A., Chapman, S., Nelson, M., Teo, B.-K., Munck, E., Hoffman, B., & Orme-Johnson, W. H. (1985) in *Nitrogen Fixation Research Progress* (Evans, H. J., Bottomley, P. J., & Newton, W. E., Eds.) p 620, Martinus Nijhoff, Boston.

McLean, P. A., True, A. E., Nelson, M. J., Chapman, S., Godfrey, M. R., Teo, B. K., Orme-Johnson, W. H., & Hoffman, B. M. (1987) *J. Am. Chem. Soc. 109*, 943–945.

Pienkos, P. T., & Brill, W. J. (1981) *J. Bacteriol. 145*, 743–751.

Rivera-Ortiz, J. M., & Burris, R. H. (1975) *J. Bacteriol. 123*, 537–545.

Roberts, G. P., MacNeil, T., MacNeil, D., & Brill, W. J. (1978) *J. Bacteriol. 136*, 267–279.

Robinson, A. C., Dean, D. R., & Burgess, B. K. (1987) *J. Biol. Chem. 262*, 14327–14332.

Shah, V. K., & Brill, W. J. (1973) *Biochim. Biophys. Acta 305*, 445-454.
Shah, V. K., & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3249-3253.
Shah, V. K., Davis, L. C., & Brill, W. J. (1972) *Biochim. Biophys. Acta 256*, 498-511.
Shah, V. K., Chisnell, J. R., & Brill, W. J. (1978) *Biochem. Biophys. Res. Commun. 81*, 232-236.
Shah, V. K., Stacey, G., & Brill, W. J. (1983) *J. Biol. Chem. 258*, 12064-12068.
Shah, V. K., Imperial, J., Ugalde, R. A., Ludden, P. W., & Brill, W. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1636-1640.
Simpson, F. B. (1985) Ph.D. Thesis, University of Wisconsin—Madison.
Smith, B. E., Bishop, P. E., Dixon, R. A., Eady, R. R., Filler, W. A., Lowe, D. J., Richards, A. J. M., Thomson, A. J., Thorneley, R. N. F., & Postgate, J. R. (1985) in *Nitrogen Fixation Research Progress* (Evans, H. J., Bottomley, P. J., & Newton, W. E., Eds.) pp 597-603, Martinus Nijhoff, Boston.
Strandberg, G., & Wilson, P. W. (1968) *Can. J. Microbiol. 14*, 25-31.
Thomas, U., Kalyanpur, M. G., & Stevens, C. M. (1966) *Biochemistry 5*, 2513-2516.
Ugalde, R. A., Imperial, J., Shah, V. K., & Brill, W. J. (1984) *J. Bacteriol. 159*, 888-893.

S-(2-Chloroacetyl)glutathione, a Reactive Glutathione Thiol Ester and a Putative Metabolite of 1,1-Dichloroethylene†

Daniel C. Liebler,* David G. Latwesen, and Thaddeus C. Reeder
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331
Received June 19, 1987; Revised Manuscript Received January 8, 1988

ABSTRACT: Conversion of the toxic vinyl halide 1,1-dichloroethylene (DCE) to *S*-(2-*S*-glutathionylacetyl)glutathione ($GSCH_2COSG$) involves sequential acylation and alkylation of two glutathione (GSH) molecules by the microsomal DCE metabolite $ClCH_2COCl$. To examine its possible role in DCE biotransformation, we synthesized the putative intermediate *S*-(2-chloroacetyl)glutathione ($ClCH_2COSG$). In aqueous buffer, $ClCH_2COSG$ did not hydrolyze to release GSH, but instead underwent a two-step rearrangement to yield a cyclic product. Product analyses by liquid secondary ion mass spectrometry and $^1H-^{13}C$ heteronuclear correlation nuclear magnetic resonance spectroscopy indicated that rearrangement involved initial transfer of the chloroacetyl moiety from the cysteinyl thiol to the γ-glutamyl α-amine. The cysteinyl thiol then displaced chloride from the 2-chloroacetyl methylene carbon to yield the cyclic product. Incubation of 2 mM $ClCH_2COSG$ with 20 mM GSH yielded approximately 4.5-fold more cyclic product than $GSCH_2COSG$. $ClCH_2COSG$ alkylated oxytocindithiol and *N*-acetyl-L-cysteine to yield *S*-[2-(alkylthio)acetyl]glutathione adducts analogous to $GSCH_2COSG$. S-2-Chloroacetylation products were absent. In reacting with thiols by alkylation and in decomposing by rearrangement, $ClCH_2COSG$ displayed properties strikingly different from those of $ClCH_2COCl$. Although much less reactive than its acyl halide precursor, $ClCH_2COSG$ may display greater selectivity in covalent modification of cellular targets in DCE intoxication.

By reacting with toxic chemicals and their metabolites, GSH[1] prevents modification of critical cellular targets and protects against tissue damage [reviewed in Larsson et al. (1983)]. Nonetheless, several recent reports implicate glutathione conjugates in chemical toxicity. GSH conjugates and their degradation products may exhibit diverse and toxicologically significant properties. *S*-(2-Haloethyl)glutathione conjugates, for example, are thought to mediate 1,2-dihaloethane covalent binding to cellular macromolecules (Koga et al., 1986; Foureman & Reed, 1987). Alternatively, some haloalkene conjugates undergo metabolic degradation to reactive intermediates (Elfarra et al., 1986; Elfarra & Anders, 1984). Glutathione conjugates derived from 2-bromohydroquinone may mediate the renal toxicity of bromobenzene (Monks et al., 1985).

Here we report studies of $ClCH_2COSG$, a putative intermediate in the biotransformation of the hepatotoxic vinyl halide DCE. Microsomal cytochromes P-450 convert DCE to Cl_2CHCHO and $ClCH_2COCl$ by a mechanism not involving an epoxide intermediate (Liebler & Guengerich, 1983). GSH-fortified microsomes and isolated rat hepatocytes convert DCE to $GSCH_2COSG$, a bis(glutathionyl) conjugate formally derived from reaction of $ClCH_2COCl$ with two GSH molecules

† This work was supported in part by USPHS Grant ES01978 and Richard Chambers Environmental Grants to D.G.L. and T.C.R. by the Oregon State University. D.C.L. is the recipient of USPHS Fellowship F32ES05309.

* Author to whom correspondence should be addressed at the Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721.

[1] Abbreviations: $ClCH_2COSG$, *S*-(2-chloroacetyl)glutathione; DCE, 1,1-dichloroethylene; DTT, dithiothreitol; FAB, fast-atom bombardment; $GSCH_2COSG$, *S*-(2-*S*-glutathionylacetyl)glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; ^1H COSY, $^1H-^1H$ correlation spectroscopy; $^1H-^{13}C$ HETCOSY, $^1H-^{13}C$ heteronuclear correlation spectroscopy; LSIMS, liquid secondary ion mass spectrometry; TEAB, triethylammonium bicarbonate.

0006-2960/88/0427-3652$01.50/0