Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6517–6521, September 1990
Biochemistry

Diastereomer-dependent substrate reduction properties of a dinitrogenase containing 1-fluorohomocitrate in the iron-molybdenum cofactor

(FeMo cofactor biosynthesis/nitrogenase)

MARK S. MADDEN*†, NICHOLAS D. KINDON‡§, PAUL W. LUDDEN*†¶, AND VINOD K. SHAH*†

*Department of Biochemistry and †Center for the Study of Nitrogen Fixation, College of Agricultural and Life Sciences, and ‡Department of Chemistry, University of Wisconsin-Madison, Madison, WI 53706

Communicated by Winston J. Brill, May 18, 1990 (received for review April 4, 1990)

ABSTRACT ***In vitro* synthesis of the iron-molybdenum cofactor (FeMo-co) of dinitrogenase using homocitrate and its analogs allows the formation of modified forms of FeMo-co that show altered substrate specificities (N_2, acetylene, cyanide, or proton reduction) of nitrogenase [reduced ferredoxin:dinitrogen oxidoreductase (ATP-hydrolyzing), EC 1.18.6.1]. The (1*R*,2*S*)-threo- and (1*S*,2*S*)-erythro-fluorinated diastereomers of homocitrate have been incorporated *in vitro* into dinitrogenase in place of homocitrate. Dinitrogenase activated with FeMo-co synthesized using *threo*-fluorohomocitrate reduces protons, cyanide, and acetylene but cannot reduce N_2. In addition, proton reduction is inhibited by carbon monoxide (CO), a characteristic of dinitrogenase from NifV⁻ mutants. Dinitrogenase activated with FeMo-co synthesized using *erythro*-fluorohomocitrate reduces protons, cyanide, acetylene, and N_2. In this case proton reduction is not inhibited by CO, a characteristic of the wild-type enzyme. Cyanide reduction properties of dinitrogenase activated with FeMo-co containing either fluorohomocitrate diastereomer are similar, and CO strongly inhibits cyanide reduction. Dinitrogenases activated with FeMo-co containing homocitrate analogs with a hydroxyl group on the C-1 position are much less susceptible to CO inhibition of cyanide reduction. However, proton and cyanide reduction by dinitrogenase containing FeMo-co activated with (1*R*,2*S*) *threo*-isocitrate is only one-third that of dinitrogenase activated with the racemic mixture of isocitrate and shows strong CO inhibition of substrate reduction. These results suggest that CO inhibition of proton and cyanide reduction occurs when the hydroxyl group on the C-1 position of analogs is "trans" to the C-2 carboxyl group (i.e., in the threo conformation). When racemic mixtures of these analogs are used in the system, it seems that the erythro form is preferentially incorporated into dinitrogenase. Finally, carbonyl sulfide inhibition of substrate reduction by dinitrogenase is dependent on the homocitrate analog incorporated into FeMo-co.**

Biological systems utilize nitrogenase [reduced ferredoxin: dinitrogen oxidoreductase (ATP-hydrolyzing), EC 1.18.6.1] to catalyze the ATP- and reductant-dependent reduction of N_2 to ammonium. Nitrogenase consists of two proteins: dinitrogenase (MoFe protein or component I) and dinitrogenase reductase (Fe protein or component II) (1, 2). Dinitrogenase reductase transfers electrons one at a time to dinitrogenase with concomitant hydrolysis of ATP. Electrons passed to dinitrogenase are channeled to a prosthetic group called the iron-molybdenum cofactor (FeMo-co), which is the ultimate site of substrate reduction.

Analysis of mutants of *Klebsiella pneumoniae* defective in nitrogen fixation (*nif*⁻) has shown that at least six gene products (*nifQ*, *nifB*, *nifN*, *nifE*, *nifH*, and *nifV*) 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 (3). Strains with mutations in *nifB*, *nifN*, and *nifE* produce an apodinitrogenase that can be activated *in vitro* by purified FeMo-co (4). Strains of *K. pneumoniae* and *Azotobacter vinelandii* with mutations in *nifH* (the structural gene for dinitrogenase reductase) also fail to synthesize FeMo-co (5, 6). An *in vitro* system for the synthesis of FeMo-co has been described that requires molybdate, at least the *nifB*, *nifN*, and *nifE* gene products (7), dinitrogenase reductase (6, 8, 9), and (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid (homocitric acid) (10).

The *in vitro* FeMo-co synthesis system (7) allows the incorporation of homocitrate and its analogs (i.e., citrate, isocitrate, homoisocitrate, and such) to produce modified forms of FeMo-co. In the absence of homocitrate or one of its analogs, no FeMo-co is formed *in vitro* as determined by acetylene and proton reduction assays and by lack of ^{99}Mo incorporation into apodinitrogenase (11). Homocitrate has been shown to be an integral part of the FeMo-co of dinitrogenase, with one mole of homocitrate found for each mole of Mo (12).

Several organic acids (Fig. 1) were tested for their ability to replace homocitrate in the FeMo-co synthesis system (11, 13). The resulting variant forms of FeMo-co exhibited altered substrate specificity and inhibitor susceptibility. Use of citrate in place of homocitrate in the *in vitro* FeMo-co synthesis system resulted in the formation of holodinitrogenase with effective proton and acetylene reduction activities but with poor N_2 reduction and no $^1H^2H$ formation. In addition, proton reduction is inhibited by carbon monoxide (CO). These results are analogous to dinitrogenase from *nifV* mutants (14, 15), and recently citrate was found in the dinitrogenase of such mutants (16). Dinitrogenase activated with FeMo-co containing homoisocitrate, isocitrate, or 1-OH citrate effectively catalyzed the reduction of protons but not the reduction of acetylene or N_2 (13). The highest stringency pertaining to substrate specificity was found for N_2 reduction, a six-electron process. ^{15}N enrichment observed in experiments where apodinitrogenase was activated with homocitrate/FeMo-co was 80 times that of background levels, compared with citrate/FeMo-co with 6.3 times background levels (13). These data suggest that none of the FeMo-co analogs tested produce a dinitrogenase capable of supporting significant diazotrophic growth.

Imperial *et al.* (13) have suggested a model that describes the general structural requirements of organic acids for incorporation of Mo into FeMo-co: (*i*) stereochemistry at the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FeMo-co, iron-molybdenum cofactor of dinitrogenase; 1-OH citrate, 1,2-dihydroxy-1,2,3-propanetricarboxylic acid.

§Present address: Fisons plc, Pharmaceutical Division, Bakewell Road, Loughborough, Leicestershire LE11 ORH, United Kingdom.
¶To whom reprint requests should be addressed.

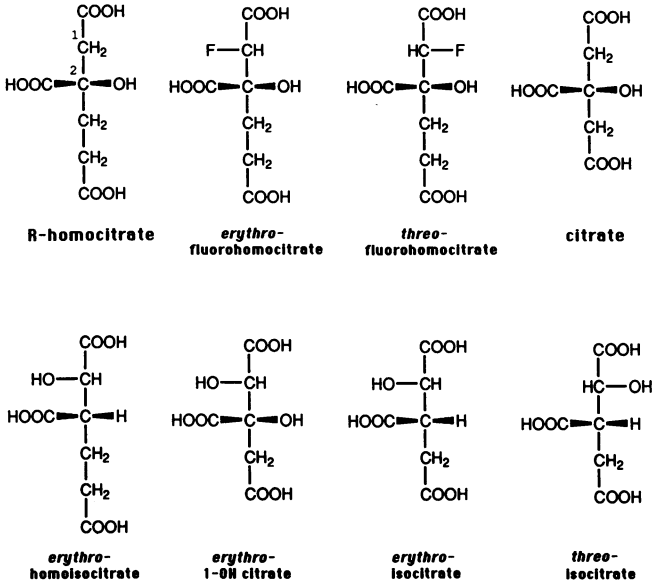

FIG. 1. Homocitrate and its analogs: homocitric acid, (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid; *erythro*-fluorohomocitric acid, (1*S*,2*S*)-1-fluoro-2-hydroxy-1,2,4-butanetricarboxylic acid; *threo*-fluorohomocitric acid, (1*R*,2*S*)-1-fluoro-2-hydroxy-1,2,4-butanetricarboxylic acid; homoisocitrate, 1-hydroxy-1,2,4-butanetricarboxylic acid; 1-OH citrate, 1,2-dihydroxy-1,2,3-propanetricarboxylic acid; isocitrate, 1-hydroxy-1,2,3-propanetricarboxylic acid; *threo*-isocitrate, (1*R*,2*S*)-1-hydroxy-1,2,3-propanetricarboxylic acid. Dinitrogenase activated with FeMo-co containing *erythro*-fluorohomocitrate shows proton, cyanide, acetylene, and N_2 reduction. Dinitrogenase activated with FeMo-co containing *threo*-fluorohomocitrate results in proton, cyanide, and acetylene reduction but not N_2 reduction. Dinitrogenases activated with FeMo-co synthesized using racemic mixtures of homocitrate analogs containing a hydroxyl group on the C-1 position are much less susceptible to CO inhibition of proton and cyanide reduction. However, proton and cyanide reduction by dinitrogenase activated with FeMo-co containing (1*R*,2*S*) *threo*-isocitrate is only one-third that of dinitrogenase activated with the racemic mixture of isocitrate and shows strong CO inhibition of substrate reduction.

C-2 chiral carbon analogous to that of the (R) isomer of homocitrate; (*ii*) a hydroxyl group on the chiral carbon atom; (*iii*) a carboxyl group on the chiral carbon atom; (*iv*) a terminal carboxyl group β to the chiral center; and (*v*) a chain length of four to six carbons containing two terminal carboxyl groups.

To elucidate this model further, we used the two diastereomers of fluorohomocitrate and expanded our studies to other substrates of nitrogenase (i.e., cyanide) as well as other inhibitors (i.e., COS). The substrate reduction properties of dinitrogenase activated by FeMo-co synthesized using the two diastereomers of fluorohomocitrate are markedly different. These differing substrate reduction properties and susceptibilities of dinitrogenase activated by FeMo-co synthesized using the diastereomers and other homocitrate analogs to inhibition by CO and COS provide insight into the functional groups on homocitrate required for active FeMo-co.

MATERIALS AND METHODS

Nitrogenase Assays. Assays were performed in the presence of excess dinitrogenase reductase. Acetylene and proton reduction assays (7) with or without CO (11) have been described. Cyanide reduction assays were analogous to those described by Hoover *et al.* (11) with the addition of 80 μl of 0.2 M KCN in 0.4 M Mops, pH 7.5. Cyanide solutions were prepared anaerobically at pH 7.5 and contained 1.7 mM dithionite. N_2 reduction was assayed by the $^{15}N_2$ fixation method (17) using 99% $^{15}N_2$ added after preincubation. Bacterial strains, preparation of crude extracts, and sources of homocitrate analogs are described in refs. 11 and 13. *threo*-Isocitrate was obtained from Sigma.

Synthesis of 1-Fluorohomocitrate and Separation of the Two Diastereomers. Both diastereomers of 1-fluorohomocitrate were synthesized by the method of Molines *et al.* (18) with minor modifications. This method utilizes di-*t*-butyl-2-oxoglutarate and the enolate of *t*-butyl fluoroacetate to form tri-*t*-butyl-1-fluorohomocitrate.‖

The diastereomers were separated using a silica gel column (4 × 20 cm) under pressure (0.3 bar; 1 bar = 10 kPa) and eluted with hexane/ether, 5:1 (vol/vol). Tri-*t*-butyl-1-fluorohomocitrate (1 g) was loaded onto the column and thirty 25-ml fractions were collected. The threo form of the fluorinated triester eluted first and separation of the two diastereomers was confirmed by ^1H NMR. The diastereomers were placed in separate 10-ml round-bottom flasks and 3 ml of 10 M HCl was added to each flask. The contents were refluxed at 90°C in an oil bath for 5 hr and left overnight at room temperature. Water (10 ml) was added to each round-bottom flask, the contents were transferred to separatory funnels, and 10 ml of dichloromethane was added to each to remove unreacted material. The water was then removed under reduced pressure. The crystals of threo diastereomer (120 mg) were dissolved in 0.5 ml of H_2O, and the solution was brought to pH 10 with 12 M NaOH, diluted with 4.5 ml of water, and stored at −20°C. The erythro diastereomer (120 mg) was similarly treated.

RESULTS AND DISCUSSION

Substrate Specificities of Dinitrogenase Activated by FeMo-co Synthesized with Fluorohomocitrate Analogs. The altered substrate reduction properties of dinitrogenase activated with FeMo-co synthesized using organic acid analogs of homocitrate have been described (11, 13). Dinitrogenase capable of proton reduction can be formed *in vitro* with many analogs of homocitrate in FeMo-co. However, more stringent structural requirements of the homocitrate analog were required to form dinitrogenase capable of acetylene reduction, and only homocitrate was able to produce dinitrogenase capable of significant N_2 reduction.

erythro- and *threo*-fluorohomocitrate were used as homocitrate analogs in the *in vitro* FeMo-co synthesis system. In preliminary experiments, different concentrations of the fluorinated analogs were tested for their ability to yield reconstituted dinitrogenase competent for acetylene reduction (data not shown). The lowest concentration generating maximum acetylene reduction was chosen for the assays in Tables 1 and 2. Dinitrogenase activated with FeMo-co synthesized *in vitro* using either fluorinated diastereomer of homocitrate reduced cyanide and protons as effectively as homocitrate-containing dinitrogenase (Table 1) and acetylene 50–60% as effectively. N_2 reduction by dinitrogenases activated by FeMo-co synthesized using the two fluorinated diastereomers was dramatically different. The fluorine atom of *threo*-fluorohomocitrate is "cis" to the hydroxyl group, and dinitrogenase activated with FeMo-co containing this analog is ineffective in N_2 reduction. In contrast, the fluorine atom of *erythro*-fluorohomocitrate is "trans" to the hydroxyl group, and dinitrogenase activated with FeMo-co containing this analog is capable of N_2 reduction 25–30% that of dinitrogenase activated with homocitrate-containing FeMo-co (Table 1). The size of the fluorine atom is similar to that of a hydrogen atom attached to carbon; therefore, steric effects from the fluorine atom of fluorohomocitrate are probably not the cause of the dramatic differences in N_2 reduction. How-

‖The experimental details of the synthesis of 1-fluorohomocitrate may be obtained from V.K.S.

Table 1. CO and COS inhibition of substrate reduction by altered forms of dinitrogenase

Organic acid	Conc., mM	Activity			
		Proton reduction*	Cyanide reduction†	Acetylene reduction‡	N_2 reduction§
		Absence of CO or COS			
Homocitrate	0.08	23	2.1	20	0.0890
erythro-Fluorohomocitrate	0.16	18	2.1	12	0.0251
threo-Fluorohomocitrate	0.16	20	2.4	10	0.0030
Citrate	8.00	9.1	0.4	7.4	0.0058
Homoisocitrate	0.80	19	1.7	1.7	ND
Isocitrate	4.00	20	1.4	4.6	ND
1-OH citrate	8.00	17	1.0	6.1	ND
threo-Isocitrate	4.00	6.6	0.5	7.2	ND
Fluorohomocitrate (racemic)	0.16	17	ND	ND	ND
		Presence of CO			
Homocitrate	0.08	30 (0)	0.2 (89)	0.2 (99)	
erythro-Fluorohomocitrate	0.16	17 (5)	0.0 (99)	0.1 (99)	
threo-Fluorohomocitrate	0.16	8.3 (59)	0.0 (100)	0.0 (100)	
Citrate	8.00	3.7 (59)	0.1 (85)	0.1 (98)	
Homoisocitrate	0.80	20 (0)	1.3 (24)	0.1 (93)	
Isocitrate	4.00	20 (0)	1.3 (9)	0.6 (86)	
1-OH citrate	8.00	18 (0)	0.8 (16)	1.0 (84)	
threo-Isocitrate	4.00	3.1 (53)	0.1 (87)	0.1 (99)	
Fluorohomocitrate (racemic)	0.16	16 (6)	ND	ND	
		Presence of COS			
Homocitrate	0.08	28 (0)	2.0 (4)	10 (48)	
erythro-Fluorohomocitrate	0.16	14 (22)	1.0 (48)	2.7 (78)	
threo-Fluorohomocitrate	0.16	7.5 (63)	1.1 (53)	0.6 (94)	
Citrate	8.00	4.6 (50)	0.3 (18)	2.8 (62)	
Homoisocitrate	0.80	19 (0)	1.7 (2)	0.9 (50)	
Isocitrate	4.00	22 (0)	1.6 (0)	3.0 (35)	
1-OH citrate	8.00	17 (0)	1.2 (0)	3.4 (44)	
threo-Isocitrate	4.00	4.0 (39)	0.5 (8)	3.8 (47)	

FeMo-co synthesis mixtures contained 0.2 ml of desalted UW45 (4 mg of protein) and 0.05 ml of a desalted NIFB preparation from strain UN1217 (9, 12), along with 0.2 ml of an ATP-regenerating mixture which contained 5 mM sodium dithionite and 0.05 mM sodium molybdate (7) and the indicated concentrations of organic acids. The reaction mixtures were incubated at 30°C for 35 min, after which 0.8 ml of additional ATP-regenerating mixture containing 5 mM sodium dithionite was added, together with purified dinitrogenase reductase (7) and assayed. ND, not determined. Addition of excess purified FeMo-co to the UW45 extracts used resulted in an activity of 44.9 nmol of ethylene formed per min per assay. Numbers in parentheses are percent inhibition by CO or COS. One hundred microliters of CO (COS) gas was injected in an assay vial containing an 8-ml gas phase. Solutions of organic acids were prepared in dilute NaOH to a final pH of 9 prior to assay. Concentrations are those present during FeMo-co synthesis.

*Expressed as nmol of hydrogen formed per min per assay.

†Expressed as nmol of methane formed per min per assay.

‡Expressed as nmol of ethylene formed per min per assay.

§Expressed as atom % ^{15}N excess as described in ref. 17. N_2 reduction assays were carried out for 1 hr.

ever, the three electron pairs of the fluorine atom may contribute to the altered substrate specificities of the dinitrogenases activated with FeMo-co containing fluorinated analogs of homocitrate.

Cyanide Reduction Properties of Altered Forms of Dinitrogenase. Cyanide reduction is a six-electron process that differs from N_2 reduction (19). Lowe *et al.* (20) have proposed that cyanide binds to a more highly oxidized form of FeMo-co

Table 2. Proton reduction by dinitrogenases containing homocitrate analogs in the presence of CO and cyanide

Organic acid	Conc., mM	Activity				
		Cyanide reduction		Proton reduction*		
		− CO	+ CO	− KCN, − CO	+ KCN, − CO	+ KCN, + CO
Homocitrate	0.08	2.1	0.3 (87)	18	3.0 (84)	23 (0)
erythro-Fluorohomocitrate	0.16	1.9	0.1 (94)	20	3.3 (84)	15 (26)
threo-Fluorohomocitrate	0.16	2.4	0.1 (95)	19	2.9 (85)	6.2 (67)
Citrate	8	0.4	0.1 (81)	8.8	0.3 (96)	1.6 (82)
Homoisocitrate	0.8	1.4	1.1 (22)	26	2.5 (90)	4.6 (82)
Isocitrate	4	1.4	1.3 (9)	22	1.8 (92)	2.6 (89)
1-OH citrate	8	0.9	0.7 (19)	21	1.8 (91)	2.4 (89)
threo-Isocitrate	4	0.5	0.1 (87)	6.3	0.3 (95)	1.2 (82)

Assay conditions were as in Table 1. Numbers in parentheses are percent inhibition by CO.

*Proton reduction was assayed in the presence of cyanide (concentration same as normal cyanide reduction assays).

than that required for H_2 evolution or N_2 reduction. Therefore, analog-activated dinitrogenases were compared for their abilities to reduce the carbon-nitrogen triple bond of cyanide.

Dinitrogenases containing *erythro*-fluorohomocitrate/FeMo-co and *threo*-fluorohomocitrate/FeMo-co reduce cyanide to methane as effectively as dinitrogenase activated with homocitrate/FeMo-co (Table 1). On the other hand, dinitrogenase containing FeMo-co synthesized with racemic mixtures of 1-hydroxyl acids (homoisocitrate, isocitrate, and 1-OH citrate) reduces cyanide to methane 50–80% as effectively as dinitrogenase activated with homocitrate/FeMo-co. These data clearly indicate that dinitrogenase containing homocitrate analogs with a fluorine atom or a hydroxyl group at the C-1 position can still catalyze effective cyanide reduction. However, cyanide reduction of citrate-containing dinitrogenase was only 19% that of dinitrogenase containing homocitrate. Since dinitrogenase containing isocitrate can effectively reduce cyanide but dinitrogenase containing citrate cannot, the distance between the hydroxyl and the C-3 carboxyl group of the analog may be critical to dinitrogenase competent for cyanide reduction. In addition, dinitrogenase containing 1-OH citrate shows cyanide reduction levels between those of dinitrogenases containing isocitrate and citrate. This result suggests that the addition of a C-1 hydroxyl group to citrate may counteract the effects associated with the C-2 hydroxyl group of citrate.

CO Inhibition of Substrate Reduction by Altered Forms of Dinitrogenase. Dinitrogenases containing homocitrate or *erythro*-fluorohomocitrate were not prone to CO inhibition of proton reduction. However, proton reduction by dinitrogenases containing either *threo*-fluorohomocitrate or citrate was inhibited 59% by CO (Table 1). These results suggest that CO inhibition of proton reduction may be associated with electronic alterations around the hydroxyl or carboxyl groups attached to the C-2 position of the homocitrate analog (Fig. 1). The fluorine atom of *threo*-fluorohomocitrate is cis with respect to the C-2 hydroxyl group and trans with respect to the C-2 carboxyl group, while the fluorine of *erythro*-fluorohomocitrate is trans with respect to the C-2 hydroxyl group and cis with respect to the C-2 carboxyl group. The fluorine atom (with its three electron pairs) may be perturbing the metal ligating properties of the C-2 hydroxyl or carboxyl oxygen atoms. Imperial *et al.* (13) have postulated that the carboxyl and hydroxyl groups of homocitrate may ligate metal atoms when incorporated into FeMo-co. It is also possible that the fluorine atom on the C-1 position of homocitrate may alter the interaction of FeMo-co to apodinitrogenase polypeptides. The hydroxyl group and the carboxyl group attached to the C-2 position of homocitrate analogs are essential to activate dinitrogenase since 1,2,3-propanetricarboxylic acid (tricarballylate), 1,2,4-butanetricarboxylic acid, and 2-hydroxy-2-methyl-1,3-propanedicarboxylic acid (β-hydroxy-β-methylglutarate) do not facilitate the incorporation of Mo into dinitrogenase or generate active FeMo-co (11, 13). Dinitrogenase could not be activated using tri-*t*-butyl fluorohomocitrate (data not shown). This result provides further evidence that the carboxyl groups are essential for enzyme activation.

Dinitrogenase containing homoisocitrate, isocitrate, or 1-OH citrate effectively reduced protons without CO inhibition. However, dinitrogenase containing *threo*-isocitrate shows CO inhibition of proton reduction similar to that of dinitrogenase containing *threo*-fluorohomocitrate or citrate. Isocitrate does not contain a C-2 hydroxyl group, yet the threo diastereomer of isocitrate contains a hydroxyl group trans to the C-2 carboxyl group and shows CO inhibition. This suggests that dinitrogenase containing homocitrate analogs with atoms having electron pairs attached to the C-1 position will show CO inhibition of proton and cyanide reduction if these atoms are trans to the C-2 carboxyl group (i.e., cis to the C-2 hydroxyl group or in the threo conformation).

The relationship between CO inhibition and the atoms attached to the C-1 carbon relative to the C-2 chiral center in homocitrate analogs was further investigated by analyzing CO inhibition of cyanide reduction with analog-activated dinitrogenases. CO effectively inhibited cyanide reduction by dinitrogenase containing homocitrate (89%), *erythro*-fluorohomocitrate (99%), *threo*-fluorohomocitrate (100%), *threo*-isocitrate (87%), or citrate (85%). Very little CO inhibition was observed for dinitrogenases activated with FeMo-co synthesized using racemic mixtures of homoisocitrate (24%), isocitrate (9%), or 1-OH citrate (16%). These results show that the presence of a hydroxyl group cis to the C-2 carboxyl group of homocitrate analogs prevents CO inhibition.

Preferential Incorporation of erythro Diastereomers. The proton and cyanide reduction activities of dinitrogenase containing the threo diastereomer of isocitrate were 33% and 37% that of the dinitrogenase activated by FeMo-co synthesized using a racemic mixture of isocitrate. In addition, dinitrogenase containing *threo*-isocitrate is susceptible to CO inhibition of proton and cyanide reduction. However, use of the racemic mixture of isocitrate for FeMo-co synthesis yields a dinitrogenase that effectively reduces protons and cyanide without CO inhibition. This suggests that the erythro diastereomer may be preferred for incorporation into FeMo-co. In addition, CO inhibition of proton reduction by dinitrogenase activated by FeMo-co synthesized using a racemic mixture of fluorohomocitrate was characteristic of the *erythro*-fluorohomocitrate-containing enzyme (Table 1). If the FeMo-co synthesis system were unable to discriminate between the two fluorinated diastereomers, then CO inhibition of proton reduction by the dinitrogenase activated by FeMo-co prepared using the racemic mixture should be about 30%. Since identical CO inhibition was observed with dinitrogenase activated by FeMo-co prepared using either *erythro*-fluorohomocitrate or the racemic mixture of fluorohomocitrate, it seems that *erythro*-fluorohomocitrate is preferentially incorporated into dinitrogenase.

Effects of CO and Cyanide on Proton Reduction by Altered Forms of Dinitrogenase. Proton reduction by dinitrogenases containing homocitrate analogs also was studied in the presence of cyanide (Table 2). In the absence of any other suitable substrate (i.e., N_2, acetylene, or cyanide) nitrogenase will reduce protons to H_2. When cyanide was added to solutions of analog-activated dinitrogenases, little or no proton reduction was observed. However, when CO was added together with cyanide, the reduction of cyanide was inhibited, and increased proton reduction activity was observed (except for the dinitrogenase containing *threo*-fluorohomocitrate, which had shown CO inhibition of proton reduction without cyanide). Dinitrogenase activated by FeMo-co synthesized using racemic mixtures of homocitrate analogs that have a hydroxyl group on the C-1 position shows little or no CO inhibition of cyanide reduction. Therefore, little or no proton reduction was observed when CO was added in the presence of cyanide.

CO completely inhibits acetylene reduction by dinitrogenases containing homocitrate, fluorohomocitrate, citrate, or *threo*-isocitrate. However, CO does not completely inhibit acetylene reduction by dinitrogenases activated by FeMo-co synthesized using racemic mixtures of homocitrate analogs with a hydroxyl group on the C-1 position; (i.e., homoisocitrate, isocitrate, and 1-OH citrate exhibit 93%, 86%, and 84% inhibition, respectively).

The degree of CO inhibition of analog-activated dinitrogenase was dependent on the nature of substrate being reduced (protons < cyanide < acetylene). Lowe *et al.* (20) have proposed that substrate reduction is dependent on the oxi-

dation state of FeMo-co. Since CO inhibition was dependent on the substrate being reduced by dinitrogenase, it would seem that the more reduced forms are more susceptible to CO inhibition.

Carbonyl Sulfide (COS) Inhibition of Substrate Reduction by Altered Forms of Dinitrogenase. Because COS is an analog of CO, and CO inhibits the reduction of all substrates except protons by dinitrogenase (21), COS was tested as a possible inhibitor to obtain further information on analog-activated dinitrogenases.

COS inhibition of proton and acetylene reduction by dinitrogenase was found to be dependent on the homocitrate analog used during *in vitro* FeMo-co synthesis (Table 1). COS did not inhibit proton or cyanide reduction by dinitrogenase containing homocitrate. However, COS inhibited proton reduction by dinitrogenases containing *erythro*-fluorohomocitrate (22%), *threo*-fluorohomocitrate (63%), *threo*-isocitrate (39%), or citrate (50%). CO strongly inhibited acetylene reduction by all forms of dinitrogenases. However, COS inhibition of acetylene reduction by dinitrogenase again was dependent on the organic acid constituent. Dinitrogenases containing homocitrate, *erythro*-fluorohomocitrate, *threo*-fluorohomocitrate, or citrate exhibited COS inhibition of 48%, 78%, 94%, and 62%, respectively.

Dinitrogenase activated with FeMo-co synthesized using the racemic mixture of C-1 hydroxyl acid analogs showed little or no COS inhibition of proton or cyanide reduction. These results are analogous to CO inhibition patterns. Inhibition by COS was generally less than CO inhibition, and the degree of COS inhibition was generally dependent on which substrate was being reduced (protons < cyanide < acetylene). However, analog-activated dinitrogenases exhibiting CO inhibition of proton reduction showed markedly different COS inhibition patterns. Dinitrogenase containing *threo*-fluorohomocitrate, *threo*-isocitrate, or citrate had nearly equal CO and COS inhibition of proton reduction. With these analogs, the inhibition of proton reduction by COS was greater than that of cyanide reduction.

It is interesting that COS inhibits cyanide reduction by dinitrogenase containing either fluorinated diastereomer of homocitrate by 50%. Proton reduction by dinitrogenase containing *erythro*-fluorohomocitrate is not inhibited by CO, but COS inhibits this reduction 22%. In addition, the highest COS inhibition of cyanide and acetylene reduction occurred when dinitrogenase was activated with FeMo-co containing either fluorohomocitrate diastereomer. It seems that COS inhibition of substrate reduction is more pronounced with dinitrogenase activated with FeMo-co containing the fluorinated homocitrate analogs compared with other analogs.

CO and COS inhibit proton reduction by analog-activated dinitrogenase to similar extents. However, dinitrogenases containing homocitrate, citrate, or *threo*-isocitrate show high CO inhibition of cyanide reduction (89%, 85%, and 87%, respectively), while COS inhibition of cyanide reduction is low (4%, 18%, and 8%). These dramatic differences suggest that CO and COS may act upon different sites on the enzyme.

SUMMARY

These results further refine the model proposed by Imperial *et al.*(13) and establish that the stereochemistry at the C-1 position of homocitrate is crucial to the function of FeMo-co in dinitrogenase. Substrate reduction properties of altered forms of FeMo-co are dependent on atoms attached to the C-1 position relative to the carboxyl group attached to the C-2 position. Fluorine substitution for hydrogen atom at the C-1 position of fluorohomocitrate dramatically alters N_2 reduction as well as CO (and COS) inhibition properties of dinitrogenase. Dinitrogenases activated with racemic mixtures of organic acids containing a hydroxyl group at the C-1 position show low CO and COS inhibition of proton and cyanide reduction. In addition, the erythro diastereomers of the homocitrate analogs (i.e., *erythro*-fluorohomocitrate or *erythro*-isocitrate) seem to be preferentially incorporated into FeMo-co over their threo counterparts.

CO (and COS) inhibition of substrate reduction is dependent on the homocitrate analog incorporated into FeMo-co. Lowe *et al.* (20) and Liang and Smith (22) have proposed that cyanide binds to a more oxidized form of FeMo-co than that required for proton or N_2 reduction. Since CO inhibition was dependent on the substrate being reduced (protons < cyanide < acetylene), it would seem that the more-reduced forms of dinitrogenase are more susceptible to CO inhibition. Finally, differences in CO and COS inhibition suggest that COS may act at a different site (or by a different mechanism) than CO.

We give special thanks to Robert Burris for his valuable help with ^{15}N experiments; Steven Burke for use of his facilities to synthesize fluorohomocitrate; Laurens Anderson for his help in determining Fischer projections of fluorohomocitrate diastereomers; and Scott Ensign, Gary Roberts, and Robert Burris for their helpful discussions and for reviewing this manuscript. We also thank Heidi Forest for assistance in preparing media and such. This research was supported by the College of Agriculture and Life Sciences at the University of Wisconsin–Madison and by U.S. Department of Agriculture Grant 8801471.

1. Bulen, W. A. & LeComte, J. R. (1966) *Proc. Natl. Acad. Sci. USA* **56**, 979–986.
2. Hageman, R. V. & Burris, R. H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2699–2702.
3. Imperial, J., Ugalde, R. A., Shah, V. K. & Brill, W. J. (1984) *J. Bacteriol.* **158**, 187–194.
4. Shah, V. K. & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3249–3253.
5. 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.
6. Robinson, A. C., Dean, D. R. & Burgess, B. K. (1987) *J. Biol. Chem.* **262**, 14327–14332
7. Shah, V. K., Imperial, J., Ugalde, R. A., Ludden, P. W. & Brill, W. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1636–1640.
8. Imperial, J., Shah, V. K., Hoover, T. R. & Ludden, P. W. (1988) in *Nitrogen Fixation: Hundred Years After*, eds. Bothe, H., de Bruijn, F. J. & Newton, W. E. (Fischer, Stuttgart, FRG), p. 128.
9. Shah, V. K., Hoover, T. R., Imperial, J., Paustian, T. D., Roberts, G. P. & Ludden, P. W. (1988) in *Nitrogen Fixation: Hundred Years After*, eds. Bothe, H., de Bruijn, F. J. & Newton, W. E. (Fischer, Stuttgart, FRG), p. 115.
10. 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.
11. Hoover, T. R., Imperial, J., Liang, J., Ludden, P. W. & Shah, V. K. (1988) *Biochemistry* **27**, 3647–3652.
12. Hoover, T. R., Imperial, J., Ludden, P. W. & Shah, V. K. (1989) *Biochemistry* **28**, 2768–2771.
13. Imperial, J., Hoover, T. R., Madden, M. S., Ludden, P. W. & Shah, V. K. (1989) *Biochemistry* **28**, 7796–7799.
14. McLean, P. A. & Dixon, R. A. (1981) *Nature (London)* **292**, 655–656.
15. Hawkes, T. R., McLean, P. A. & Smith, B. E. (1984) *Biochem. J.* **217**, 317–321.
16. Liang, J., Madden, M., Shah, V. K. & Burris, R. H. (1990) *Biochemistry*, in press.
17. Burris, R. H. & Wilson, P. W. (1957) *Methods Enzymol.* **4**, 355–366.
18. Molines, H., Massoudi, H., Cantacuzene, D. & Wakselman, C. (1983) *Synthesis*, 222–224.
19. Burgess, B. K. (1985) in *Molybdenum Enzymes*, ed. Spiro, T. (Wiley-Interscience, New York), pp. 161–220.
20. Lowe, D. J., Fisher, K., Thorneley, R. N. F., Vaughn, S. A. & Burgess, B.K. (1989) *Biochemistry* **28**, 8460–8466.
21. Rivera-Ortiz, J. M. & Burris, R. H. (1975) *J. Bacteriol.* **123**, 537–545.
22. Liang, J. & Smith, B. E. (1984) in *Advances in Nitrogen Fixation Research*, eds. Veeger, C. & Newton, W. E. (Nijhoff/Junk, Dordrecht, The Netherlands), p. 155.