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Substrate Reduction Properties of Dinitrogenase Activated in Vitro Are Dependent upon the Presence of Homocitrate or Its Analogues during Iron-Molybdenum Cofactor Synthesis†

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ABSTRACT: (*R*)-2-Hydroxy-1,2,4-butanetricarboxylic acid [(*R*)-homocitrate] has been recently reported to be an integral constituent of the otherwise thought to be inorganic iron-molybdenum cofactor of dinitrogenase [Hoover, T. R., Imperial, J., Ludden, P. W., & Shah, V. K. (1989) *Biochemistry 28*, 2768-2771]. Different organic acids can substitute for homocitrate in an in vitro system for iron-molybdenum cofactor synthesis and incorporation into dinitrogenase [Hoover, T. R., Imperial, J., Ludden, P. W., & Shah, V. K. (1988) *Biochemistry 27*, 3647-3652]. Dinitrogenase activated with homocitrate-FeMo-co was able to reduce dinitrogen, acetylene, and protons efficiently. Homoisocitrate and isocitrate dinitrogenases did not reduce dinitrogen or acetylene, but showed very high proton reduction activities. Citrate and citramalate dinitrogenases had very low dinitrogen reduction activities and intermediate acetylene and proton reduction activities. CO inhibited proton reduction in both these cases but not in the case of dinitrogenases activated with other homocitrate analogues. By use of these and other commercially available homocitrate analogues in the in vitro system, the structural features of the homocitrate molecule absolutely required for the synthesis of a catalytically competent iron-molybdenum cofactor were determined to be the hydroxyl group, the 1- and 2-carboxyl groups, and the *R* configuration of the chiral center. The stringency of the structural requirements was dependent on the nitrogenase substrate used for the assay, with dinitrogen having the most stringent requirements followed by acetylene and protons.

Nitrogenase catalyzes the ATP-[1] and reductant-dependent reduction of N_2 to ammonia (Bulen & LeComte, 1966), in addition to the reduction of other triple-bonded molecules. In the absence of any other substance, nitrogenase catalyzes the reduction of protons to H_2. Nitrogenase consists of two proteins: dinitrogenase (or MoFe protein) and dinitrogenase reductase (or Fe protein) (Bulen & LeComte, 1966; Hageman & Burris, 1978). Substrate reduction occurs on dinitrogenase. A unique prosthetic group, the iron-molybdenum cofactor (FeMo-co) containing Mo, Fe, S (Shah & Brill, 1977), and homocitrate (Hoover et al., 1989), has been proposed as the site for substrate reduction (Shah & Brill, 1977; Rawlings et al., 1978; Hawkes et al., 1984; Hoover et al., 1988a, 1989). The structure and biosynthetic pathway of FeMo-co remain undefined. A system for in vitro synthesis of FeMo-co that requires molybdate, ATP, at least the *nifB*, *nifN*, and *nifE* gene products (Shah et al., 1986), the *nifH* gene product (Robinson et al., 1987; Shah et al., 1988), and (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid (homocitrate) (Hoover et al., 1987) has been described. Unmodified homocitrate can be recovered from FeMo-co in a stoichiometric ratio of 1 homocitrate to 1 Mo (Hoover et al., 1989).

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[1] Abbreviations: FeMo-co, iron-molybdenum cofactor of dinitrogenase; ATP, adenosine 5'-triphosphate; homocitrate acid, (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid; homoisocitric acid, 1-hydroxy-1,2,4-butanetricarboxylic acid; citramalic acid, 2-hydroxy-2-methylbutanedioic acid; tricarballylic acid, 1,2,3-propanetricarboxylic acid.

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Synthesis and accumulation of homocitrate in *Klebsiella pneumoniae* require a functional *nifV* gene product (Hoover et al., 1986). NifV$^-$ mutants synthesize a modified form of FeMo-co (Hawkes et al., 1984) with altered substrate reduction and inhibitor susceptibility properties. Dinitrogenase from NifV$^-$ mutants effectively reduces acetylene but not N_2. In addition, the H_2 evolution by the NifV$^-$ enzyme is inhibited by CO (MacLean & Dixon, 1981; MacLean et al., 1983). Substitution of citrate for homocitrate in the in vitro FeMo-co synthesis system results in a dinitrogenase with substrate specificity identical with that of dinitrogenase from NifV$^-$ mutants (Hoover et al, 1988a), and recently, citrate was recovered from purified NifV$^-$ dinitrogenase (Liang et al., unpublished data). Other organic acids have been tested for their ability to promote synthesis of aberrant forms of FeMo-co (Hoover et al., 1988a,b). We have extended these original observations to include a large number of homocitrate analogues and determined the minimum structural requirements for synthesis and incorporation of a catalytically competent FeMo-co into dinitrogenase.

Materials and Methods

Reagents. Homocitric acid lactone (Sigma Chemical Co.) was converted to the free acid form by preparation of solutions in dilute NaOH. *cis*-Aconitic acid, (*R*)-citramalic acid, D-glutamic acid, glutaric acid, D- and L-2-hydroxyglutaric acid, 3-hydroxy-3-methylglutaric acid, D,L-isocitric acid, D- and L-malic acid, oxalacetic acid, 2- and 3-oxoadipic acid 2-oxocaproic acid, 2- and 3-oxoglutaric acid, and tricarballylic acid were obtained from Sigma. *trans*-Aconitic acid, (*S*)-citramalic acid, 2-hydroxy-2-methylbutyric acid, and D-saccharic acid were obtained from Aldrich Chemical Co. 1,2,4-Butanetricarboxylic acid was from Chem Service (West Chester, PA). Homoisocitric acid was obtained from Research Plus (Bayonne, NJ), and 3-fluoro-2-oxoglutaric acid was a gift from Charles Grissom.

Bacterial Strains, Preparation of Crude Extracts, and in Vitro FeMo-co Synthesis Assays. These were as in Hoover et al. (1988a, 1989).

Nitrogenase Assays. Assays were performed with an excess of dinitrogenase reductase (6–10 mol/mol of dinitrogenase). Assays for acetylene reduction (Shah et al., 1986) and proton reduction with or without CO (Hoover et al., 1988a) have been described. N_2 reduction was assayed by the $^{15}N_2$ fixation method (Burris & Wilson, 1957) using 99% $^{15}N_2$ added after preincubation for FeMo-co synthesis.

Results and Discussion

Various organic acids were used as homocitrate analogues in the in vitro FeMo-co synthesis system. In vitro formed FeMo-co and FeMo-co analogues were incorporated into apo[FeMo-co-less] dinitrogenase, and dinitrogenase activities were assayed in the presence of an excess of dinitrogenase reductase (Table I). In preliminary experiments, different concentrations of analogues were tested for their ability to yield reconstituted dinitrogenase competent for acetylene reduction (data not shown). The lowest concentration generating maximum acetylene reduction activity was chosen for the assays in Table I.

Among the organic acids tested, (*R*)-homocitrate, the naturally occurring organic moiety of FeMo-co (Hoover et al., 1988a), gave the highest activities, even at very low concentrations. However, at concentrations of analogues 10–100-fold higher than those required for homocitrate, the biosynthetic system showed little specificity, and a variety of organic acids could be used to generate FeMo-co analogues, capable of being incorporated into dinitrogenase and competent for alternate substrate reduction. Substrate specificity of dinitrogenase assembled with FeMo-co analogues was dependent upon the organic acid used in the FeMo-co synthesis system.

Table I: Substrate Reduction Activity of Dinitrogenase Activated in Vitro in the Presence of Various Organic Acids[a]

organic acid	concn[b] (mM)	N_2 reduction activity[c]	C_2H_2 reduction activity[d]	proton reduction activity[d] −CO	proton reduction activity[d] +CO[e]
none		0.0042	1.3	3.3	3.4
homocitrate	0.08	0.3279	38.9	46.7	47.3
citrate	8	0.0240	18.4	23.1	8.5 (63)
(*R*)-citramalate	2	0.0241	16.3	24.6	7.4 (70)
cis-aconitate	8	0.0248	20.3	13.1	4.7 (64)
2-hydroxycitrate	0.8	0.0131	7.8	27.4	26.7
D-malate	8	0.0124	6.2	14.2	16.5
2-oxoglutarate	1.6	0.0110	4.4	15.0	14.9
isocitrate	1.6	0.0097	6.2	33.3	37.0
homoisocitrate	0.3	0.0052	2.3	36.3	39.4
3-oxoglutarate	1.6	0.0075	5.1	11.3	9.2 (19)
L-malate	8	0.0077	3.2	13.2	13.2
D-saccharate	8	0.0073	3.2	10.9	15.0
trans-aconitate	16	0.0069	5.5	6.6	4.0 (40)
3-fluoro-2-oxoglutarate	0.8	ND[f]	5.3	12.3	15.1
oxaloacetate	16	0.010	4.7	6.5	5.8
(*S*)-citramalate	1.6	0.0073	3.5	5.4	5.0
D-2-hydroxyglutarate	16	0.0050	3.0	5.2	5.4
3-hydroxy-3-methylglutarate	16	ND	2.8	4.1	4.1
2-(hydroxymethyl)butyrate	1.6	ND	1.7	3.0	3.0
glutarate	16	ND	2.2	3.1	2.8
L-2-hydroxyglutarate	16	ND	1.9	3.4	2.8
2-oxocaproate	16	ND	0.8	2.3	2.6
L-glutamate	16	0.0058	2.4	2.3	2.6
tricarballylate	16	ND	2.7	3.1	3.1
1,2,4-butanetricarboxylate	16	0.0052	2.6	2.3	2.6

[a] The 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 UN1100 (Shah et al., 1988; Hoover et al., 1989), along with 0.2 mL of an ATP-regenerating mixture which contained 5 mM sodium dithionite and 0.05 mM sodium molybdate (Shah et al., 1986) and the indicated concentrations of organic acids. The reaction mixtures were incubated at 30 °C for 30 min, after which time 0.8 mL of additional ATP-regenerating mixture containing 5 mM sodium dithionite was added, together with purified dinitrogenase reductase (Shah et al., 1986) and assayed. [b] Solutions were prepared in dilute NaOH to a final pH of 9 prior to the assay. Concentrations are those present during FeMo-co synthesis. [c] Activities expressed as atom % ^{15}N excess as described (Burris & Wilson, 1957). N_2 reduction assays were carried out for 1 h. [d] Activities expressed as nanomoles of acetylene or protons reduced per minute per assay. [e] Figures in parentheses are percentage inhibition of proton reduction by CO. [f] ND, not determined.

The highest stringency was found for N_2 reduction. Only FeMo-co synthesized with homocitrate (homocitrate–FeMo-co) gave high N_2 reduction activity. Much lower activities were observed with citrate–, (*R*)-citramalate–, and *cis*-aconitate–FeMo-co [approximately 6% of (*R*)-homocitrate–FeMo-co activity]. Activities significantly above background were also observed with D-malate–, 2-hydroxycitrate–, 2-oxoglutarate–, and isocitrate–FeMo-co (Table I). On the basis of substrate specificity and inhibitor susceptibility, Hoover et al., (1988a), suggested that NifV$^-$ mutants incorporate citrate–FeMo-co into dinitrogenase, and citrate has been recovered from purified NifV$^-$ dinitrogenase (Liang et al., unpublished data). NifV$^-$ mutants of *K. pneumoniae* show a clear Nif$^-$ phenotype (Roberts et al., 1978). Taken together, these data suggest that none of the FeMo-co analogues would result in a dinitrogenase capable of supporting significant diazotrophic growth.

Many of the FeMo-co analogues were competent for acetylene and proton reduction. With acetylene as substrate, activities up to 50–70% of the homocitrate–FeMo-co (wild

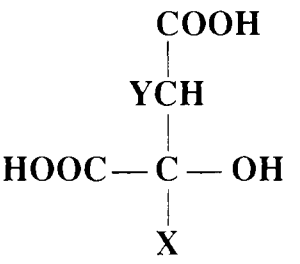
type) were found for citrate–, (*R*)-citramalate–, and *cis*-aconitate–FeMo-co. D-Malate–, 2-hydroxycitrate–, isocitrate–, and *trans*-aconitate–FeMo-co, as well as 2- and 3-oxoglutarate–FeMo-co, resulted in dinitrogenases with 10–20% of the wild-type acetylene reduction activities.

Proton reduction activities for some of the FeMo-co analogues were very high: 70–80% of the homocitrate–FeMo-co (wild type) for isocitrate and homoisocitrate; 40–60% for citrate, 2-hydroxycitrate, and (*R*)-citramalate; approximately 20% for D-malate, L-malate, 2- and 3-oxoglutarate, D-saccharate, and *cis*-aconitate; and significantly above background for *trans*-aconitate, (*S*)-citramalate, and oxalacetate. MacLean and Dixon (1981) showed that proton reduction by $NifV^-$ dinitrogenase is inhibited by CO, in contrast to the situation found with the wild-type enzyme. Hoover et al. (1988) confirmed these results for dinitrogenase containing citrate–FeMo-co. We found strong CO inhibition of proton reduction by dinitrogenases reconstituted with citrate–, (*R*)-citramalate–, and *cis*-aconitate–FeMo-co. CO inhibition of proton reduction was also observed for 3-oxoglutarate–, but not for 2-oxoglutarate–FeMo-co.

Our data suggest that the machinery for FeMo-co synthesis and insertion into dinitrogenase can utilize a variety of organic acids to yield aberrant forms of FeMo-co and dinitrogenase. Since some of the organic acids are common metabolites, this raises the question of how cells avoid synthesis of aberrant forms of FeMo-co. The above results and those of Hoover et al. (1988) show that the FeMo-co biosynthetic system has a 10–100-fold higher affinity for homocitrate than for alternative organic acids. Hoover (1988) found concentrations of homocitrate in the culture media of nitrogen-fixing cells as high as 0.2 mM. This suggests that the cell synthesizes a large excess of (*R*)-homocitrate to avoid synthesis of aberrant iron–molybdenum cofactors. Only when homocitrate is not available (such as in $NifV^-$ mutants of *K. pneumoniae*) can other FeMo-co forms be synthesized. Citrate was found as a component of $NifV^-$ dinitrogenase (Liang et al., unpublished data), and it appears that under normal nitrogenase derepressing conditions for *K. pneumoniae* only citrate is available in high enough concentration to serve effectively as a substrate for synthesis of modified FeMo-co or other FeMo-co analogues are not stable.

A comparison of different analogues revealed a strong correlation between the stringency of structural requirements for synthesis of an active FeMo-co and the complexity of the reaction catalyzed. More analogues yielded cofacotrs competent for proton reduction at high specific activities. Acetylene reduction (a two-electron process) followed. For N_2 reduction only homocitrate–FeMo-co showed high activity.

The data presented here suggest a model for the general structure required of the organic moiety of iron–molybdenum cofactors competent for substrate reduction (Figure 1). The *R* isomer seems to be required; (*R*)-citramalate and D-malate showed high activities, whereas (*S*)-citramalate and L-malate showed little or no activity. Only the *R* isomer of homocitrate is synthesized in biological systems (Hoover et al., 1987). The hydroxyl group appears to be essential, both for incorporation of molybdenum into dinitrogenase and for activity; 1,2,4-butanetricarboxylate or tricarballylate did not induce incorporation of ^{99}Mo into dinitrogenase (Hoover et al., 1988a; J. Imperial, unpublished data), and they did not generate active FeMo-co (Table I). Additional, indirect evidence is provided by the fact that the lactone form of (*R*)-homocitrate also showed no activity in the FeMo-co synthesis assay (Hoover et al., 1987). No homocitrate analogue was available without



(*R*)-isomer

FIGURE 1: Model for the minimum organic moiety required for synthesis of the iron–molybdenum cofactor. X = H, D-malate; X = CH_3, (*R*)-citramalate; X = CH_2COOH, citrate; X = CH_2CH_2COOH, (*R*)-homocitrate. Y = H, homocitrate; Y = OH 2-hydroxycitrate.

the 2-carboxyl group. Substitution of the equivalent group in citrate, as in 3-hydroxy-3-methylglutarate, resulted in complete loss of activity in the FeMo-co synthesis system. This supports the idea that the 2-carboxyl group is required for FeMo-co synthesis or acivity. The same seems true for the 1-carboxyl group, as (*R*)-2-hydroxy-2-methylbutyrate (which resembles citramalate except that the 1-carboxyl group is replaced with a methyl group) had no activity. Substitution of the 1-carboxyl and 2-methylene groups by a proton (L-2-hydroxyglutarate) also resulted in loss of the activity, further suggesting the necessity of the 1-carboxyl group for activity. Neither the 4-carboxyl group nor the C-3,4 tail of the homocitrate molecule was absolutely required for synthesis of a FeMo-co analogue, because citrate (X = CH_2COOH), (*R*)-citramalate (X = CH_3), and D-malate (X = H) were active in the acetylene and proton reduction assay (Figure 1, Table I). However, the catalytic activity was dramatically affected, especially for dinitrogen reduction. Among these three analogues, D-malate showed much lower activities for all three substrates. It is interesting to note that strong CO inhibition of proton reduction was observed with citrate, (*R*)-citramalate, and *cis*-aconitate FeMo-co. This suggests that the C-chain length or the 4-carboxyl group may be important for resistance to CO inhibition.

Moving the hydroxyl group from C-2 to C-1 had a dramatic effect on activity. Homoisocitrate–FeMo-co was completely inactive for N_2 and acetylene reduction but nearly as active as homocitrate–FeMo-co for proton reduction. It appears that homoisocitrate is as effeciently utilized in FeMo-co synthesis, since the levels of homoisocitrate required for incorporation of ^{99}Mo into dinitrogenase are as low as those of homocitrate (Hoover et al., 1988a; J. Imperial, unpublished data). Interpretation of the results with isocitrate is complicated by the presence in the extracts of aconitate hydratase, which can generate citrate. The effect of isocitrate was similar to that of homoisocitrate. N_2 reduction and acetylene reduction were decreased with respect to citrate, but proton reduction activity was higher than that for citrate–FeMo-co. 2-Hydroxycitrate showed activities intermediate between those of citrate and isocitrate. Proton reduction mediated by 2-hydroxycitrate– and isocitrate–FeMo-co was not inhibited by CO. This suggests that the distance between the hydroxyl group and the terminal carboxyl group may be important in determining CO inhibition of proton reduction with these analogues.

Homocitrate forms complexes with Fe^{3+} and MoO_4^{2-} (Hoover et al., 1987). The metal-chelating properties of citrate in biological systems have been extensively studied (Neilands, 1981). The results with homocitrate analogues suggest that the inability of these organic acids to form appropriate bi- or tridentate complexes with Fe or Mo in FeMo-co may be re-

sponsible for their ineffectiveness in N_2 reduction.

Two sets of data do not fit into the model. The *cis*- and *trans*-aconitate analogues showed higher activities than expected from their structures. Because *trans*-aconitate preparations are usually contaminated by *cis*-aconitate and since the extracts have an active aconitate hydratase (Alexander & Wilson, 1956), it is possible that products of their transformation are responsible for the observed activities. Two oxoglutaric acids, 2- and 3-oxoglutarate, were active in the assay, generating cofactor analogues that showed low but reproducible substrate reduction activities for dinitrogen, acetylene, and protons. Oxoglutarates with a longer C-chain (2-oxoadipate, 3-oxoadipate, 2-oxocaproate) were inactive, whereas oxalacetate generated some acetylene and proton reduction activity. The strongest deviations from the model were observed when proton reduction was being measured; low activities were found with the *S* analogues, L-malate and (*S*)-citramalate. This suggests that the structural requirements of the organic moiety for assembly of an organometallic cluster and its insertion into dinitrogenase are not stringent. However, it cannot be ruled out that unknown contaminants present in the commercial preparations of organic acids used may be responsible for some of the very low levels of activity observed. No incorporation of ^{99}Mo into dinitrogenase was observed in the absence of added organic acids or in the presence of either tricarballylate or 1,2,4-butanetricarboxylate (Hoover et al., 1988a; J. Imperial, unpublished data). Addition of homocitrate at the end of in vitro FeMo-co synthesis reactions lacking homocitrate resulted in very low activities. This suggests that homocitrate is not incorporated into a performed inorganic portion of FeMo-co, but is required during FeMo-co synthesis.

The results presented here strongly suggest that homocitrate is either very close to the substrate reduction site in dinitrogenase or that is dictates the structure of the iron–molybdenum cofactor of dinitrogenase. Small changes in the molecule result in major changes in substrate specificity and even acquisition of new properties (CO inhibition of proton reduction). This can be interpreted in one of two ways: homocitrate participates in substrate binding or electron or proton transfer during substrate reduction, or it acts as a scaffolding molecule for correct orientation of the metal centers. The evidence thus far does not allow discrimination among these possibilities. Our results show that it is feasible to design homocitrate analogues and place them at the active site of dinitrogenase. ^{19}F NMR and ^{17}O and ^{13}C ENDOR experiments under way with homocitrate derivatives should provide more detailed information on the mechanism of catalysis.

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