Crystallographic Analysis of the MoFe Protein of Nitrogenase from a nifV Mutant of Klebsiella pneumoniae Identifies Citrate as a Ligand to the Molybdenum of Iron Molybdenum Cofactor (FeMoco)*

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The x-ray crystal structure of NifV–Klebsiella pneumoniae nitrogenase MoFe protein (NifV–Kp1) has been determined and refined to a resolution of 1.9 Å. This is the first structure for a nitrogenase MoFe protein with an altered cofactor. Moreover, it is the first direct evidence that the organic acid citrate is not just present, but replaces homocitrate as a ligand to the molybdenum atom of the iron molybdenum cofactor (FeMoco). Subsequent refinement of the structure revealed that the citrate was present at reduced occupancy.

Nitrogenase catalyzes the reduction of dinitrogen to ammonia according to the following reaction.

\[
N_2 + 8H^+ + 16MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i
\]

The wild-type molybdenum-containing enzyme consists of two components; the smaller, component 2 or the Fe protein, is a 60-kDa homodimer containing a [4Fe4S] cluster and is the obligate electron donor to the larger component. This latter protein, component 1 or the MoFe protein (designated Kp1 for the Klebsiella pneumoniae protein) is a 225-kDa αβ2 tetramer that contains two types of unique iron-sulfur metal centers. Each αβ half of the “wild-type” protein possesses an [8Fe7S] P-cluster and a [1Mo7Fe9S:homocitrate] FeMoco1 center, the latter being the site at which substrate reduction occurs (1).

The nifV mutant of K. pneumoniae produces an enzyme that has a wild-type Fe protein but an altered MoFe protein (NifV–Kp1) with a modified FeMoco. This mutant nitrogenase can reduce acetylene, but its N2 reduction activity is only ~7% that of the wild type. Furthermore, unlike the wild type, its H2 evolution activity is inhibited in the presence of CO (2, 3). The wild-type Kp1 protein and NifV–Kp1 protein apparently possess the same metal constituents (1); however, it is thought that the organic acid constituent of the FeMoco differs.

The wild-type Kp1 has homocitrate ligated to the molybdenum atom of the FeMoco (4). Homocitrate is synthesized by the nifV-encoded homocitrate synthase (NifV) condensation of acetyl-CoA and α-ketoglutarate (5). R-homocitrate is the only isomer produced in vivo (6), and in an in vitro FeMoco assembly system (7), only the R-isomer is incorporated from racemic mixtures of L- and R-isomers. The incorporation of 17 different di- and tricarboxylic acids into the cofactor has been tested, but only citrate, which differs from homocitrate by a single methylene group (see Fig. 1), yielded a FeMoco analogue capable of producing MoFe protein with C2H2 reduction activity comparable to that of the wild type (7). Furthermore, NMR spectroscopy of the extracted organic moiety from NifV–Kp1 identified citrate but no homocitrate (8). The authors reasonably concluded that the citrate was bound in place of homocitrate, although these experiments did not prove its association with the FeMoco. Here we report the crystal structure of the NifV–Kp1 protein at 1.9 Å resolution demonstrating that citrate occupies the site of homocitrate in the altered protein.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—NifV–Kp1 was purified from K. pneumoniae strain UNF1613 (derived from the wild-type strain M5a1) (3) using a protocol based on that used for wild-type Kp1 (4) but at pH 7.4 and 8 °C. The enzyme activity of the preparation was estimated from its ability to reduce acetylene (9), which gave a specific activity of ~930 nmol acetylene-reduced min−1 mg−1 protein. The metal content of the final protein preparation was determined by inductively coupled plasma analysis on samples wet-ashed as described elsewhere (9). This revealed a full occupancy of the metal sites (2.1 molybdenum and 30.1 iron per protein tetramer). The purified protein was dialyzed overnight against 10 mM NaCl in 25 mM Tris-HCl, pH 7.4, concentrated to 10.1 mg/ml and stored as 30–50-μl aliquots in liquid nitrogen.

Crystallizations were set up under anaerobic conditions using the capillary liquid-liquid diffusion technique, essentially as for the wild-type protein (4). However, a lower final protein concentration of 1.3 mg/ml and a pH value of 7.4 was used in this case. Crystals appeared after 6–8 weeks and were manipulated as described (4) before flash-cooling in liquid nitrogen using a cryoprotectant comprised of mother liquor containing 25% ethylene glycol and 20 mM sodium dithionite.

Data Collection and Structure Solution—The NifV–Kp1 crystals diffracted x-rays to high resolution and belonged to the monoclinic space group C2 with cell parameters of a = 204.1 Å, b = 75.1 Å, c = 164.3 Å, and β = 124.0°, being essentially isomorphic with the wild-type Kp1 (4). A 1.9 Å resolution data set was collected using an ADSC Quantum 4 CCD detector on beamline ID14–4 at the European Synchrotron Radiation Facility in Grenoble, France (λ = 0.936 Å).

X-ray data were processed with DENZO and scaled and merged using SCALEPACK (10). All subsequent downstream processing and statistical analysis was effected using programs from the CCP4 suite (11) unless otherwise stated. Data collection and processing statistics are summarized in Table I.

Model Building and Refinement—Starting phases for the NifV–Kp1 structure were obtained from the model of dithionite-reduced wild-type Kp1 (PDB accession number 1QGU). All non-protein atoms were removed from the coordinate file, and then rigid body and simulated annealing refinement were carried out using the program X-PLOR (12). Model building was performed by interactive computer graphics using the program O (13) by inspection of SIGMAA-weighted (14) 2mFobs – Fcalc maps.
RESULTS AND DISCUSSION

The protein components of the NiF\textsuperscript{-}Kp1 model were closely superposable with the wild-type Kp1 structure, giving a root mean square deviation of only 0.27 Å over all protein atoms. A difference electron density map at 1.9 Å resolution calculated using phases derived from a model containing FeMoco without an organic acid substituent, clearly indicated residual density at the molybdenum end of the cofactor. However, homocitrate gave a relatively poor fit to this density, whereas citrate gave a better fit but did not account for all the residual density. An additional lobe of density was present extending away from the shortened (with respect to homocitrate) carboxylate arm of the citrate (Fig. 2), which was consistent with the presence of an additional atom. Furthermore, the average temperature factor for the citrate refined to a significantly higher value than that for the molybdenum of the FeMoco, 32 Å\textsuperscript{2} versus 17 Å\textsuperscript{2}, respectively, strongly suggesting that the citrate was present at reduced occupancy. By comparison, after refinement of the wild-type dithionite-reduced structure at 1.6 Å resolution, the fully occupied homocitrate and the molybdenum had comparable temperature factors of 12 Å\textsuperscript{2} and 11 Å\textsuperscript{2}, respectively (4).

Attempts to model alternative organic acids into the residual electron density were less convincing than the citrate, whereas the placement of a water molecule in the extra lobe of density yielded an improbable hydrogen bond length of 1.6 Å with the citrate. Similarly, a monovalent cation, such as sodium, would have been too close to the carboxylate group. Nevertheless, the putative water molecule was capable of hydrogen bonding to the backbone NH group of Ile-423 (α245 in Av1\textsuperscript{2} (Azotobacter vinelandii nitrogenase component 1)), an interaction mimicking that seen in the wild-type protein between the long carboxylate arm of homocitrate and this residue (Fig. 3). Because we have a full complement of metals (see “Experimental Procedures”), we therefore propose that the crystal comprised a mixture of protein containing FeMoco with citrate bound, and protein containing FeMoco without an organic ligand (referred to as MoFe cluster, see below). In the absence of the citrate, a network of water molecules most likely occupies this region and may mimic some of the interactions made by the citrate with both the protein and the molybdenum. Therefore, the citrate and the original water molecule were assigned half-occupancy, and two additional half-occupancy water molecules were introduced, overlapping the carboxyl oxygen (O-5) and the hydroxyl oxygen (O-7) of the citrate that coordinate the molybdenum. After the final refinement, the citrate had an overall temperature factor of 17 Å\textsuperscript{2}, and the three half-occupancy waters had an average temperature factor of 16 Å\textsuperscript{2}, as compared with the overall value of 17 Å\textsuperscript{2} for the rest of the cofactor. As a further test, the refinement was repeated using a half-occupancy homocitrate in place of the citrate, which resulted in a slightly higher overall temperature factor of 20 Å\textsuperscript{2} for the organic acid moiety. Nevertheless, averaging the temperature factors of the atoms at the end of the long pendant arm (–CH2–COO\textsuperscript{–}) of the refined homocitrate gave a value of 26 Å\textsuperscript{2} as compared with the

\*\*Kp1 residue numbering is used throughout. For a comparison of Kp1 and Av1 sequences see Ref. 4.
FeMoco biosynthetic system has a 10-FeMoco protein. It exhibited the same characteristic properties as the wild-type protein.

Furthermore, the spectroscopic properties of MoFe cluster preparations were virtually indistinguishable from the wild-type protein.

The spectroscopic properties of MoFe cluster preparations were virtually indistinguishable from the wild-type protein. The longer carboxylate arm of the homocitrate in the wild-type structure can clearly be seen protruding from the density, whereas the citrate and water molecule of the NiFe cluster are more consistent with the density. For clarity, the bonds from the organic acids to the molybdenum atoms and the additional half-occupancy water molecule of the NiFe− structure have been omitted. This figure was produced using the programs O and OPLOR (13).

These observations would suggest that if our preparation of NiFe− had 50% of the MoFe protein containing citrate and 50% with no organic acid, then spectroscopically it would be impossible to distinguish the two species. This interpretation is consistent with the recent mass spectrometric analysis of NiFe− Av1, where the citrate concentration was only 30% that of the molybdenum (23).

If acetylene reduction by MoFe protein requires a FeMoco containing an organic acid but the activity is essentially the same with either homocitrate or citrate, then a 50% occupancy of citrate could account for the activity of the NiFe− preparation, which has half the activity of the wild-type protein: 930 nmol min⁻¹ mg⁻¹ (this work) versus 1800 nmol min⁻¹ mg⁻¹, respectively (4).

Explanations for the effects of the nifV mutation on H₂ evolution and N₂ reduction activities are more difficult to deduce. There is good spectroscopic evidence that MoFe protein binds CO at at least two sites on wild-type FeMoco (24–26).

This result is inhibition of the reduction of most substrates, but not H₂ evolution. However, CO does inhibit H₂ evolution by the NiFe− nitrogenase (2). This behavior is also observed with a Gln-190 Lys mutant (27, 28). Gln-190 (a191 in Av1) is hydrogen-bonded to one of the carboxyl groups of homocitrate (Fig. 3). These data imply a relationship between modification of homocitrate and CO inhibition of H₂ evolution. It is conceivable that such modifications perturb proton supply to FeMoco or possibly cause slight changes in the molybdenum coordination sphere, which allow CO to bind.

The site of binding of other substrates to FeMoco has been investigated using targeted mutagenesis of the residues surrounding it in the protein. There is strong evidence for two sites for acetylene binding, one of high and the other of lower affinity. The evidence (29) indicates that the high affinity site is on a central FeMoco face of FeMoco close to Val-69 (a70 in Av1). When this site is perturbed by mutating the preceding residue, Gly-69 (69 in Av1), to a Ser, acetylene becomes a competitive inhibitor of N₂ reduction. These data suggest that N₂ binds to the lower affinity acetylene site (29), but the location of this site is unclear. Several other mutations, in such disparate positions as Gln-190 (28) (near homocitrate) and Arg-275 (a277 in Av1) (30) (close to Cys-273 or a275 in Av1), which binds the tetrahedral ion at the opposite end of FeMoco), eliminate N₂ reduction activity and thus give no clue to the N₂ binding site or its mechanism of reduction. One explanation of the above phenomenon is that the environment of FeMoco is critically important in accessing the lower redox levels. On the above model, level E4 of the Lowe-Thorneley scheme (31), which
binds N\textsubscript{2}, can be accessed in the wild-type. However, the above substitutions of amino acids, and possibly homocitrate, in the FeMoco environment perturb critical hydrogen bonds such that only level E2 can be reached and thus only H\textsuperscript{+} and acetylene reduced.

There have been numerous attempts to model the binding and reduction of substrates by the FeMoco, as reviewed in Lawson and Smith (32), but only one model specifically addresses the role of the organic acid in catalysis. In this model, based on the Av1 structure (33), citrate and homocitrate are thought to bind to the molybdenum atom in an analogous fashion through alkoxy and carboxylato groups (34). During catalysis, after dissociation of the carboxylate ligand from molybdenum, the flexible pendant arm of the homocitrate is proposed to hydrogen-bond to the Ne\textsuperscript{2} of His-\epsilon440 (\epsilon442 in Av1). This hydrogen bond would impose an imidazolate character on the His, effectively increasing the electron density within the cluster and thus promoting N\textsubscript{2} binding. By contrast, the shorter arm of citrate could be unable to make the same hydrogen bond to the His, with the result that N\textsubscript{2} affinity of the cluster is decreased.

In conclusion, through x-ray crystallography we have identified citrate bound to the molybdenum atom of the FeMoco in NifV\textsuperscript{−} Kp1. This observation is consistent with a body of biochemical and spectroscopic data, although in these studies the association of citrate with the cofactor itself was not proven. In addition, our study shows that the citrate is only present in approximately half of the cofactors, being substituted by water molecules in the remainder. This may account for the halving of acetylene reduction activity for this mutant relative to the wild-type enzyme.

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REFERENCES
1. Hawkes, T. R., McLean, P. A., and Smith, B. E. (1984) Biochem. J. 217, 317–321
2. McLean, P. A., and Dixon, R. A. (1981) Nature 292, 655–656
3. McLean, P. A., Smith, B. E., and Dixon, R. A. (1983) Biochem. J. 211, 589–597
4. Mayer, S. M., Lawson, D. M., Gormal, C. A., Roe, S. M., and Smith, B. E. (1999) J. Mol. Biol. 292, 871–891
5. Zheng, L. M., White, R. H., and Dean, D. R. (1997) J. Bacteriol. 179, 5963–5966
6. Hoover, T. R., Robertson, A. D., Cerny, R. L., Hayes, R. N., Imperial, J., Shah, V. K., and Ludden, P. W. (1987) Nature 329, 855–857
7. Imperial, J., Hoover, T. R., Madden, M. S., Ludden, P. W., and Shah, V. K. (1989) Biochemistry 28, 7796–7799
8. Liang, J., Madden, M., Shah, V. K., and Burris, R. H. (1990) Biochemistry 29, 8577–8581
9. Eady, R. R., Smith, B. E., Cook, K. A., and Postgate, J. R. (1972) Biochem. J. 128, 655–675
10. Otto, W., and Minor, W. (1997) Methods Enzymol. 276, 307–326
11. Collaborative Computational Project, No. 4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
12. Brünger, A. T. (1992) A System for X-ray Crystallography and NMR, X-PLOR version 3.1, Yale University Press, New Haven, CT
13. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. D Biol. Crystallogr. 47, 110–119
14. Read, R. J. (1986) Acta Crystallogr. Sect. A 42, 140–149
15. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
16. Brünger, A. T. (1993) Acta Crystallogr. Sect. D Biol. Crystallogr. 49, 24–36
17. Lamatin, V. S., and Wilson, K. S. (1993) Acta Crystallogr. Sect. D Biol. Crystallogr. 49, 129–147
18. Strassman, M., and Ceci, L. N. (1964) Biochim. Biophys. Res. Commun. 14, 262–267
19. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
20. Hoover, T. R., Shah, V. K., Roberts, G. P., and Ludden, P. W. (1986) J. Bacteriol. 167, 999–1003
21. Ma, L., Gavini, N., Liu, H. I., Hedman, B., Hodgson, K. O., and Burgess, B. K. (1994) J. Biol. Chem. 269, 18007–18105
22. Ma, L., Brosius, M. A., and Burgess, B. K. (1996) J. Biol. Chem. 271, 10526–10532
23. Newton, W. E., Vichitphan, K., and Fisher, K. (2002) in 13th International Congress on Nitrogen Fixation (Finnan, T., Layzell, D., O’Brien, M. R., Vessey, K., and Newton, W. E., eds) CABI Publishing, Wallingford, Oxford, UK
24. Pollock, R. C., Lee, H. I., Cameron, L. M., Derose, V. J., Hales, B. J., OrmeJohnson, W. H., and Hoffman, B. M. (1995) J. Am. Chem. Soc. 117, 8696–8697
25. Lee, H. I., Cameron, L. M., Hales, B. J., and Hoffman, B. M. (1997) J. Am. Chem. Soc. 119, 1021–1028
26. George, S. J., Ashby, G. A., Wharton, C. W., and Thorneley, R. F. W. (1997) J. Am. Chem. Soc. 119, 6439–6451
27. Scott, D. J., May, H. D., Newton, W. E., Brigle, K. E., and Dean, D. R. (1990) Nature 343, 188–190
28. Scott, D. J., Dean, D. R., and Newton, W. E. (1992) J. Biol. Chem. 267, 20002–20010
29. Christiansen, J., Seefeldt, L. C., and Dean, D. R. (2000) J. Biol. Chem. 275, 36104–36107
30. Shen, J., Dean, D. R., and Newton, W. E. (1997) Biochemistry 36, 4884–4894
31. Thorneley, R. N. F., and Lowe, D. J. (1985) in Molybdenum Enzymes (Spiro, T. G., ed), pp. 221–284, John Wiley & Sons, Inc., New York
32. Lawson, D. M., and Smith, B. E. (2002) in Molybdenum and Tungsten: Their Roles in Biological Processes (Sigel, A., and Sigel, H., eds) Vol. 39, pp. 75–119, M. Dekker, Inc., New York
33. Peters, J. W., Stowell, M. H. B., Salis, S. M., Finnegan, M. G., Johnson, M. K., and Rees, D. C. (1997) Biochemistry 36, 1181–1187
34. Gronberg, K. L. C., Gormal, C. A., Durrant, M. C., Smith, B. E., and Henderson, R. A. (1998) J. Am. Chem. Soc. 120, 10613–10621
35. Cruickshank, D. W. J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 583–601
36. Laskowski, R. A., Macarthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Cryst. 26, 283–291