Assignment of protonated R-homocitrate in extracted FeMo-cofactor of nitrogenase via vibrational circular dichroism spectroscopy

Lan Deng¹,⁵, Hongxin Wang²,⁵, Christie H. Dapper³, William E. Newton³, Sergey Shilov⁴, Shunlin Wang⁴, Stephen P. Cramer² & Zhao-Hui Zhou¹

Protonation of FeMo-cofactor (FeMo-co) is important for the process of substrate hydrogenation. Its structure has been clarified as Δ-Mo*Fe₇S₉C(R-homocit*)(cys)(Hhis) after the efforts of nearly 30 years, but it remains controversial whether FeMo-co is protonated or deprotonated with chelated ≡C − O(H) homocitrate. We have used protonated molybdenum(V) lactate¹ and its enantiomer as model compounds for R-homocitrate in FeMo-co of nitrogenase. Vibrational circular dichroism (VCD) spectrum of ¹ at 1051 cm⁻¹ is attributed to ≡C − O₄⁺ vibration, and molybdenum(VI) R-lactate at 1086 cm⁻¹ is assigned as ≡C − Oα-alkoxy vibration. These vibrations set up labels for the protonation state of coordinated α-hydroxycarboxylates. The characteristic VCD band of NMF-extracted FeMo-co is assigned to ν(C − O₄⁺), which is based on the comparison of molybdenum(VI) R-homocitrate. Density functional theory calculations are consistent with these assignments. To the best of our knowledge, this is the first time that protonated R-homocitrate in FeMo-co is confirmed by VCD spectra.
Nitrogenase, as a catalytic enzyme that reduces atmospheric dinitrogen to bioavailable ammonia in nature, has attracted widespread attentions from researchers. Molybdenum nitrogenase consists of FeMo- and Fe-proteins, where FeMo-cofactor (FeMo-co) in FeMo-protein is the active site of substrate binding and reduction. Through long-term exploration, the structure of FeMo-co has been clarified as $\text{Mo}^\ast\text{Fe}_{5}\text{S}_{6}\text{C}(\text{R-homocit})^\ast(\text{cys})(\text{Hhis})^\ast$–$\text{H}_2\text{homocit} = \text{homocitric acid}, \text{Hcys} = \text{cysteine}, \text{Hhis} = \text{histidine}$, and the absolute configuration of molybdenum is assigned as $\Delta^1$. Homocitrate has been suggested to chelate with metal molybdenum via the oxygen atoms of $\alpha$-alkoxy and $\alpha$-carboxy groups. Nevertheless, besides the metal oxidation states of FeMo-co are ambiguous, the precise local structure of chelated homocitrate is controversial. With a computational study for a proposed protonated state of FeMo-co, structural comparisons of oxidomolybdenum(IV) with the local structures of FeMo-cofactors in Protein Data Bank (PDB) gave an indirect evidence for the protonation of $\alpha$-alkoxy group in $\Delta$-homocitrate. Comparisons of infrared spectroscopy (IR) between FeMo-co and a series of model compounds provided a direct evidence for the two possibilities of $\alpha$-alkoxy and/or $\alpha$-hydroxy groups in $\Delta$-homocitrate. Moreover, quantum mechanics/molecular mechanics calculations recommended protonation of $\alpha$-alkoxy group in FeMo-co. But $\equiv\text{C}–\text{O}(\text{H})$ coordination with molybdenum of chelated $\Delta$-homocitrate is still suspicious in view of protons are almost always invisible in limited resolutions of crystal structures, and severe lack of experimental evidence in addition to computational researches. The proton step confers a certain degree of lability to the homocitrate ligation to the cofactor. Most reported homocitrate and its homologues bind to molybdenum via $\alpha$-alkoxy, $\alpha$-carboxy, and/or $\beta$-carboxy groups. Several molybdenum $\alpha$-hydroxycarboxylates were isolated coordinating via $\alpha$-hydroxy (protonated) and $\alpha$-carboxy groups. Less concern is on molybdenum complexes with $\alpha$-hydroxycarboxylate and imidazole-like ligand simultaneously. Here mixed-ligand molybdenum(V) compounds with lactate/glycolate and 1,2,4-triazole $\equiv\Delta\text{Mo}^\ast\text{O}_2\text{(H}\text{lact})^\ast\text{O}(\text{R-Hlact})^\ast\text{O}^\ast$–$\frac{1}{2}\text{H}_2\text{O}$ (1, $\text{H-lact} = \text{lactic acid}, \text{trz} = 1,2,4$-triazole), $\equiv\Delta\text{Mo}^\ast\text{O}_2\text{(H}\text{glyc})^\ast\text{O}^\ast$–$\frac{1}{2}\text{H}_2\text{O}$ (2, $\text{H-glyc} = \text{glycolic acid}$. Chiral centers and structural comparisons. The local coordination modes in 1–3 are similar to those of homocitrate and histidine residue in FeMo-co as shown in Fig. 1, where lactate or glycolate imitates homocitrate, and 1,2,4-triazole imitates imidazole residue of histidine respectively. Although there is a large gap between the model compounds and the cofactor of Mo-nitrogenase, the protonated $\alpha$-hydroxy groups in chelated $\alpha$-hydroxycarboxylates should be suitable for the local study of FeMo-co, which possess two chiral centers. Namely chiral metal center Mo in $\Delta$-configuration and chelated homocitrate in $R$.
configuration as shown in Fig. 1b, or the suggested NMF-extracted structure of FeMo-co in Fig. 1c. Due to the influence of the chirality of amino acid residues in MoFe protein, VCD is only used for extracted FeMo-co.

Analyzes of vibrational circular dichroism spectroscopy. Spectra of vibrational circular dichroism are a powerful tool to investigate chiral molecules in the infrared region, which use different absorptions of left- and right-polarized infrared lights. The obtained VCD signals of 1 and 2 were mirror images in the regions of 1800–800 cm⁻¹ as shown in Fig. 2. This corresponds to the absolute configurations of Δ/Δ-R and Δ/Δ-S molybdenum lactate with chiral Mo(V) and a-carbon of lactate, respectively. IR spectra obtained are listed below for comparisons. From the VCD spectra, we can see that 1 and 2 show obvious positive and negative Cotton effects. That is, the enantiomers of chiral molybdenum lactates have their chiral characteristics. The chirality of R or S-lactates couples strongly with the chiral molybdenum centers in dinuclear oxidomolybdenum complexes, respectively. Typical coupled peaks around 1652 and 1377 cm⁻¹ in peak numbers a and b are assigned to the asymmetric and symmetric stretching vibrations of CO₂⁻, respectively. The signs of CO₂⁻ were opposite to each other for both Δ/Δ-R-lactate: +/− and Δ/Δ-S-lactate: −/+. The vibrational bands above 1800 cm⁻¹ are assigned to C–H, N–H, and O–H stretching modes. The peaks (numbered as c) in the region of 967–977 cm⁻¹ indicate the existence of Mo=O bonds, which are consistent with the values observed in IR spectra. The bands in the range of 1120–1170 cm⁻¹ for peak numbers d are associated with C–N vibrations of triazole compared with IR spectra of free 1,2,4-triazole in Supplementary Fig. 23.30,31

The C–O stretching vibrations in alcohols produce bands in the region of 1100–1000 cm⁻¹. In our previous report, IR spectra of some alcohols, α-hydroxycarboxylic acids have been compared with Spectral Database for Organic Compounds (SDBS), where peak 1047 cm⁻¹ is assigned to C–O(H) vibration for lactic acid in Supplementary Fig. 24.30 Here strong VCD peaks at 1051 in 1 and 1053 cm⁻¹ in 2 are assigned to the protonated C–OH stretching vibrations numbered as e in Fig. 2, respectively. These are in agreement with those obtained from IR spectra simultaneously. Compared to peaks c, peaks a ~ d are not very strong. This is because these characteristic vibrations are located far away from the chiral carbon, and triazole is not chiral. Likewise, VCD and IR spectra of deprotonated [Na₂[MoO₄]₂(R-lact⁻)₂]₂, 13H₂O, [Na₂[MoO₄]₂(S-lact⁻)₂]₂, 13H₂O38 are shown in Supplementary Fig. 10. By comparing with VCD spectra of deprotonated [Na₂[MoO₄]₂(R-lact⁻)₂]₂, 13H₂O with Δ/Δ-R configuration in protonated dimer 1 in Fig. 3, we can draw the conclusion that protonated C–OH stretching vibrational frequencies are generally lower than those of deprotonated C–O stretching vibrations, where strong vibrations at 1086 cm⁻¹ for [Na₂[MoO₄]₂(R-lact⁻)₂], 13H₂O is assigned to the deprotonated C–O₆-carboxy group. More diagnostic peaks are shown in Supplementary Fig. 11. In the other words, ν(C–OH) will shift from high wave number to low wave number, when R-lactate undergoes protonation coordination. This is also consistent with the auxiliary infrared spectra. The protonation weakens the strength of C–O bond, resulting in the red-shift of vibrational frequency. Combined with C–O(H) bond distances discussed in Supplementary Tables 10 and 11, the conclusion of red-shift is also in compliance with Hooke’s law. The same result also appears in Δ/Δ-molybdenum S-lactate 2 and [Na₂[MoO₄]₂(S-lact⁻)₂]₂, 13H₂O38, which can be seen in Supplementary Fig. 12.

To identify the protonation state of homocitrate in FeMo-co, we have measured the VCD spectrum of extracted FeMo-co Δ-Mo₆Fe₆S₆[C(R(H)homocit)₃] (NMF)₂ (20) purified from nitrogenase in Azotobacter vinelandii, where coordination sites of histidine and cysteine residues have been substituted by N-methylformamides. The VCD spectrum of FeMo-co is compared with that of the synthetic deprotonated molybdenum(VI) R-homocitrate K₂[Δ,Δ,Δ-Mo₉O₄(OH)₂(R-Hhomocit)₃] Cl·5H₂O (21)33 in Fig. 4, where the film spectrum of extracted FeMo-co 20 exhibits absorption peaks at 1657, 1622, 1608, 1527, 1479, 1429, 1383, 1327, 1271, 1189, 1153, 1106, and 957 cm⁻¹, respectively. Qualitative assignments of 20 and 21 are given in Supplementary Table 12. The vibrations of the following groups CO₂⁻, C–C, C–O(H), Mo=O, and NMF are identified. IR spectrum of pure NMF is listed in Supplementary Fig. 25 for comparison. The C–O stretching frequencies of molybdenum R-homocitrate 21 can be served as references for C–OH vibrations in FeMo-co 20, and also those from the previous assignments of infrared spectra20. Here VCD spectra are much more sensitive only for the local environments around the chiral organic component of R-homocitrate and metal center of molybdenum atom than those from infrared spectra. We can assign the peak of 1068 cm⁻¹ as C–OH vibrational frequency for FeMo-co, which are similar to the assignments of C–OH vibrations in 1, 2, VCD peaks around 1657 cm⁻¹, 1622 cm⁻¹ in 20, and 1616 cm⁻¹ in 21 are assigned to the asymmetric vibration νₐ(CO₂⁻) for R-homocitrate, respectively, while 1705 cm⁻¹ for free y-carboxylic acid group in 21. The peaks at 1479 and 1383 cm⁻¹ for 20 should belong to the symmetric carboxy vibrations. Those at 1608 and 1327 cm⁻¹ in FeMo-co 20 are assigned to the asymmetric and
symmetric vibrations $\nu(-\text{NHCO})$ for NMF absorptions, while the peak at 1527 cm$^{-1}$ should belong to N-H vibration of N-methylformamide, which disappeared in 21 referred to IR spectra of free NMF (Supplementary Fig. 25)\textsuperscript{32,52}. The peaks at 1189 cm$^{-1}$ and 1153 cm$^{-1}$ for 20 are assigned to C-C vibrations, and the peak at 953 cm$^{-1}$ in 21 is assigned to Mo=O vibration.

Most notably, the peak at 1068 cm$^{-1}$ in the VCD spectrum of extracted FeMo-co 20 is assigned to C–OH vibration as mentioned above. Similar absorptions at 1051 cm$^{-1}$ in 1, 1053 cm$^{-1}$ in 2 are also observed for C–OH vibrations respectively. While the peak at 1084 cm$^{-1}$ in 21 is assigned to the deprotonated C–O vibration, which is in similar tendency for C–O vibration of 1086 cm$^{-1}$ in [Na$_2$\{M–Mo$^\text{V}$O$_2$(R-lact$^\text{\alpha}$)$_2$\}]$_3$·$\Delta$H$_2$O\textsuperscript{38}. The $\equiv$C–O(H) peaks in compounds Na$_3$(Hhomocit)·H$_2$O\textsuperscript{33}, K$_2$[Mo$^\text{V}$O$_4$(R,S-$\Delta$-homocit)$_2$]2H$_2$O\textsuperscript{35}, and Na$_2$[Mo$_3$SO$_5$(R,S-lact)$_2$(im)$_3$]·10H$_2$O\textsuperscript{19} shown in Supplementary Figs. 26, 28 are used for references. When homocitrate undergoes protonation, C–OH vibration will shift from high wave number to low wave number, which is consistent with the red-shift observed for protonated R-lactate described above. Unlike VCD vibrations of molybdenum R- or S-lactates coordinated with $\alpha$-alkoxy (1086, 1078 cm$^{-1}$) or $\alpha$-hydroxy groups (1051, 1053 cm$^{-1}$), the difference is smaller between $\alpha$-alkoxy (1084 cm$^{-1}$) and $\alpha$-hydroxy groups (1068 cm$^{-1}$) for molybdenum R-homocitrate. This is attributed to their electron-drawing effects from side chains of $\beta$- and $\gamma$- carboxy groups in R-homocitrate. Therefore, protonated homocitrate in extracted FeMo-co has been evaluated through the comparisons of VCD spectra with molybdenum complexes of chiral protonated and deprotonated R, S-lactates and R-homocitrate. That is, coordinated R-homocitrate in FeMo-co of Mo-nitrogenase should protonate at resting state, as those of N$_2$-coordinated FeMo-co with free or coordinated $\alpha$-hydroxy groups\textsuperscript{30}.

**Density functional theory calculations.** Theoretical calculations are useful to identify the vibrational modes of the chiral molecule...
for its VCD assignments. The optimized structures of molybdenum 
L-lactate 1 and NMF-extracted FeMo-co 20 have been 
calculated by density functional theory (DFT) at the B3LYP/def2-SVP level. Figure 5 shows and calculated experimental spectra of 1. The vibrational modes of the experimental VCD 
signals agreed well with those calculated. The calculated ν(C=O) and ν(C=O) peaks of 1 appeared at 1653 and 1371 cm⁻¹. This is close to the observed bands with the signs of peaks a, b in 1. The calculated signals of N─H and Mo─O vibrations modes agree with 
the experimental ones. The ν(C=O) peak that we care about mostly 
appears in a similar position at 1054 cm⁻¹, where 1051 cm⁻¹ is for 
the experimental VCD of 1. This further increases the accuracy of 
aforementioned assignments. Optimized molecular structure of 1 is 
shown in Supplementary Fig. 13. Specific peaks for identification can be 
seen in Supplementary Table 13.

We further studied the experimental and theoretical VCD spectra of extracted FeMo-co 20 synchronously shown in Fig. 6. We can draw a conclusion that the vibrational signals of VCD 
observed from experiment are basically consistent with the calculated values. 1058 cm⁻¹ is identified as ν(C=O-H) peak. Optimized molecular structure of 20 is shown in Supplementary Fig. 14. More data for peak identification can be seen in 
Supplementary Table 14.

The upshots of theoretical calculations tell us that protonation will 
afflict the VCD signal of the entire chiral structure. These further 
confirm the accuracy of the VCD analysis for different protonated model compounds, indicating that coordinated 
R-homocitrato in the extracted FeMo-co is protonated. Therefore, we 
suggested the protonation of α-alkoxy groups in R-
homocitrato of Mo-nitrogenase.

Conclusions. We describe a study that examines the protonation 
state of homocitrato in FeMo-co through a comparative spec-
troscopic analysis of NMF-extracted FeMo-co with chiral 
molybdenum α-hydroxyoxycarboxylates. Protonated dinuclear 
molybdenum(V) lactates [Δ/Δ-Mo–O₂(S)(μ-S)(R-
Hlact–)₂(trz)₂(trz)]·½H₂O (1), [Δ/Δ-Mo–O₂(S)(μ-S)(S-
Hlact–)₂(trz)₂(trz)] (2), and glycolate [Mo₂O₃(S)(μ-S)(O₂) 
(Hglyc)₂(trz)₂(H₂O)] (3) have been used as mimic compounds for 
the local chelated environment of R-homocitrato in FeMo-
cofactor. The lactates/glycolates in 1–3 chelate to molybdenum 
(V) atoms with α-hydroxy and α-carboxy groups as bidentate ligands respectively. The longer distances of Mo–O̵̅hydroxy 2.243 
(Å) in 1, 2.246(5) in 2, and 2.284(7) in 3) are comparable with 
that of FeMo-co 20 (Mo–O̵̅hydroxy 2.263 Å). Most importantly, the protonation state of R-homocitrato in FeMo-co 
has been suggested based on comparisons of VCD spectra of 
NMF-extracted FeMo-co with 1, 2 and molybdenum R-homo-
citrato K₃[Δ/Δ,Δ–A–A–Mo–O₂(S)(R-Hhomocit*)₂]Cl·5H₂O. The VCD 
spectra can be served as a strong evidence to the protonation of 
R-homocitrato in FeMo-co. IR and DFT calculations have increased 
credibility of the distributive results. The presence of protonation 
implies connections regarding the nature of protonated FeMo-co 
redox states as well as for potential substrate reduction 
mechanisms for hydrogenation. VCD spectroscopy for the 
protonation state of R-homocitrato opens up an exciting direction 
in investigating the molecular-mechanism of biological nitrogen fixation.

Methods

Preparations of [Δ/Δ-Mo–O₂(S)(μ-S)(R-Hlact–)₂(trz)₂(trz)]·½H₂O (1), Na₂MoO₄·2H₂O (1.21 g, 5.0 mmol) and excess R-lactic acid (1.0 mL, 13.0 mmol) were dissolved in 9.0 mL water. The pH value of the solution was adjusted to 4.0 with the addition of NaOH. The mixture was heated at 60 °C for 24 h and cooled to 
room temperature. Excess Na₂S₂O₄ (0.871 g, 5.0 mmol) and 1,2,4-triazole (0.691 g, 10.0 mmol) were added and the pH was controlled to 2.0–3.0 with concentrated hydrochloric acid. The mixture was then heated at 80 °C for 24 h and cooled to 
room temperature. Product 1 was isolated as yellow plates immediately in 40.9% yields (0.682 g) based on molybdenum. Elemental analysis (calc. for C₁₅H₂₀Mo₂O₇Na₅N₅: C, 21.6; H, 3.0; N, 18.9%. Found: C, 21.4; H, 3.0; N, 18.7%. mp: 474 °C 
for the local chelated environment of 
α-alkoxy distances (Å) for 
different metal-valence molybdenum lactates, glycolates are 
shown in Supplementary Figs. 18, 22, respectively. Analyses and electron paramagnetic resonance spectra for three 
nuclearmagnetism of biological nitrogen fixation.

Cell growth and purification of nitrogenase proteins. The Av wild-type strain 
was grown in the absence of a fixed-nitrogen source in a 24-L fermenter at 30 °C in 
a modified liquid Burk medium. All cultures contained 20 mM FeCl₃ and 10 mM 
Na₂MoO₄ and were grown to a final cell density of 250 Klett units recorded on a 
Klett–Summerson meter equipped with a number 54 filter. All manipulations of 
nitrogenase proteins were performed anaerobically using either a Schlenk line or an 
aerobic glovebox operating at less than 1 ppm of O₂. After harvesting, cell 
extracts were prepared by diluting the whole cells with an equal amount of 50 mM 
Tris pH 8.0 buffer prior to passing through a French pressure cell and a centrifuge 
at 98,000 × g for 90 min. Nitrogenase component proteins were separated by 
aerobic Q-Sepharose anion exchange column chromatography using a linear 
NaCl concentration gradient. Av₂ was purified to homogeneity by fractionation 
from a second Q-Sepharose column. Av₁ was further purified by Sephacryl S-200 
gel filtration and phenyl-Sepharose hydrophobic-interaction chromatography. 
The purified nitrogenase proteins were concentrated individually using an Amicon 
microfiltration pressure concentration before buffer exchange to 25 mM HEPES pH 
7.5, 100 mM NaCl, 10 mM MgCl₂, and 2 mM Na₂S₂O₄ by dialysis at 4 °C. Purified 
protein was used for a series of tests of Av₁ and Av₂ activity assays. Av₁ was purified as above 
through the gel-filtration step, yielding protein with a specific activity of about 1000 
mmol of H₂ per g·atom per mol of Av₁. After diafiltration to lower the NaCl concentration, the Av₁ was loaded onto a DE-52 
cellulose column that had been washed with 50 mM Tris pH 7.4 buffer containing 
2 mM Na₂S₂O₄. The bound protein was washed with N,N-dimethylformamide

Other spectral and data analyses. IR spectra of 1–3 in different 
regions, solid diffused reflectance UV-Vis spectra are shown in 
Supplementary Figs. 15, 17, respectively. Solid-state ¹H and ¹³C 
NMR spectra, thermogravimetric analysis, differential calorimetry 
and electron paramagnetic resonance spectra for three 
complexes are shown in Supplementary Figs. 18, 22, respectively. 
Theoretical bond valence calculations data can be seen in 
Supplementary Table 15. Comparisons of Mo–O̵hydroxy/α-alkoxy 
Mo–O̵carboxyl, C=O–hydroxy/α-alkoxy distances (Å) for 1–3 with 
different metal-valence molybdenum lactates, glycolates are 
shown in Supplementary Table 10. The characteristic bond dis-
tances (Å) for 49 reported structures of FeMo-cofactors in the 
RCSB Protein Data Bank are listed in Supplementary Table 11. 
From the contradistinctive consequences we can see the protona-
tion state of α-alkoxy groups have more obvious influences in 
bond distances than those from oxidation state of metal 
or ligand types.

Extraction of FeMo-co from Av₁ and activity assays. Av₁ was purified as above 
through the gel-filtration step, yielding protein with a specific activity of about 1000 
mmol of H₂ per g·atom per mol of Av₁. After diafiltration to lower the NaCl concentration, the Av₁ was loaded onto a DE-52 
cellulose column that had been washed with 50 mM Tris pH 7.4 buffer containing 
2 mM Na₂S₂O₄. The bound protein was washed with N,N-dimethylformamide
containing 50 mM 2,2'-bipyridine, 5 mM phosphate buffer pH 8, with 2 mM Na2S2O4 and water (ca. 5% v/v) until the non-cofactor iron was completely eluted. The concentrated FeMo-co was then washed with N-methylformamide (NMF) containing 5 mM phosphate buffer pH 8, with 2 mM Na2S2O4 and water (ca. 5% v/v), and FeMo-co was then eluted with NMF that contained 500 mM tetraethylammonium chloride, 5 mM phosphate buffer pH 8, with 2 mM Na2S2O4 and water (ca. 5% v/v). The eluted FeMo-co was concentrated approximately 20-fold by distilling off the NMF under vacuum at 40 °C. FeMo-co was assayed by reconstitution of the D142 As strain90, which has a deletion for the FeMo-co biosynthetic genes nfxB/NX. The FeMo-co used in this study activated a D142 crude extract and produced 75 nmol of H2 (min·mg protein)−1. The extracted FeMo-co can be used to reactivate apo-nitrogenase, activation of the FeMo-co-deficient MoFe protein by NMF-extracted FeMo-co were determined as document described and will not affect recombination activities57–59. The extraction process has nothing to do with the redox state including protonation state of FeMo-co60. Moreover, hydrocarboxin formation like CH4, C2H6, C3H8 by solvent-extracted cofactors proved CO can be reduced by cofactors in the presence of strong chemical reductants without the assistance of corresponding protein scaffold61,62. But protein environment still is a major contributor in view of the activity of FeMo-co63. Namely, the catalytic activity of NMF-extracted FeMo-co can maintain in CO and N2 reductions with the chelated mode of R-homocitractate, regarding the recent structures bound with CO and N2 substrates64,65.

Physical measurements. Na2MoO4·2H2O, 0, lactic acid, 1,2,4-triazole and Na2S2O4 were purchased from Sigma. All solvents and chemicals were of commercially analytical grade. pH value was determined by PHB-8 digital pH meter. The X-ray crystallographic coordinates for structures reported in this Article and its Supplementary Information and/or from the corresponding authors on reasonable request. The X-ray crystallographic coordinates for structures reported in this Article have been deposited at the Cambridge Crystallographic Data Centre (CCDC), under deposition numbers 1938986–1938988. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/.

Theoretical calculations. All optimizations for the electronic structures were calculated at B3LYP/def2-SVP level for the two systems. VCD spectra for comparison with experimentally observed frequencies, under the direction of Computational Chemistry Comparison and Benchmark Database (CCHDB)66,67. The dispersion correction was conducted by Grimme D3 version with BJ damping68. All quantum calculations were done with Gaussian16a software.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data that support the findings in this study are available within the article and its Supplementary Information and/or from the corresponding authors on reasonable request. The x-ray crystallographic coordinates for structures reported in this Article have been deposited at the Cambridge Crystallographic Data Centre (CCDC), under deposition numbers 1938986–1938988. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/data_request/cif.

Code availability. The Gaussian 09 package, Revision A.03 was employed for all the DFT calculations69. The software and instructions for its use are available at http://gaussian.com/.

Received: 29 April 2020; Accepted: 29 September 2020; Published online: 28 October 2020

References
1. Burgess, B. K. & Lowe, D. J. Mechanism of molybdenum nitrogenase. Chem. Rev. 96, 2983–3012 (1996).
2. Smith, B. E. Catalysts for Nitrogen Fixation (Kluwer Academic Publishers, 2004).
3. Ribbe, M. W. Nitrogen Fixation (Humana Press, 2011).
4. Burén, S., Jiménez-Vicente, E., Eichaverrí-Erasun, C. & Rubio, L. M. Bioisynthesis of nitrogenase cofactors. Chem. Rev. 120, 4921–4968 (2020).
5. van Stappen, C. et al. The spectroscopy of nitrogenases. Chem. Rev. 120, 5005–5081 (2020).
6. Kim, J. & Rees, D. C. Structural models for the metal centers in the nitrogenase molybdenum-iron protein. Science 257, 1677–1682 (1992).
7. Einsle, O. et al. Nitrogenase MoFe-protein at 1.1 Å resolution: a central ligand in the FeMo-cofactor. Science 345, 1620–1623 (2014).
8. Spatzal, T. et al. Evidence for interstitial carbon in nitrogenase MoFe coector. Science 334, 940 (2011).
9. Lancaster, K. M. et al. X-ray emission spectroscopy evidences a central carbon in the nitrogenase-iron-molybdenum cofactor. Science 334, 974–977 (2011).
10. Zhou, Z., Yan, W. B., Wan, H. L. & Tsai, K. R. Synthesis and characterization of homochiral polycyclic S-malato molybdate(VI): toward the potentially stereospecific formation and absolute configuration of iron-molybdenum cofactor in nitrogenase. J. Inorg. Biochem. 90, 137–143 (2002).
11. Schmid, B. et al. Structure of a cofactor-deficient nitrogenase MoFe protein. Science 296, 352–356 (2002).
12. Yoo, S. J. et al. Møssbauer study of the MoFe protein of nitrogenase from Azotobacter vinelandii using selective 57Fe enrichment of the M-centers. J. Am. Chem. Soc. 122, 4926–4936 (2000).
13. Bjornsson, R. et al. Identification of a spin-coupled Mo(III) in the nitrogenase molybdenum-iron cofactor. Chem. Sci. 5, 3096–3100 (2014).
14. Jin, W. T., Yang, M., Zhu, S. S. & Zhou, Z. H. Bond-valence analyses of the crystal structures of FeMoV cofactors in FeMoV proteins. Acta Crystallogr. Sect. D 76, 428–437 (2020).
15. Cao, Z. X., Jin, X., Zhou, Z. H. & Zhang, Q. E. Protonation of metal-bound α-hydroxy carboxylate ligand and implication for the role of homocitractate in nitrogenase: computational study of the oxygen-bidentate chelate ring opening. Int. J. Quantum Chem. 106, 2161–2166 (2006).
16. Wang, S. Y. et al. Preliminary assignment of protonated and deprotonated homocitractates in extracted FeMo-cofactors by comparisons with molybdenum (IV) lactates and oxidovanadyl glycylates. Inorg. Chem. 58, 2523–2532 (2019).
17. Harris, T. V. & Szilagyi, R. K. Comparative assessment of the composition and charge state of nitrogenase FeMo-cofactor. Inorg. Chem. 50, 4811–4824 (2011).
18. Benediktsson, B. & Bjornsson, R. QM/MM study of the nitrogenase MoFe protein resting state: broken-symmetry states, protonation states, and QM region convergence in the FeMoco active site. Inorg. Chem. 56, 13417–13429 (2017).
19. Cao, L. L., Caldararu, O. & Ryde, U. Protonation states of homocitractate and nearby residues in nitrogenase studied by computational methods and quantum refinement. J. Phys. Chem. B 121, 8242–8262 (2017).
20. Siegbahn, P. E. M. A major structural change of the homocitractate ligand of probable importance for the nitrogenase mechanism. Inorg. Chem. 57, 1090–1095 (2018).
21. Szilagyi, R. K., Musaev, D. G. & Morokuma, K. Theoretical studies of biological nitrogen fixation. Part II. Hydrogen bonded networks as possible reactant and product channels. J. Mol. Struct. 506, 131–146 (2000).
22. Szilagyi, R. K., Musaev, D. G. & Morokuma, K. Theoretical studies of biological nitrogen fixation. Part I. Density functional modeling of the Mo-site of the FeMo-cofactor. Inorg. Chem. 40, 766–775 (2001).
23. Thorhallsson, A. T., Benediktsson, B. & Bjornsson, R. A model for dinitrogen binding in the Ε state of nitrogenase. Chem. Sci. 10, 11110–11124 (2019).
24. Siegbahn, P. E. M. The mechanism for nitrogenase including all steps. Phys. Chem. Chem. Phys. 21, 15747–15759 (2019).
25. Dance, I. The pathway for arterial proton supply to the active site of nitrogenase: enhanced density functional modeling of the Grothuss mechanism. Dalton Trans. 44, 18167–18186 (2015).
26. Kang, W. et al. Structural evidence for a dynamic metallocofactor during N2 reduction by Mo-nitrogenase. Science 368, 1381–1385 (2020).
27. Wang, S. Y. & Zhou, Z. H. Molybdenum imidazole citrate and bipyridine homocitractates in different oxidation states—balance between coordinated α-hydroxy and α-alkoxy groups. RSC Adv. 9, 519–528 (2019).
28. Zhou, Z. H. et al. Structure and spectroscopy of a bidentate bis-homocitrate dioxo-molybdate(VI) complex: insights relevant to the structure and properties of the FeMo-cofactor in nitrogenase. J. Inorg. Biochem. 118, 100–106 (2013).
33. Zhou, Z. H. et al. Syntheses, spectroscopies and structures of molybdenum(VI) complexes with homocitrate. Inorg. Chem. 45, 8447–8451 (2006).

34. Zhang, R. H. et al. Crystalline and solution chemistry of tetrameric and dinamic molybdenum(VI) citrate complexes. Inorg. Chem. Acta 406, 27–36 (2013).

35. Zhou, Z. H. et al. N-heterocyclic chelated oxomolybdenum(VI) and V complexes with bidentate citrate. Dalton Trans. 252, 2475–2479 (2008).

36. Zhou, Z. H. et al. Dimeric oxomolybdenum(VI) and oxomolybdenum(V) complexes with citrate at very low pH and neutral conditions. Inorg. Chem. 44, 6912–6914 (2005).

37. Li, D. M. et al. Synthesis and characterization of binuclear molybdynium--polycarboxylate complexes with sulfur bridges. J. Inorg. Biochem. 99, 1602–1610 (2005).

38. Zhou, Z. H. et al. Synthesis and spectral properties of molybdenum(VI) citrate monomeric raceme and dimer, K₃[MoO₃(cit)]·2H₂O and K₄[MoO₄(OH)(cit)·2H₂O. Inorg. Chem. 39, 59–64 (2000).

39. Xing, Y. H. et al. A new dinuclear molybdenum(VI)-sulfur complex containing citrate ligand: synthesis and characterization of K₂[N₃S₂H₃][Mo₂O₇S₄]·5H₂O. Eur. J. Inorg. Chem. 35, 745–756 (1998).

40. Knobler, C. B. et al. Molybdenum(VI) complexes with malic acid: their inter-relationships, and the crystal structure of dicaesium bis[(S)-malato(2-)]-cis-dioxomolybdate(VI)-water (1:1). J. Chem. Soc. Dalton Trans. 7, 1299–1303 (1983).

41. Takuma, M., Okhi, Y. & Tatsumi, K. Molydbenum carbonyl complexes with citrate and its relevant carboxylates. Organometallics 24, 1344–1347 (2005).

42. Sokolov, M. N. et al. Complexes of Mn(S₄)⁺⁺ (M = Mo, W) with chiral a-hydroxy and aminoo acids: synthesis, structure and solution studies. Inorg. Chem. 395, 11–18 (2013).

43. Freedman, T. B., Cao, X., Dukor, R. K. & Nafe, L. A. Absolute configuration determination of chiral molecules in the solution state using vibrational circular dichroism. Chirality 15, 743–758 (2003).

44. Crawford, T. D. Ab initio calculation of molecular chiroptical properties. Theor. Chem. Acc. 115, 227–245 (2000).

45. Strum, F. J., Devlin, F. J. & Pan, J. F. Determination of the absolute configurations of chiral molecules using vibrational circular dichroism (VCD) spectroscopy. Chirality 20, 643–663 (2008).

46. Koenis, M. A. J. et al. Taming conformational heterogeneity in and with vibrational circular dichroism spectroscopy. Chem. Sci. 10, 7680–7689 (2019).

47. Halls, M. D., Velkovski, J. & Schlegel, H. B. Harmonic frequency scaling factors for Hartree-Fock, S-VWN, B-LYP, B3-LYP, B3-PW91 and MP2 with the Sadlej pVTZ electric property basis set. Theor. Chem. Acc. 105, 413–421 (2001).

48. Huang, R. et al. Harmonic vibrational frequencies: scaling factors for HF, B3LYP, and MP2 methods in combination with correlation consistent basis sets. J. Phys. Chem. A 108, 9213–9217 (2004).

49. Laury, M. L., Carlson, M. J. & Wilson, A. K. Vibrational frequency scaling factors for density functional theory and the polarization consistent basis sets. J. Comput. Chem. 33, 2380–2387 (2012).

50. Grunze, S., Ehrlich, S. & Goerigk, L. Effect of the damping function in dispersion corrected density functional theory. J. Comput. Chem. 32, 1456–1465 (2011).

51. Frisch, M. J. et al. Gaussian 16, Revision A.03 (Gaussian, Inc., Wallingford, 2016).

Acknowledgements
We thank the support from the National Natural Science Foundation (21773196) for the generous financial supports and thank Fan Yao from Shiyanjia Lab (www.shiyanjia.com) for the VCD calculations.

Author contributions
L.D. and H.W. performed the syntheses, spectral characterizations, and structure analyses. C.J.W., E.W.N. extracted FeMo-co. S.S. and S.W. conducted VCD spectroscopic measurements. S.P.C. designed VCD experiment of FeMo-co. L.D. and Z.H.Z. conducted VCD experiments and prepared the manuscript with feedback from the other authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s42004-020-00392-z.

Correspondence and requests for materials should be addressed to S.P.C. or Z.-H.Z.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.