Evidence for Interstitial Carbon in Nitrogenase FeMo Cofactor

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The enzyme nitrogenase is the only known biological system able to break the triple bond of dinitrogen to yield bioavailable ammonium (*1*). Its active site, the FeMo cofactor, is a [Mo:7Fe:9S:X]:homocitrate cluster, the largest and most complex biological metal center known to date. The exact mode and position of N_2 binding to the FeMo cofactor is unknown. The identification of a light atom X (C, N, or O) at its center in a 1.16 Å resolution crystal structure (*2*) gave rise to contradictory mechanistic proposals that require clarification. Our analysis of the diffraction data indicated that, although an unambiguous assignment could not be made, X was most plausibly a nitrogen species. Through further optimization of protein isolation and crystallization, we have obtained an improved structural model at 1.0 Å resolution (Fig. 1A) (*3*). In the 1.16 Å resolution structure, the central atom was obscured by the geometry of the FeMo cofactor. Our suggestion of a nitrogen species was based on integrating electron density (ED) at the cofactor center using a probe radius of 1.4 Å, the approximate van der Waals radius of a candidate atom. We now varied this probe radius on a very fine ED grid and performed a statistical analysis for all light atoms in the structure. A plot of ED versus probe radius for the average C, N, and O atoms shows that these can be distinguished (Fig. 1B), with the distinction clearer for smaller radii. The corresponding curves for the two central atoms in the two copies of the FeMo cofactor in the asymmetric unit overlay perfectly with the curve obtained for all other carbons. A slight deviation to higher values was seen at a radius of 1.4 Å, indicating that the influence of the surrounding Fe atoms is already noticeable, and this in part biased our analysis of the earlier structure toward nitrogen. In Fig. 1B, plotted values do not represent integrated ED but rather the average ED within the sphere of given radius. Alternatively, we have plotted the average of EDs for each exact atom position versus its B factor (Fig. 1C and fig. S2). Again, the different light atoms group into distinct areas of the plot, with the interstitial atoms close to the center of the carbon distribution. Although the ρ_0 plot indicated the atom to be a carbon both with the 1.16 Å and with the 1.0 Å resolution data, the ED/radius plot was far more ambiguous at the lower resolution and did not allow us to distinguish between C and N for the central atom (*3*). The seemingly modest improvement in resolution obtained with the new data set corresponds to an increase of the data-parameter ratio (with an anisotropic U_{ij} temperature factor model) of 50%, from 4.3 (1.16 Å) to 6.6 (1.0 Å), underlining that the improved resolution was essential for assigning the identity of the central atom.

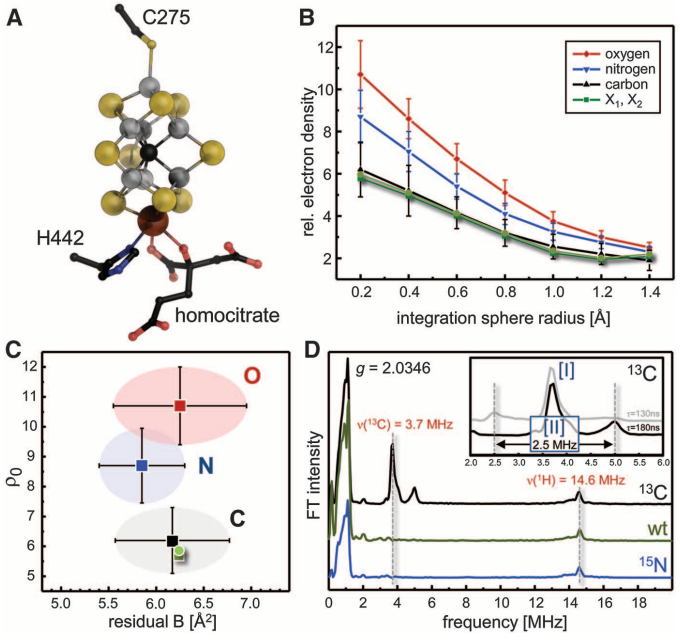

Fig. 1. Carbon in the center of the FeMo cofactor. (**A**) The [Mo:7Fe:9S:C]:homocitrate FeMo cofactor. (**B**) Average ED in a sphere of given radius for all carbon (black), nitrogen (blue), and oxygen (red) atoms in the structure. The two central atoms in the asymmetric unit (green) closely follow the trace for carbon. (**C**) ED at the atomic positions (ρ_0) versus residual B factors shows C, N, and O to occupy distinct areas. The central atoms (green) fall within the carbon area. (**D**) X-band three-pulse ESEEM frequency domain spectra of wild-type (WT) (green), U-^{15}N–labeled (blue), and U-^{13}C–labeled (black) nitrogenase. (Inset) ^{13}C resonances recorded with two different τ values. FT, Fourier transform; *g*, g-tensor.

To complement the diffraction data, we produced *Azotobacter vinelandii* MoFe protein labeled with ^{13}C or ^{15}N, respectively, for resonance spectroscopy. The isolation procedure (*3*) was optimized to yield complete incorporation of the isotopes, far exceeding the labeling ratio of ~5% reported earlier (*4*). Inspection of wild-type and two uniformly isotope-labeled (U-^{15}N and U-^{13}C) nitrogenases (*3*) by electron spin echo envelope modulation (ESEEM), a powerful electron paramagnetic resonance technique for detection of weak hyperfine couplings (hfcs) in paramagnetic moieties such as the clusters of nitrogenase (*3*), revealed for the U-^{13}C–labeled (nuclear spin quantum number = 1/2) sample an additional spectral pattern, centered at the free ^{13}C Larmor frequency (3.7 MHz, Fig. 1D) with a splitting of 2.5 MHz, that is not detected in the other samples. Two types of resonances can be discriminated, one originating from ^{13}C atoms that are very weakly coupled to the paramagnetic FeMo cofactor (Fig. 1D, [I]) and the other from a more strongly coupled ^{13}C hfc with significant unpaired electron spin density (Fig. 1D, [II]). The latter is expected for a carbon nearby or within the FeMo cofactor. This observation is consistent with X being C, because the crystal structure does not reveal any carbons within the first coordination sphere of the cluster that could account for such a large hfc.

References and Notes

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3. Materials and methods are available as supporting material on *Science* Online.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/334/6058/940/DC1
Materials and Methods
Figs. S1 and S2
Table S1
References (*5*–*8*)

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