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Competitive ^{15}N Kinetic Isotope Effects of Nitrogenase-Catalyzed Dinitrogen Reduction

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The ^{15}N kinetic isotope effect (KIE) for dinitrogen reduction with *Azotobacter vinelandii* nitrogenase (*Av*) was measured using the natural distribution of $^{15}N^{14}N/^{14}N_2$ (masses 29/28). The value of *V*/*K* ^{15}N KIE, also noted as $^{15}(V/K)$, was determined to be 1.7% ± 0.2% (KIE = 1.017 ± 0.002). The error in our experiments is sufficiently small that the different proposed reaction mechanisms can be distinguished. Most significantly, the observed KIE suggests that the initial binding of N_2 to the pre-reduced enzyme (and presumably to the very reactive over-reduced FeMoco) is reversible. Irreversible binding would commit every bonded N_2 to react (regardless of its isotopic composition), resulting in no observed KIE (infinite forward commitment to catalysis).[1,2] Another critical mechanistic finding is that the KIE also indicates that, following N_2 binding, the irreversible step precedes the transfer of an additional electron by the Fe protein. If the transfer of an additional electron from the Fe protein was required, then the slow association and dissociation steps[3,4] would mask the observed *V*/*K* KIE, as in the case of k_{cat} KIE (these two kinetic conclusions are further discussed below).[2] These findings impose critical restrictions on any theoretical model that attempts to calculate/simulate the nitrogenase-catalyzed N_2 reduction.

This is interesting because a lot is known about biological nitrogen fixation but very little about how nitrogen is being fixed (chemically reduced). N_2 is the most stable molecule in the atmosphere, it is almost an inert gas, and its reduction to useful (fixed) nitrogen is fascinating from the chemical point of view. The enzyme nitrogenase catalyzes that reaction under ambient conditions. We chose to study an Mo-nitrogenase because it is the most extensively studied nitrogenase and the only one for which detailed structural information is available.[5−7] Yet, the methodology presented here can be applied to any N_2 reducing catalyst. The *Av* nitrogenase consists of the Fe protein and the MoFe protein. The Fe protein is the only agent that can reduce the MoFe protein productively. The MoFe protein contains two large complex metal clusters, the P-cluster and the FeMo-cofactor (FeMoco), which presumably provides the substrate binding/reduction site. Despite these structural data, the reaction mechanism for N_2 reduction remains elusive. To date, the details of N_2 binding and reduction have been addressed either by coordination model compounds or by theoretical calculations (over 35 theoretical models since 1992).[8] The lack of experimental data that address the reaction coordinate (from N_2 binding to its chemical reduction) makes it impossible to assess the relevance of these different models to the enzymatic mechanism. Although most of these models contradict each other (different binding sites, head-on vs side-on N_2 binding, different sequences of reductive protonations, etc.),[8] they are all equally valid/invalid since no experimental data are available to distinguish between them. Clearly, there is a need for an experimental approach that, together with the crystal structure and theoretical models, will address the core question of biological nitrogen fixation: the N_2 chemical reduction step. Here, we present new research aimed at probing the change in the nitrogen bond order along the enzymatic reaction coordinate via ^{15}N KIE.

KIEs can serve as useful probes for the nature of chemical reactions and enzyme mechanisms.[9,10] Practically, measurements of the KIE for the N_2 reduction might be masked by the fact that the chemical step is not rate limiting (cf. kinetic complexity).[9] The Thorneley and Lowe kinetic model is the most detailed kinetic model for nitrogenase so far.[3,4] This model comprises eight Fe protein cycles that are required to complete a single MoFe protein cycle, thus providing the eight electrons necessary to reduce $N_2 + 8H^+$ to $2NH_3 + H_2$. Each electron-transfer step involves the association and dissociation of the Fe protein and MoFe protein complex. These association and dissociation steps (under low and high electron flow conditions, respectively) are the overall rate-limiting steps in the catalytic cycle.

Since the N_2 reduction step is far from being rate limiting for the overall cycle, KIE measurements on the catalytic turnover (V_{max} or k_{cat}) are not likely to result in measurable isotope effects. The method presented here affords a direct examination of the changes in N_2 bond order along the enzymatic reaction coordinate by measuring competitive ^{15}N KIEs on the second-order rate constant *V*/*K*. This rate constant is only sensitive to kinetic steps ranging from the initial binding of the N_2 to the pre-reduced enzyme until the first irreversible step.

Interestingly, one of the earliest usages of an isotope ratio mass spectrometer (Burris and Miller, 1941)[11] was an attempt to measure ^{15}N KIEs on nitrogen fixation with a culture of free-living *Av* bacterium under a $^{15}N_2$-enriched atmosphere, but the experimental errors were too large to identify any isotope effect.

Our experimental design involves measurements using the purified wild-type *Av* nitrogenase and naturally abundant ^{15}N (0.37%). The enrichment of $^{15}N^{14}N$ relative to $^{14}N_2$ (masses 29 and 28 amu, respectively) in the remaining N_2 during the course of the reaction, and the fractional conversion, allowed calculation of the ^{15}N KIE (see below). The first step in each experiment involves preparation of a reaction mixture with N_2 substrate, ATP, ATP recycling system (creatine kinase/creatine phosphate), $MgCl_2$, and dithionite in a pH = 7.4 buffer, under common reaction conditions.[12] Various N_2 concentrations are achieved by mixing desired volumes of reaction mixture saturated with N_2 with the same mixture fully degassed. The final N_2 concentration of interest is determined by the ratio between these two solutions. The mixture is transferred

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into a deflatable reaction chamber (a gas-impermeable Tedlar bag from Midan Co., equipped with a septum and magnetic stirrer), which is then attached to the vacuum line and kept in a water bath at a constant temperature (25 °C in the current work). The deflatable bag is essential because liquid samples need to be withdrawn for analysis without changing the pressure. Gas-phase (headspace or bubbles) formation in the reaction chamber poses a serious problem, not only due to the isotopic liquid/gas fractionation ($^{15}K = 1.00071$), but also because accurate determination of fractional conversion relies on homogeneous distribution of N_2 in the chamber.

The reaction is initiated by injecting the enzymes (the Fe and MoFe proteins) in the desired molar ratio. It is important to note that converting a large percentage of N_2 is a slow process even under ideal conditions of 25:1 Fe/MoFe protein ratios and 1 atm of N_2. The K_M of nitrogenase for N_2 is close to 0.09 atm of N_2 at a high Fe/MoFe protein ratio and is smaller at a lower Fe/MoFe protein ratio (e.g., 0.05 atm at 4:1 ratio).[12] Thus, a major challenge was to achieve a significant fractional conversion (to ensure sufficient enrichment of ^{15}N in the remaining N_2). The ratio of Fe/MoFe protein in the reaction mixture was adjusted to enhance the reaction rate and to keep the obligatory H_2 production to a minimum. Under the common conditions of 4:1 Fe/MoFe protein ratios and 1 atm of N_2, H_2 bubbles start to form after 20% conversion. Higher fractional conversions could only be reached by lowering the N_2 concentration (pressure) to 0.8 atm and increasing the Fe/MoFe protein ratio to 25:1.

Aliquots were withdrawn at various time points and quenched in 85% phosphoric acid at various fractional conversions ranging from 20% to 64%.[8] A stream of helium pre-purified over Getter alloy was used as carrier gas to purge the remaining N_2 from the acid. Special care was taken to ensure complete quantitative isolation of nitrogen from the reaction samples after quenching. The purification steps are described in the Supporting Information and include ethanol/dry ice and liquid nitrogen traps to remove water, CuO trap (700 °C) to oxidize H_2, and organic compounds. The N_2 was then quantitatively trapped on silica gel at liquid nitrogen temperature. The relative N_2 molar amount was measured using a pressure transducer at a constant volume (6 mL) and temperature (100 °C), and finally the N_2 was sealed in sample tube over silica gel for isotope ratio analysis. The enrichment of the residual dinitrogen with ^{15}N was analyzed by isotope ratio mass spectrometry (IRMS).[8]

Each 29/28 ratio was defined as R_t. Fractional conversion (f) was calculated from the change in pressure at 100 °C relative to its value at the zero time point (t_0). The initial isotopic ratio R_0 (at t_0) was determined by withdrawing and analyzing several aliquots prior to injection of the nitrogenase enzymes. The V/K ^{15}N KIE was calculated using[13]

$$\mathrm{KIE} = \frac{\ln(1-f)}{\ln[(1-f)(R_t/R_0)]}$$

The resulting V/K ^{15}N KIE was 1.7% ± 0.2% (for raw data and calculations, see Supporting Information). We were able to obtain this value in a consistent and reproducible fashion. Errors do not result from the IRMS inherent errors (±0.0001) but reflect the distribution of results from many independent experiments. The error in our experiments is common in KIE measurements for similar gaseous substrates.[14,15] Importantly, this KIE is independent of fractional conversion (at 18−64% conversion range), which is an important indication that the experiment is free of many possible artifacts.[13]

In most of the proposed mechanistic models for nitrogenase,[8] the chemical reduction is the first irreversible step. Yet, it is possible that a slow kinetic step will partially mask the intrinsic KIE, such that the observed value is the lower limit of the intrinsic KIE. Experiments that compare the observed $^{15}(V/K)$ KIEs in H_2O vs D_2O are under way. These experiments should reveal whether the reductive protonation and the cleavage of the N_2 bond occur at the same step or stepwise,[16] and will facilitate calculations of intrinsic KIEs.[17] Additionally, similar studies under D_2 may reveal whether the HD exchange phenomenon (observed only while reducing N_2)[12] occurs before, at, or after the N_2 reduction irreversible step.

In summary, a unique experimental methodology was developed that enables a direct examination of the N_2 reduction step within the complex kinetic cascade of the nitrogenase catalysis. Competitive ^{15}N KIEs were used to demonstrate that the change in N_2 bond order between the free substrate in solution and the reaction's transition state can be measured along the reaction coordinate. Together with the crystal structure, the KIE value reported here (1.7% ± 0.2%) should be taken into consideration by any mechanistic model attempting to explain the nitrogenase-catalyzed N_2 fixation. This is of special importance as, so far, almost all theoretical models have been based solely on the FeMoco X-ray structure (at ground state). A vibrational analysis of the transition states that were proposed by these models (or of the most relevant intermediates) will allow a prediction of a V/K KIE. This new experimental data should facilitate the assessment of these models by supporting or contradicting their proposed transition states. Finally, the similarities and differences in the N_2 reduction step between various mutants of the *Av* nitrogenase or nitrogenases from other organisms may now be investigated.

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Supporting Information Available: Examples of theoretical models, detailed description of the reaction preparation, the experimental apparatus, raw data, and their analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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