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Measurement of Kinetics and Active Site Distances in Metalloenzyme Using Paramagnetic NMR with ^{13}C Hyperpolarization

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Abstract

Paramagnetic relaxation enhancement (PRE) conjoint with hyperpolarized NMR reveals structural information on the enzyme-product complex in an on-going metalloenzyme catalyzed reaction. Substrates of pseudouridine monophosphate glycosidase are hyperpolarized using the dynamic nuclear polarization (DNP) method. Time series of ^{13}C NMR spectra are subsequently measured with the enzyme containing diamagnetic Mg^{2+} or paramagnetic Mn^{2+} ions in the active site. The differences of the signal evolution and line widths in the Mg^{2+} vs. Mn^{2+} reactions are explained through PRE in the enzyme-bound product, which is in fast exchange with its free form. Here, a strong distance dependence of the paramagnetically enhanced relaxation rates enables the calculation of distances from product atoms to the metal center in the complexed structure. The same method can be used to add structural information to real-time characterizations of chemical processes involving compounds with naturally present or artificially introduced paramagnetic sites.

Keywords

Dissolution dynamic nuclear polarization; nuclear magnetic resonance; paramagnetic relaxation effect; metalloenzyme catalysis; non-equilibrium structure

Structural properties, including of complexed and non-equilibrium configurations, play a pivotal role in biological functions of macromolecules. Nuclear magnetic resonance (NMR) spectroscopy has long been applied to probe molecular structures,^{1,2} however, due to an intrinsically low sensitivity, is often limited to proton nuclei and equilibrium conditions that

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Supporting Information. The Supporting Information including hyperpolarized ^{13}C -NMR experiments, effect of free Mn^{2+} , kinetic model and fitting, and distance determination based on PRE, is provided.

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permit signal averaging. Hyperpolarization, especially dissolution dynamic nuclear polarization (D-DNP),³ provides signal enhancement allowing real-time NMR for the determination of kinetics and mechanisms in chemical processes, including enzymatic reactions.^{4,5}

Paramagnetic relaxation enhancement (PRE) has been used to characterize metalloenzymes containing paramagnetic metal ions naturally or by substitution using conventional NMR.^{2,10,11} Distance restraints based on PRE can range up to ~35 Å.^{6–9} However, the potential of PRE in assisting structural characterizations in real-time hyperpolarized NMR to date remains untapped. In this paper, we demonstrate that structural constraints in the active site of an enzyme can be measured along with kinetic parameters in an on-going reaction, using PRE of hyperpolarized ¹³C signals. The metalloenzyme pseudouridine monophosphate glycosidase (ΨMPG) was chosen for this purpose. ΨMPG is active with several diamagnetic or paramagnetic metal ions, such as Mn²⁺, Fe²⁺, Co²⁺, and Mg²⁺.¹² In bacteria, this enzyme reversibly catalyzes the cleavage of the C–C glycosidic bond in pseudouridine monophosphate (ΨMP), thereby recycling pseudouridine, the most abundant nucleoside in RNA modifications.¹³

In the present experiments, we aimed to measure the PRE by comparing the signal evolution under catalysis by ΨMPG with diamagnetic Mg²⁺ and paramagnetic Mn²⁺ in the active site. ¹³C hyperpolarized substrates 4,5-¹³C₂-uracil and ribose-5-phosphate (R5P) were rapidly mixed with a solution of the purified ΨMPG-Mg²⁺ or ΨMPG-Mn²⁺ complex. The catalyzed reaction resulted in the formation of ΨMP, in the thermodynamically favored reaction direction, which is the reverse of the *in vivo* pathway (Figure 1a). Real-time ¹³C NMR spectra were recorded using successive small-flip angle excitations. An individual spectrum taken from each time series at 2.34 s is plotted in Figure 1b, and the entire time series of spectra are shown in the supporting information (SI), Figure S1. Owing to the large chemical shift range of ¹³C, reactant and product peaks are readily separated. An increase in polarization of ~ 5000 times was calculated by comparing the measured spectra to a thermal equilibrium spectrum of a reference compound. This enhancement also allowed observation of signals from R5P at natural ¹³C isotope abundance in the first scans (Figure S1), which were however not further analyzed.

Spectra show signals of reactant and product at nearly identical chemical shifts in Mg²⁺ and Mn²⁺ reactions (Figure 1c). It is apparent, however, that the product peaks have larger line widths in the reaction with Mn²⁺ than with Mg²⁺. This line broadening cannot be explained by dissociated free Mn²⁺ alone (SI part 2), and may instead be explained by PRE occurring in enzyme complexed product in fast exchange with its free form. The time evolution of integrated signals of the labeled reactant and product peaks is shown in Figure 2. Signal intensities change due to the combined effects of spin relaxation, reaction kinetics, and radio frequency pulses.¹⁴ The reactant peaks decay more rapidly, and the product peaks reach their maxima earlier and also decay faster in the reaction with Mn²⁺. This difference *prima facie* would be caused by increased reaction and relaxation rates in the presence of paramagnetic Mn²⁺. Differences in reaction rates with other ions in the active site of ΨMPG have in fact previously been observed, and have been attributed to a dependence on the ionic radius.¹²

A kinetic model is proposed in Scheme 1 to explain the observed signal evolution quantitatively, and to determine kinetic rate constants and spin relaxation rates that can be used for distance calculation. Signal intensities and product line widths from datasets with three different enzyme concentrations were fitted simultaneously using this model (see SI part 3 for full explanations). This procedure is similar to ref.¹⁵, albeit using different input data.

The analysis results in relaxation rates and kinetic rate constants of detailed steps in both forward and reverse directions in the ΨMPG catalyzed reaction. The fitted time evolution of integrals and line widths is plotted in Figure 3. The best fit parameters are summarized in Tables S1 and S2. Fitting errors were estimated from the Jacobian matrix from the fitting procedure, as well as from a Monte Carlo simulation considering an additional ~ 5% uncertainty in the concentrations of the enzyme and substrate arising out of the injection procedure. The parameters $k_1 = (2.23 \pm 0.12) \cdot 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_2 = (4.13 \pm 0.29) \text{ s}^{-1}$ for the reaction catalyzed by ΨMPG-Mn²⁺ (95% confidence intervals from Monte Carlo simulation) are in remarkably good agreement with the kinetic rate constants determined previously using quench-flow experiments at the room temperature, $k_1 = (2.2 \pm 0.36) \cdot 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_2 = (3.74 \pm 0.14) \text{ s}^{-1}$.¹² In the ΨMPG-Mg²⁺ reaction, $k_1 = (2.17 \pm 0.24) \cdot 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_2 = (2.46 \pm 0.26) \text{ s}^{-1}$, are obtained. The dissociation rate constant of the enzyme-product complex k_3 is $(2.29 \pm 0.35) \cdot 10^4 \text{ s}^{-1}$ in the ΨMPG-Mg²⁺ reaction and $(0.45 \pm 0.08) \cdot 10^4 \text{ s}^{-1}$ in the ΨMPG-Mn²⁺ reaction, consistent with the fast exchange assumption that $k_{\text{ex}} = k_3 + k_{-3} [\text{E}] \gg \nu$ (the frequency difference between free and bound forms, ν , is estimated as ~ 40 Hz from the product peak shifts in the Mg²⁺ and Mn²⁺ reactions and the calculated proportions of the bound product). The association rate k_{-3} is $(9.25 \pm 1.28) \cdot 10^7$ and $(0.85 \pm 0.15) \cdot 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ in the Mg²⁺ and Mn²⁺ reactions respectively, which is within the diffusion-limited range of $\sim 10^7 - 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$.¹⁶ A Gibbs free energy change of $G = -23 \text{ kJ/mol}$ for the formation of the product is further calculated, in both the reactions with Mg²⁺ and Mn²⁺. This value compares to the literature value of -21 kJ/mol in the absence of enzyme,¹³ indicating that the kinetic fitting correctly reproduces the overall equilibrium of the reaction. The small reverse reaction rate constants and the negative G of the product formation are concordant with the fact that the formation of the C-C glycosidic bond is favored.

Application of the paramagnetic relaxation theory to the extracted relaxation rates leads to the determination of distances between individual atoms of the enzyme-bound product and the active site metal. The differences of R_1 and R_2 rates from the enzyme-bound product in the reactions with Mn²⁺ vs. Mg²⁺ are expected primarily due to the paramagnetic effect, *i.e.* $R_{1,PRE}^b = R_{1,Mn}^b - R_{1,Mg}^b$ and $R_{2,PRE}^b = R_{2,Mn}^b - R_{2,Mg}^b$ (the superscript *b* denotes the enzyme bound form). Based on Equations S(10) and S(11) from the dipolar interaction mechanism of PRE,^{8,17,18} distances between the metal center and C_{4'} and C_{5'} atoms in the active site are derived (SI part 4). Figure 4 shows that the results are within error limits of distances measured in two crystal structures of related complexes (PDB ID: 4GIL and 4GIM),¹² although it should be noted that the crystal structures do not represent exactly the same complex: The product ring is open in 4GIL and the enzyme is mutated in 4GIM (Figure S4). The distances calculated are subject to several sources of error. Firstly, $R_{1,J}^b$ is assumed to be the same in EU and EP in the model, since the enzyme bound forms are not

directly observable. Thus $R_{1,PRE}^b$ derived distances for EP are average values between EU and EP along the reaction coordinate. Secondly, as in any measurement of the PRE, additional metal binding on the complex would cause further relaxation, which however would only be possible for weak affinity or a longer distance from the product atoms based on the magnitude of observed relaxation rates. The distances in principle are also averaged over an ensemble of dynamic states.

The combination of PRE and DNP advances the available methodology for the characterization of molecular structure and dynamics in two respects. Owing to the $10^3 - 10^4$ fold signal enhancement by DNP, paramagnetic NMR measurements can be achieved at low concentration. The real-time ability of DNP also provides PRE experiments with time resolution, which may be exploited in the study of dynamic processes. As reported here, the measurements of PRE in Ψ MPG for the first time characterize the actual complex arising in the process of the reaction, and not under equilibrium. Meanwhile, paramagnetic relaxation based methods add a capability for structural characterization to D-DNP experiments. This capability offers additional information beyond the measurement of kinetics from real-time DNP-NMR. The combined method is applicable to reactions involving compounds with naturally present or artificially introduced paramagnetic sites, including many enzyme catalyzed reactions, polymerizations such as with radical intermediates, and others.

In summary, the high sensitivity from hyperpolarization by D-DNP enables real-time paramagnetic NMR measurements. Hyperpolarized ^{13}C spectra were measured in a metalloenzyme catalyzed reaction with diamagnetic and paramagnetic metal substitution. Through a quantitative analysis of time-dependent NMR intensities and line widths, kinetic and nuclear spin relaxation rate constants were determined. Examining the paramagnetically enhanced R_1 and R_2 relaxation values of carbon nuclei in the enzyme complexed product, long-range distance information in the active site was obtained. This approach therefore provides conformational information during an ongoing reaction, without the use of stabilized intermediates.

Experimental Methods

Hyperpolarized NMR experiments.

Ψ MPG was expressed and purified as previously described.¹² The stock enzyme concentration was determined from absorbance at 280 nm using UV-Vis spectroscopy using the calculated extinction coefficient of $9,970 \text{ M}^{-1} \text{ cm}^{-1}$. For hyperpolarization, a 30 μL solution of 21 mM 4,5- $^{13}\text{C}_2$ -uracil, 375 mM ribose-5-phosphate, and 454 mM acetone was prepared with 15 mM OX063 radical and 1 mM Gd-DTPA in ethylene glycol/ H_2O (3:2, v/v). The sample was hyperpolarized in a HyperSense system (Oxford Instruments, Abingdon, U.K.) by irradiating 60 mW 93.974 GHz microwaves at 1.4 K in a 3.35 T magnetic field for 3 hours. After dissolution and rapid injection¹⁹ with preheated 50 mM Tris buffer (pH 7.5), ^{13}C NMR spectra were measured using successive small-flip angle excitations on a 400 MHz NMR spectrometer (Bruker Biospin, Billerica, MA) at 298 K. Detailed experimental procedures are described in SI part 1.

Data analysis.

To obtain the fitting parameters, minimization was performed on the sum of squared residues from the observed and calculated NMR signals based on the model. In the model, the NMR signal of spins of reactant, product, and intermediates evolve according to rate equations for the reactions and for spin relaxation. These processes are represented by a set of equations for NMR signals S_n and concentrations $[N_n]$, combined with the equations for mass balance (Equations (1) and (2)). Line widths were derived from R_2 , which is the average over free and bound forms, weighted by the respective calculated mole fractions. Distances from the product atoms to the metal center in the active site were calculated based on the fitted R_1 and R_2 values using the paramagnetic relaxation theory. The error ranges for distances were derived via propagation of uncertainty. Details about fitting and distance determination are provided in SI part 3 and 4, respectively.

$$\frac{dS_i}{dt} = \left(\sum_{n \in \text{precursors}} k'_n \times S_n \right) - \left(\sum_{n \in \text{products}} k'_n \times S_i \right) - R_{1,i} \times S_i \quad (1)$$

$$\frac{d[N_i]}{dt} = \left(\sum_{n \in \text{precursors}} k'_n \times [N_n] \right) - \left(\sum_{n \in \text{products}} k'_n \times [N_i] \right) \quad (2)$$

In Equations (1) and (2), precursors and products are relative to the species i , and $R_{1,i}$ is the spin-lattice relaxation rate of i . The first order rate constants k'_n are $k'_n = k_n [E]$ for the second order steps in Scheme 1, and otherwise $k'_n = k_n$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Reaction of ΨMPG, *in vitro* occurring left to right, coupling uracil (1) and R5P (2) to form ΨMP (3). b) Individual spectra from the time point at 2.34 s, of reactions with Mg^{2+} (red) and Mn^{2+} (blue, scaled $\times 2$). A total of 96 scans were acquired over a total acquisition time of 39 s. The peaks labeled with '†' are from the internal reference acetone. '*' indicates the peak of the glassing solvent ethylene glycol used for DNP polarization, initially suppressed by a selective pulse followed by a pulsed field gradient. c) Enlarged reactant and product peaks. In all spectra, chemical shifts are calibrated using the acetone peak at 215 ppm. The concentrations of uracil (^{13}C labeled for observation), R5P (added in excess), and enzyme were 560 μM , 10 mM, and 83 μM respectively.

Figure 2. Time-dependent integrals in ΨMPG catalyzed reactions with Mg²⁺(red) and Mn²⁺(blue). a) Labeled positions in the reactant uracil (4) and in the product ΨMP (4'); b) Labeled positions 5 and 5'. Open and filled 'O' represent reactant and product peaks respectively. The integrals, $I_{rel.}$, were normalized to the maximum signal.

Figure 3.

Time-dependent NMR signals in reactions with $\Psi\text{MPG-Mg}^{2+}$ or $\Psi\text{MPG-Mn}^{2+}$, including the fitted curves. From top to bottom, the enzyme concentrations are 120, 83, and 56 μM respectively. a) Integrals of reactant and product peaks, I_{rel} , normalized to the maximum signal. The curves are shifted to avoid overlapping. b) Line widths of the product peaks. '●' and '▲' represent $\text{C}_{4'}$ and $\text{C}_{5'}$, respectively.

Figure 4.

Distances between $C4'$ / $C5'$ and the metal ion in the enzyme-product complex. From $R^b_{1,PRE}$, $d(C4'-M) = 15.1 \pm 3.2$ Å and $d(C5'-M) = 15.5 \pm 3.3$ Å; from $R^b_{2,PRE}$, $d(C4'-M) = 10.6 \pm 2.3$ Å and $d(C5'-M) = 11.8 \pm 2.6$ Å. For distances from two crystal structures, the error bars specify the resolutions. In 4GIL, $d(C4'-M) = 13.1 \pm 2.5$ Å and $d(C5'-M) = 12.8 \pm 2.5$ Å; in 4GIM, $d(C4'-M) = 12.0 \pm 1.8$ Å and $d(C5'-M) = 10.9 \pm 1.8$ Å. For distances based on $R^b_{1,PRE}$ and $R^b_{2,PRE}$, the values were calculated using the median values of R^b_1 and R^b_2 from the Monte Carlo simulation. The error bars indicate the 95% confidence intervals derived from the distribution of R^b_1 and R^b_2 in the simulation. From the best fit, the obtained distance ranges are: $d(C4'-M) = 15.3 \pm 3.2$ Å and $d(C5'-M) = 15.5 \pm 3.3$ Å via $R^b_{1,PRE}$; $d(C4'-M) = 10.6 \pm 2.4$ Å and $d(C5'-M) = 11.9 \pm 2.8$ Å via $R^b_{2,PRE}$.

Scheme 1.

Simplified unireactant mechanism with fast exchange of the product to the enzyme ^a

^a U, E, and P stand for uracil, ΨMPG, and the product ΨMP respectively. EU is the enzyme-reactant complex, and EP is the enzyme-product complex. k_1 and k_{-3} are second-order rate constants, and k_{-1} , k_2 , k_{-2} , and k_3 are first-order rate constants.