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Kinetic and Equilibrium Constants of Phytic Acid and Ferric and Ferrous Phytate Derived from Nuclear Magnetic Resonance Spectroscopy

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Inositol phosphates are metabolically derived organic phosphates (P) that increasingly appear to be an important sink and source of P in the environment. Salts of *myo*-inositol hexakisdihydrogen phosphate (IHP) or more commonly phytate are the most common inositol phosphates in the environment. IHP resists acidic dephosphorylation and enzymatic dephosphorylation as ferric or ferrous IHP. Mobility of IHP iron complexes is potentially pH and redox responsive, making the time scale and environmental fate and transport of the P associated with the IHP of interest to the mass balance of phosphorus. Ferric and ferrous complexes of IHP were investigated by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and enzymatic dephosphorylation. Ferrous IHP was found to form quickly and persist for a longer period then ferric IHP. Dissociation constants derived from ^{1}H NMR experiments of chemically exchanging systems at equilibrium were 1.11 and 1.19 and formation constants were 0.90 and 0.84 for ferric and ferrous IHP, respectively. The recovery of P from enzymatic dephosphorylation of ferric and ferrous IHP was consistent with the magnitude of the kinetic and equilibrium rate constants.

KEYWORDS: Inositol hexakisdihydrogen phosphate; phytic acid; proton nuclear magnetic resonance; organic phosphate; ferric phytate; ferrous phytate; IHP; kinetic rate constants

INTRODUCTION

The primary transport processes of the environmental phosphorus cycle involve nonpoint source movement from soil systems to surface waters. The major nonpoint source transport mechanisms are believed to be overland flow processes. Erosion-driven movement of sediment-bound phosphorus and runoff containing dissolved phosphates (P) are believed to contribute the majority of phosphorus to surface waters (*1, 2*). A third possible nonpoint source transport process involves leaching of phosphorus in soil pore water to drainage waters (*3*). Factors controlling phosphorus movement into the dissolved phase include the oxidation−reduction potential of the soil, the amount and type of clay present in the soil, and the fraction of phosphorus present as an organic phosphorus species (*2*−*9*).

myo-Inositol-1,2,3,4,5,6-hexakisdihydrogen phosphate (IHP) (phytate as a salt or phytic acid when free acid) is acknowledged to be the major form of organic phosphorus in soil systems and manures (*9*−*14*). The contribution of IHP to the phosphorus mass balance may be underestimated in some circumstances such as high levels of organics or less stringent acid digests (*15*). Measurement of the kinetic and thermodynamic constants of organic phosphorus and its common environmental complexes is a critical component of understanding the environmental role and impact of IHP on the phosphorus cycle.

IHP is known to be enzymatically dephosphorylated by phytases from plant, microbial, and animal sources. *Aspergillus ficuum* is a soil fungus that produces 3-phytase (EC 3.1.3.8). This phytase acts on the C_3 phosphate group initially but eventually dephosphorylates five of the six P on the IHP molecule (*8, 16, 17*). The final dephosphorylation is achieved by acid or alkali phosphamonoesterases (EC 3.1.31 and EC 3.1.3.2), which are known to be present in commercial sources of phytase (*8*). IHP and phytases are supplied naturally and also anthropogenically, entering terrestrial systems in manure and as plant decomposition byproduct (*18*). IHP is not responsive to oxidation reduction reactions itself but can form complexes with metals that are redox responsive. Recent research verified that speciation of IHP is both strongly and predictably pH dependent in the presence and absence of metal ions (*19*).

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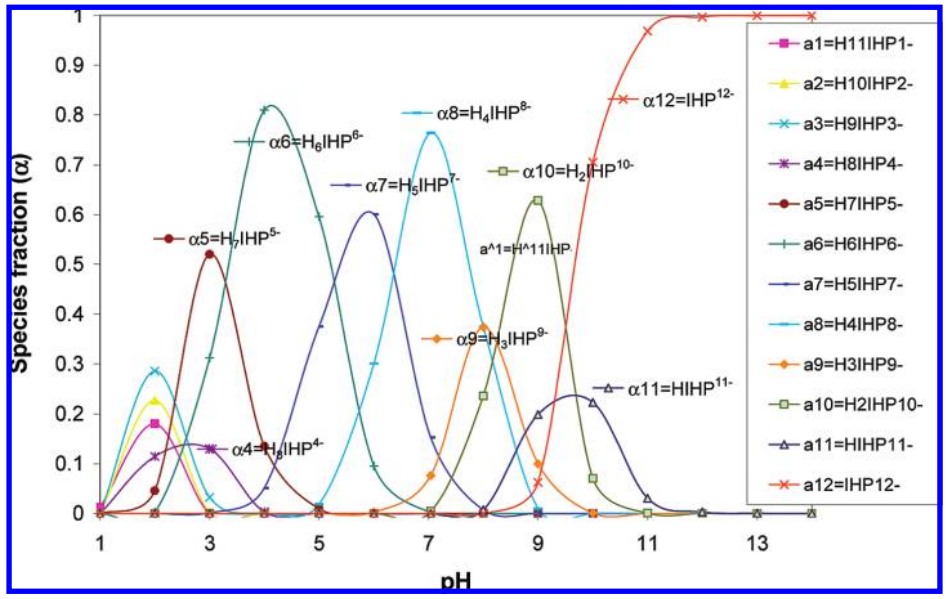

Figure 1. Speciation of *myo*-inositol hexakisdihydrogen phosphate (IHP) derived from acid dissociation constants.

Multiple species are present at relevant environmental pH (**Figure 1**) (*19*).

Proton nuclear magnetic resonance (^{1}H NMR) may be used to monitor the formation and dissociation ratios of ferric and ferrous complexes of IHP. Several groups have successfully investigated IHP bound to the internal cavity of hemoglobin using ^{1}H NMR, thus indicating that ^{1}H NMR can be used to measure IHP in the presence of paramagnetic metal ions (*20, 21*). Additionally, the magnitude of inorganic P recovered from an enzymatic digestion of each of the complexes should be consistent with the NMR data providing an external check of the kinetic and equilibrium data.

MATERIALS AND METHODS

Nuclear Magnetic Resonance. Phytic acid dodecasodium salt *x*-hydrate ($C_6H_6Na_{12}O_{24}P_6 \cdot xH_2O$), with an anhydrous formula weight of 923.82 purchased from Sigma Chemical (St. Louis, MO), was prepared at 5 mM concentration in deuterium oxide (D_2O) purchased from Aldrich Chemical (St. Louis, MO) and deionized water. The deionized water and D_2O were prepared in equal parts by volume. IHP solutions were pH adjusted to 4.0, 6.0, and 8.0 using deuterium chloride (DCl) and sodium deuteroxide (NaOD), both from Aldrich Chemical.

^{1}H NMR spectra were collected on a 300 MHz proton nuclear magnetic resonance instrument using 3 mm 400 series NMR tubes purchased from Aldrich. Complexes of ferric and ferrous iron were prepared using 5 mM IHP solution, which was pH adjusted to 4.5 using the same procedure used to prepare the noncomplexed IHP solution. The pH of the experiment was based on the speciation of IHP (**Figure 1**), the observed solubility of ferric IHP, and the activity of the enzyme (*8*). The complexes were prepared at ratios of 15 mM ferrous or ferric iron to 5 mM IHP. Iron solutions were prepared from iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) from Aldrich Chemical and iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) supplied from Sigma-Aldrich Chemical (St. Louis, MO). The ferrous complex was protected from oxidation by nitrogen (N) gas. The NMR tube was permanently sealed by melting the open end of the tube with a blow torch.

Kinetic and Equilibrium Calculations from NMR Spectra. The ratios of kinetic constants (k) for hydrogen and ferric and ferrous complexes of IHP were derived by monitoring the spectral shift of each complex with ^{1}H NMR. The 12 protons (H) associated with the 6 P groups have acid dissociation constants that participate in trace metal chelation but do not produce the ^{1}H NMR spectra. The P-associated H peaks are indistinguishable from the solvent reference peak. The ^{1}H NMR spectrum of IHP was obtained using D_2O solvent, near the solubility limit of IHP in order to get structural information as a frame of reference for the analysis of iron and IHP complexes in DHO solvent reference system. A direct measurement of the ratio of kinetic rate constant for the metal−IHP complex can be obtained by comparing the peak shifts of the uncomplexed IHP to the peak shifts of the IHP complexes of ferrous and ferric iron in the DHO solvent reference system according to eq 1 (*22, 23*). Line shape broadening has been fully explained by matrix density theory (*24*).

The kinetic formation constant was measured as the chemical shift of the highest peak. Although the iron−IHP complex peaks moved in reference to the uncomplexed IHP peaks, the distance between the maxima of the two broad peaks did not differ in relation to each other, in either ferric or ferrous IHP complexes.

$$AB + CD \underset{k_r}{\overset{k_f}{\leftrightarrow}} AC + BD \quad (1)$$

Fragments A, B, C, and D represent H, deuterium, and ferric or ferrous iron, and the anion was IHP or solvent in a chemically exchanging system at equilibrium. The mean lifetime of exchanges is τ (s) for each species in equilibrium, and it is defined as the rate of formation (F) to its concentration (C) by eq 2.

$$1/\tau_c = F/C \quad (2)$$

The lifetime of exchange can then be described as

$$1/\tau_{AB} = k_f(CD),\ 1/\tau_{CD} = k_f(AB)$$

$$1/\tau_{AB} = k_r(BD),\ 1/\tau_{CD} = k_r(AC)$$

$$k_f/k_r = (AC)(BD)/(AB)(CD)$$

The frequency of the NMR is $1/\tau$ (s^{-1}). The concentrations of the species in the exchanging system were kept constant during each separate NMR experiment. The thermodynamic constants (K_f, eq 3, and K_d, eq 4) were derived from the ratios of the H and metal kinetic constants. By definition, activities of the exchanging system are measured in equilibrium in a NMR experiment (*22*−*24*). The thermodynamic equilibrium constant is described in eq 5

$$K_f = K_{metal}/k_{H+} \quad (3)$$

$$K_d = k_{H+}/k_{metal} \quad (4)$$

$$K = K_f/K_d \quad (5)$$

Enzymatic Analysis. Ferric and ferrous complexes of IHP were incubated at pH 4.5 and 24 °C with 0.03 units/mL of phytase from *A. ficuum* (EC 3.1.3.8) (CAS Registry No. 37288-11-2) purchased from Sigma. The phytase has a specific activity of 3.5 units mg^{-1}. One unit

could liberate 1 mmol of inorganic P from 4.2×10^{-2} M phytate per minute at pH 2 and 37 °C. IHP and metal−IHP complexes were prepared in millimolar ratios of IHP to iron of 1:0, 1:0.6, 1:0.3, and 1:0.1. Incubation occurred over a time course of 15, 30, 60, 90, 1080, 1440, and 2880 min. The highest concentration treatment of ferric and ferrous IHP was governed by the solubility of the ferric IHP treatment. The 1 mM IHP and 0.6 mM ferric iron were allowed to equilibrate at pH 4.5 overnight to ensure that no visible precipitate formed. Controls and samples were prepared just prior to the addition of the enzyme solution.

Each ratio was incubated with enzyme present and enzyme absent, representing samples and controls, respectively. The enzyme was denatured by boiling for 15 min, acidified by adding of 200 μL of 1 M hydrochloric acid to prevent precipitation of ferric P immediately after boiling, and frozen until analysis. Liberated P was analyzed colorimetrically by an ascorbic acid molybdate reaction (*25*). Controls were subtracted from matching samples. The ferrous complexes were protected from oxidation by a blanket of N gas and sealed in polyethylene tubes. Controls and samples were prepared and analyzed in triplicate.

Statistical Analysis. The program SAS 5.1 (*26*) was used to test for statistical differences in phosphate concentration across treatments for the reaction times of 1080, 1440, and 2880 min. Analysis of covariance (ANCOVA) was used to assess differences through time and among treatments. The reaction time 2880 min was chosen to calculate the percent recovery of the 1 mM IHP treatment. Analyses were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Constants Derived from Nuclear Magnetic Resonance Spectra. ^{1}H NMR spectra generated from experiments that include iron are expected to peak broaden and lose structural detail (*20*−*22*). This was observed (**Figures 2** and **3**). Additionally, decrease in baseline resolution and structural resolution should be expected due to the decrease in deuterium concentration in the solvent system (*20*). The following information was obtained for a 250 mM solution of IHP in 100% D_2O (300 MHz, D_2O): δ 4.48 (qd, 2, $J = 8$ Hz, $HPO_4C\ \underline{C}HPO_4CHPO_4$), 4.231 (m, 4, $J = 5.2$ Hz, $HPO_4C\ \underline{C}HPO_4\ CHPO_4$) (**Figure 2**).

Changing the solvent system to HDO and lowering the concentration of IHP to 5 mM produced the expected peak broadening and loss of structural information as well as expected peak shifts due to the change in the solvent. The quadruplet and multiplet were replaced by two broad bands that were consistent at pH 4, 6, and 8 (300 MHz, DHO): δ 4.685 (s, $J =$ 1.3 Hz, $HPO_4C\ \underline{C}HPO_4CHPO_4$), 4.75 (s, $J = 3$ Hz, $HPO_4C\ \underline{C}HPO_4CHPO_4$) (**Figure 3b**). The loss of structural information does not allow for definitive assignment of H, but the intensities of the peaks were equal, indicating an assignment of three H contributing to each broad peak.

The ^{1}H NMR at pH 4.5 for ferrous IHP was as follows (300 MHz, DHO): δ 3.86 (s, 2, $J = 200$ Hz, $HPO_4C\ \underline{C}HPO_4CHPO_4$), 4.09 (s, 4, $J = 200$ Hz, $HPO_4C\ \underline{C}HPO_4CHPO_4$). The ^{1}H NMR at pH 4.5 for ferric IHP was as follows (300 MHz, DHO): δ 4.24 (s, 2, $J = 190$ Hz, $HPO_4C\ \underline{C}HPO_4CHPO_4$), 4.46 (s, 4, $J =$ 250 Hz, $HPO_4C\ \underline{C}HPO_4CHPO_4$) (**Figure 3a**). The same factors that contributed to decreased resolution and peak broadening for the uncomplexed IHP are present in the complexed IHP, but additional peak broadening is attributable to the presence of ferric or ferrous metal.

Changes in pH did not affect the ^{1}H NMR spectra of IHP, making it an appropriate method for monitoring changes in kinetic rate constants (**Figure 3b**). Spectral shifts were attributed to the cation associated with the IHP molecule. The kinetic rate constants for IHP, ferric IHP, and ferrous IHP were 4.69×10^{-6}, 4.21×10^{-6}, and 3.95×10^{-6} s^{-1}, respectively. The experimental equilibrium rate constant for the dissociation of ferric IHP (K_d) was 1.11, and the equilibrium dissociation constant (K_d) for ferrous IHP was equal to 1.19. The experimental formation constants (K_f) are 0.90 and 0.84 for ferric and ferrous IHP, respectively. The experimental kinetic constants derived from ^{1}H NMR indicated that ferrous IHP forms more rapidly and dissociates at a slower rate then ferric IHP, although they are of the same order of magnitude.

Enzymatic Dephosphorylation. Significant differences existed among all of the treatments with the exception of ferric IHP with a treatment concentration 0.6 mM and ferrous IHP with a treatment concentration of 0.3 mM (**Table 1**: ANCOVA $F = 84.16$; $P < 0.0001$). The P model was significant (ANCOVA $r^2 = 0.989$; $F = 343.63$; $P < 0.0001$). Treatment and time covariant with treatment were significant (ANCOVA $F = 84.16$ and 12.47, respectively; $P < 0.0001$). The independent time variable was not significant (ANCOVA $F = 3.45$; $P = 0.0691$). **Table 1** summarizes the average ($n = 3$) P (μg/mL) liberated by the enzyme phytase over the series of incubation times and gives the percent recoveries for time = 2880 min. IHP with a concentration of 1 mM was 98% dephosphorylated at 2880 min. The nonenzyme controls are not reported in **Table 1**. The average ± standard deviation of the control data was 3 ± 1 ($N = 147$). The percent recovery of P

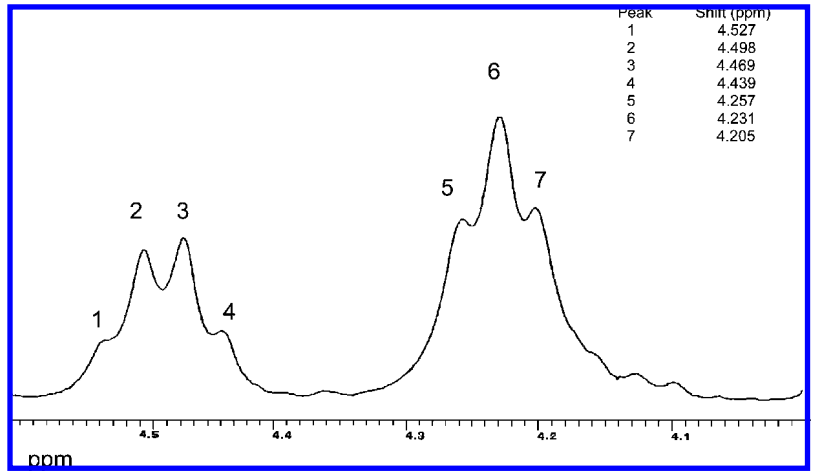

Figure 2. *myo*-Inositol hexakisdihydrogen phosphate (IHP) 250 mM at native pH in D_2O solvent system. The spectrum represents the six hydrogen atoms associated with the six carbon atoms in the ring structure of IHP. The 12 possible acidic protons are associated with the phosphate groups and do not contribute to the ^{1}H NMR.

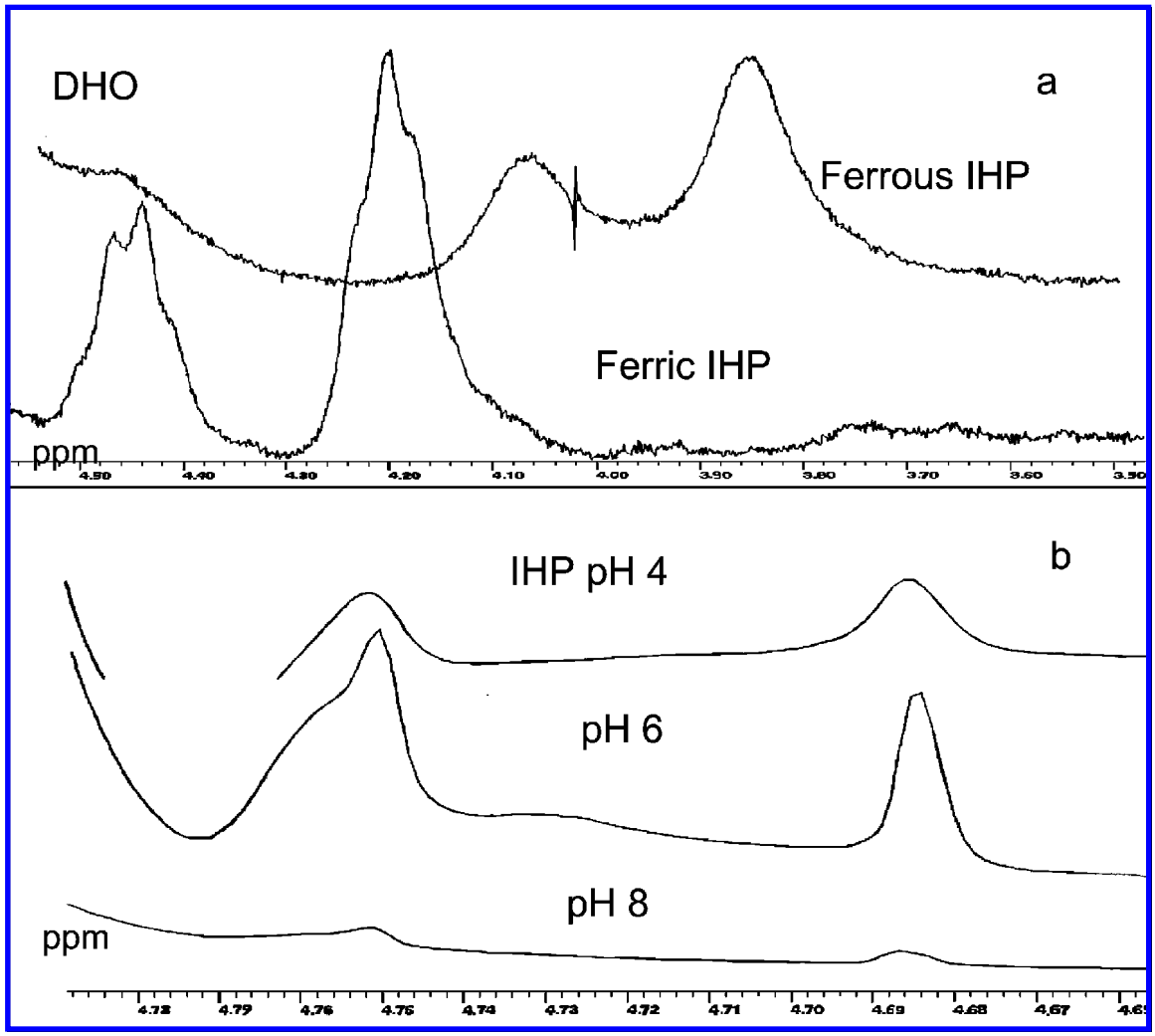

Figure 3. (**a**) ^{1}NMR spectra of 5 mM ferric and ferrous IHP are chemically shifted in the DHO solvent system when compared to (**b**) IHP (5 mM) at pH 4, 6, and 8 in the DHO solvent system. The spectra of IHP and ferric and ferrous IHP lose structural detail and chemically shift when compared to 250 mM IHP at native pH in D_2O solvent system (**Figure 2**).

Table 1. Seven Treatments Were Prepared, Each Contained 1 mM of IHP[a]

treatment	concn (mM) in each treatment	concn (μg/mL) of phosphate recovered	% HPO^{2-} recovered at 2880 min
IHP	1.00a	56.0 ± 0.5	98
ferric IHP	0.1b	38.6 ± 2	68
ferrous IHP	0.1 c	34.6 ± 1	61
ferric IHP	0.3d	30.5 ± 2	54
ferrous IHP	0.3e	19.1 ± 0.5	33
ferric IHP	0.6e	17.2 ± 3	30
ferrous IHP	0.6f	9.7 ± 1	17

[a] Three of the six treatments contained ferrous IHP in the mM concentrations listed. The remaining three treatments contained ferric IHP in the concentrations listed. Recoveries of enzymatically dephosphorylated phosphate ion are in μg/mL. The Tukey group letters denote statistical difference. Treatments with the same letter are not statistically different. The concentration of recovered phosphate in (μg/mL) is a 10-fold dilution of the total concentration.

from the enzymatic dephosphorylation of IHP must account for the 6 mM P per 1 mM IHP.

Ferric and ferrous iron form complexes with IHP that can persist in solution and resist dephosphorylation by the enzyme phytase. The ^{1}H NMR-derived findings are supported by the overall enzymatic dephosphorylation recoveries of P over a 2-day period. The P recovered from the enzymatic dephosphorylation of ferrous IHP was significantly less than the P recovered from ferric IHP and IHP alone. The P dephosphorylated from ferric IHP was also significantly less than the P dephosphorylated from uncomplexed IHP.

IHP has a large charge to mass ratio and an ability to chelate ferrous and ferric iron in solution. Iron is ubiquitous in many soils that receive phosphorus-rich manure inputs, making iron−IHP complexes likely to occur in soil systems that contain iron oxides. In solution, the ferrous IHP complex exhibits a higher equilibrium constant and is soluble at relevant soil pH, implicating subsurface leaching of ferrous IHP complexes as a likely P transport mechanism. Ferric IHP is much less soluble than ferrous IHP at environmentally relevant pH values and may be an important binding site for IHP in iron-containing oxidized soil systems. Soil systems prone to seasonal or periodic soil pore water anoxia may support cycles of fixation and mobility of iron-associated IHP complexes.

ABBREVIATIONS USED

IHP, *myo*-inositol hexakisdihydrogen phosphate; P, phosphate; N, nitrogen; ^{1}H NMR, proton nuclear magnetic resonance; H, proton; D, deuterium; NaOD, sodium deuteroxide; DCl, deuterium chloride; D_2O, deuterium oxide; k, kinetic rate constant; K, thermodynamic rate constant; τ, mean lifetime of exchanges in a chemically exchanging system; ANCOVA, analysis of covariance.

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