Using NMR spectroscopy to elucidate the role of molecular motions in enzyme function

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Abstract

Conformational motions play an essential role in enzyme function, often facilitating the formation of enzyme-substrate complexes and/or product release. Although considerable debate remains regarding the role of molecular motions in the conversion of enzymatic substrates to products, numerous examples have found motions to be crucial for optimization of enzyme scaffolds, effective substrate binding, and product dissociation. Conformational fluctuations are often rate-limiting to enzyme catalysis, primarily through product release, with the chemical reaction occurring much more quickly. As a result, the direct involvement of motions at various stages along the enzyme reaction coordinate remains largely unknown and untested. In the following review, we describe the use of solution NMR techniques designed to probe various timescales of molecular motions and detail examples in which motions play a role in propagating catalytic effects from the active site and directly participate in essential aspects of enzyme function.

1. NMR methods and theory

Molecular motions are critical in the function of enzymes. Enzymes change their conformation multiple times during the catalytic cycle and these motions must occur with timescales commensurate with the rate constants that define the reaction mechanism. Characterization of these motions is essential to understanding their role in enzyme chemistry. Solution NMR spectroscopy is the experimental method of choice for analyzing molecular motions over an enormous timescale range. Solution NMR can be performed under a wide range of aqueous conditions including those that approximate physiological pH and [salt]. Moreover the incorporation of spin-1/2 nuclei into proteins is minimally perturbative and is relatively straightforward. Further, NMR spectroscopy maintains protein integrity, and the experimental library for the study of protein structure and dynamics is constantly improving.

Biomolecular NMR has historically been restricted to proteins smaller than 50 kDa, but novel TROSY[1] methods and ^{13}C -methyl labeling strategies[2-5] now enable the study of much larger proteins through enhanced signal-to-noise (S/N) and resolution.

Here, we briefly review the NMR methods designed to characterize macromolecular motions followed by examining several examples from the authors' work that illustrate some of the biological insight that can be obtained through the use of this powerful technique.

1.1. Picosecond-to-nanosecond motions (ps – ns)

NMR techniques are powerful for the study of atomic-resolution protein dynamics over an enormous time scale ranging from picoseconds (ps) to seconds (s) (**Error! Reference source not found.**).[6-9] Motions on the fast end (ps – ns) of this continuum, which are faster than the overall rotational diffusion of the protein under study, reflect stochastic equilibrium fluctuations in the bond vectors of individual atoms. These stochastic motions modulate the chemical shift anisotropy and dipolar interactions between the nuclei. The identity of nuclei and frequency of the motions determine the rate at which Boltzmann equilibrium is established.[10-14]. A comprehensive review of the theoretical aspects of NMR spin-relaxation is given by Palmer et al.[15-17] Here, we present a shortened overview of the spin-relaxation formalism. The mathematical expressions below are useful for describing a heteronuclear spin-1/2 pair system such as the amide proton-nitrogen (^1H - ^{15}N) located in the protein backbone. The longitudinal, transverse magnetization and cross-relaxation of the ^{15}N heteronucleus relax (R_1 , R_2 , σ_{IS}) or return to their Boltzmann equilibrium state as described by Abragam,[18]

$$R_1 = (d^2 / 4) \left[J(\omega_I - \omega_S) + 3J(\omega_S) + 6J(\omega_I + \omega_S) \right] + c^2 J(\omega_S) \quad (1)$$

$$R_2 = (d^2 / 8) \left[4J(0) + J(\omega_I - \omega_S) + 3J(\omega_S) + 6J(\omega_I) + 6J(\omega_I + \omega_S) \right] + (c^2 / 6) \left[4J(0) + 3J(\omega_S) \right] + R_{\text{ex}} \quad (2)$$

$$S_{IS} = \left(d^2 / 4 \right) \left[J(\omega_I + \omega_S) - J(\omega_I - \omega_S) \right] \quad (3)$$

where ω_I and ω_S are the Larmor frequencies of the I (^1H) and S (^{15}N) nuclei and c is the chemical shift anisotropy coupling constant = $D_{SW_S} / \sqrt{3}$, in which $\Delta\sigma$ is the chemical shift anisotropy value of the S nucleus. R_{ex} is the additional contribution to R_2 that results from conformational exchange motions that occur with μs – ms frequency and is often equal to zero.

The dipolar coupling constant d is described by Equation (4),

$$d = \left(m_0 h g_S g_I / 8 \rho^2 \right) \langle r_{IS}^{-3} \rangle \quad (4).$$

Here, μ_0 is the permeability of free space, h is Planck's constant, γ_I and γ_S are the gyromagnetic ratios of nuclei I and S, and $\langle r_{IS} \rangle$ is the average internuclear bond length for the I and S atoms.

The spectral density function, $J(\omega)$, is the cosine Fourier transform of the autocorrelation function of the I-S bond vector motion.[18]

$$J(\omega) = 2 \int_0^\infty C(t) \cos \omega t \, dt \quad (5)$$

When internal bond vector fluctuations are uncorrelated with the macromolecular rotational diffusion, the total autocorrelation function is obtained as the product of the autocorrelation functions of the overall (C_o) and the internal (C_i) motions.[19]

$$C(t) = C_o(t) C_i(t) \quad (6)$$

For isotropic rotational diffusion, as with a spherical macromolecule, C_o decays as a single exponential as shown in Equation (7);

$$C_o(t) = \frac{1}{5} e^{-t/\tau_c} \quad (7)$$

where τ_c is the rotational correlation time of the protein. The spectral density function describing this motion is expressed in Equation (8).

$$J(\omega) = \frac{2}{5} \frac{\tau_c}{(1 + \omega^2 \tau_c^2)} \quad (8).$$

Thus, for bond vector motions due to rotational tumbling of the protein and internal motions, the spectral density function becomes

$$J(\omega) = \frac{2}{5} \left(\frac{S^2 \tau_c}{1 + (\tau_c \omega)^2} + \frac{(1 - S^2) \tau}{1 + (\tau \omega)^2} \right) \quad (9)$$

with $\tau = \tau_c^{-1} + \tau_e^{-1}$. In Equation (9), τ_e is the effective correlation time for internal motions and S^2 is a generalized order parameter describing the amplitude of internal bond vector motions. S^2 values range from 0 to 1, where lower values indicate enhanced flexibility. Thus, measurement of R_1 , R_2 , and σ_{IS} allow sufficient sampling of the spectral density function to subsequently determine S^2 on a per-residue basis.[20] With some caveats, the magnitude of S^2 obtained by NMR relaxation experiments is also a measure of the configurational entropy of the individual bond vectors in the protein under consideration.[21-23] In addition to the ^{15}N labeled protein backbone, C_α positions as well as sidechain dynamics on the ps – ns timescale can be characterized by NMR spectroscopy.[24-30]

1.2. Microsecond-to-millisecond motions (μs – ms)

Several of the most critical functions of biomolecules occur over the μs – ms time regime (Figure 1) and many of the useful NMR experiments designed to probe the dynamics of these processes are called relaxation dispersion techniques. Relaxation dispersion NMR has been used to characterize protein motions,[31-35] protein folding,[36-38] ligand binding,[39, 40] and enzymatic mechanisms.[41-46] The collection and interpretation of NMR relaxation data depends on conformational fluctuations due to dynamic processes, which are derived and discussed in detail in the following sections.

Molecular motions (μs – ms) involving multiple atoms, amino acids, or elements of protein secondary structure require traversing significant energy barriers. If such motions also involve changes to the magnetic environment of the spin-1/2 nuclei involved in the motional

process then these motions can be studied due to the elevated R_2 value at these flexible sites.

Generically these conformational exchange motions occur between conformations A and B,

where $A \xrightleftharpoons[k_{-1}]{k_1} B$ with forward (k_1) and reverse (k_{-1}) rates constants and fractional populations

p_A and p_B , respectively. The sum of these rate constants is called k_{ex} . As noted above,

fluctuations in the local magnetic field experienced by each NMR-active nuclei due to motional

exchange broaden the resonances and result in an additional contribution (R_{ex}) to the

transverse relaxation rate constant, R_2 , where Equation (1) can be recast as;

$$R_2 = R_2^0 + R_{ex} \quad (10)$$

R_2^0 is determined by ps – ns motions at frequencies near the nuclear Larmor frequency, which include internal bond vector fluctuations and overall rotational diffusion of the protein.

Molecular motions exceeding $\sim 5000 \text{ s}^{-1}$, are best characterized by the off-resonance $R_{1\rho}$ experiment, which is capable of providing insight into motional processes up to $\sim 10^5 \text{ s}^{-1}$. [31] In the off-resonance $R_{1\rho}$ experiment (Equation (17)) [47] an effective field (ω_e) is used to irradiate the protein where modulation of ω_e affects the contribution of molecular motions to the measured relaxation rate. [48]

$$R_{1\rho} = R_1 \cos^2 q + R_2 \sin^2 q + \frac{\sin^2 q p_A p_B D w^2 k_{ex}}{W_{Ae}^2 W_{Be}^2 / W_e^2 + k_{ex}^2} \quad (11).$$

Because the resonance frequencies differ for the nuclei involved in the conformational

exchange process, the effective magnetic fields for sites A and B are expressed as $\omega_{Ae/Be}^2 =$

$\omega_{A/B}^2 + \omega_1^2$ and $\omega_e^2 = \omega_{iso}^2 + \omega_1^2$. The frequency offsets from the RF carrier of site A, site B, and

population-averaged resonances are ω_A , ω_B , and ω_{iso} , respectively. The RF field strength is ω_1

with a tilt angle $\theta = \arctan(\omega_1 / \omega_{iso})$. In the case of fast conformational exchange where $k_{ex} >$

$\Delta\omega$, characterization of motions from the $R_{1\rho}$ experiment is simplified and uses Equation (18), in

which

$$R_{1r} = R_1 \cos^2 q + R_2 \sin^2 q + R_{ex} \quad (12)$$

where R_{ex} is:

$$R_{ex} = \frac{f_{ex}}{k_{ex}^2 + W_e^2} \sin^2 q \quad (13)$$

and $\Phi_{ex} = p_a p_b \Delta \omega^2 / k_{ex}$. Kinetic processes occurring with rate constants between a few hundred to 5000 s^{-1} are characterized by the relaxation-compensated Carr-Purcell-Meiboom-Gill (rcCPMG) experiment.[32],[49] Relaxation dispersion of the CPMG type utilizes the fact that when 180° radio frequency (RF) refocusing pulses are applied to a system undergoing conformational exchange, the dephasing of the ensemble of spins due to visitation of different magnetic environments is disrupted by these RF pulses. The extent of this modulation depends on the relationship between the pulsing frequency (τ_{cp}^{-1}) and k_{ex} . This modulation manifests as τ_{cp} -dependent differences in the exchange contribution to R_2 . [50-52] These CPMG experiments can be performed on single (SQ), [32, 49] double, [53] zero, [53] or multiple quantum (MQ) [54] coherences. Standard indirect-detection probes typically enable shorter ^{13}C pulses than ^{15}N pulses, thus the duty cycle for ^{13}C CPMG experiments can often be greater than those for ^{15}N -based experiments. Below we describe the SQ and MQ experiments as they are more frequently used.

The basis of the single-quantum rcCPMG experiment is given by Equation (20) where the measured transverse relaxation rate ($R_2(1/\tau_{cp})$) varies as the 180° pulse spacing (τ_{cp}) is altered, [50-52]

$$R_2(1/\tau_{cp}) = \frac{1}{2} \left(R_{2A}^0 + R_{2B}^0 + k_{ex} - \frac{1}{\tau_{cp}} \cosh^{-1} \left[D_+ \cosh(h_+) - D_- \cosh(h_-) \right] \right) \quad (14)$$

$$D_{\pm} = \frac{1}{2} \left[\pm 1 + \frac{Y + 2D\omega^2}{(Y^2 + Z^2)^{1/2}} \right]; \quad h_{\pm} = \frac{t_{cp}}{\sqrt{2}} \left[\pm Y + (Y^2 + Z^2)^{1/2} \right]^{1/2};$$

$$Y = (R_{2A}^0 - R_{2B}^0 - p_A k_{ex} + p_B k_{ex})^2 - D\omega^2 + 4p_A p_B k_{ex}^2; \quad Z = 2D\omega(R_{2A}^0 - R_{2B}^0 - p_A k_{ex} + p_B k_{ex})$$

in which R_{2A}^0 and R_{2B}^0 are the intrinsic transverse relaxation rates of the two sites in the absence of conformational exchange.

The expression described by Equation (14) is useful when conformational motion is in slow to intermediate exchange ($k_{ex} \leq \Delta\omega$). Equation (14) simplifies under fast exchange conditions ($k_{ex} > \Delta\omega$). In this exchange regime, conformational exchange processes characterized by the rcCPMG experiment are described by Equation (21).[55]

$$R_2(1/t_{cp}) = R_2^0 + f_{ex} / k_{ex} [1 - 2 \tanh(k_{ex} t_{cp} / 2) / (k_{ex} t_{cp})] \quad (15)$$

where $\phi_{ex} = p_A p_B \Delta\omega^2$. A full characterization of the exchange parameters for a particular motional process necessitates acquisition of experimental NMR data at two or more static magnetic fields and/or multiple temperatures.[56-58]

rcCPMG NMR was first applied to proteins for the study of backbone amide groups,[32] but there have been several adaptations to other spin-1/2 pairs.[54, 59] Another variant of this experiment for characterization of sidechain methyl groups such as Ile, Leu, Val, and Ala residues is denoted as the multiple quantum (MQ) relaxation dispersion experiment.[54, 59] If sufficiently high-quality data are obtained, the MQ dispersion experiment has the added benefit of providing $\Delta\omega_H$ and $\Delta\omega_C$, which could yield additional structural information on the lowly populated conformation.

In total the CPMG and $R_{1\rho}$ experiments report on the kinetics of the conformational motion from $10^2 - 10^5 \text{ s}^{-1}$, provide the equilibrium populations of the interconverting conformations, and elucidate structural information about ground and higher energy conformations through analysis of $\Delta\omega$ values.[60-62] Incorporation of TROSY enhancement into

any of these experiments enables studies of very large proteins or macromolecular assemblies (≥ 100 kDa).[1] Below, applications of these NMR experiments for the study of enzyme function are reviewed. Only three examples are covered here, yet there are many excellent studies in the literature to which the interested reader is referred for additional information. [46, 63-95]

2. Ribonuclease A (RNase A)

RNase A is a 14 kDa monomeric enzyme that catalyzes the transphosphorylation of single stranded RNA with specificity for pyrimidines on the 3' side of the bond to be cleaved and purines on the 5' side of this bond. The slow step in the enzyme-catalyzed reaction has been demonstrated to be product release[96] while biophysical experiments have provided evidence of conformational changes in the enzyme that accompany ligand binding at the active site.[97]

2.1. Fast dynamics

Changes in ps – ns motions contribute to the configurational entropy changes in proteins [21-23, 98] and there are often compensating changes in S^2 values upon ligand binding.[99] To address the role of these fast timescale motions in RNase A function, measurement of the temperature and ligand dependence of S^2 was performed.[100] To achieve this, the backbone dynamics of RNase A were characterized in the apo state and bound to a non-cleavable, high-affinity substrate analog, 5'-phosphothymidine(3',5') pyrophosphate adenosine 3'-phosphate (pTppAp). pTppAp binds to the RNase A active site and is a mimic of the pre-catalytic ES complex. With few exceptions, the S^2 values in RNase A increase when bound to pTppAp (Figure 2). Thus, RNase A pays an entropy penalty, in the form of reduced amplitude of backbone motion, upon binding the high-affinity substrate analog. The global effect of restricted motion upon binding pTppAp stands in contrast to the local perturbations of NMR chemical shifts, which primarily occur at the active site. Additional insight comes from the temperature dependence of S^2 values.[86] The overall average S^2 values for apo and liganded RNase A have the same temperature dependence ($dS^2/dT = 0.0014 \text{ K}^{-1}$). This indicates no global

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4 contribution to the overall heat capacity change due to changes in dynamics. However, on an
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6 individual residue basis there are significant differences in the temperature dependence of S^2
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8 (Figure 3). Thus, the differing $d(1-S)/dT$ values are offset such that dynamical changes to ΔC_p
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10 $= 0$. Interestingly, the temperature dependence of S^2 for the individual secondary structure units
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12 becomes more uniform when RNase A is bound to pTppAp compared to apo RNase A (Figure
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14 4). This would suggest that binding of pTppAp facilitates a more collective dynamic behavior
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16 across the protein with a more uniform shape of the protein energy landscape for RNase A in
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18 the bound state.
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24 25 **2.2. Slow timescale motions ($\mu s - ms$)**

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27 Early work using CPMG relaxation dispersion studies identified a two-site conformational
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29 exchange process at multiple regions throughout the enzyme occurring at a rate of exchange
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31 (k_{ex}) of $1700s^{-1}$. [41] These results were intriguing because this conformational exchange rate
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33 constant is identical to the RNase A k_{cat} value and to the product release rate constant (k_{off})
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35 determined by NMR lineshape analysis. [42] Product release is known to be rate-limiting in
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37 RNase A [101] and thus this initial work suggested that the conformational change measured by
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39 NMR might be coupled to the rate-limiting step in enzyme turnover. To examine the role of ms
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41 motions in RNase A function, NMR relaxation dispersion experiments were performed on
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43 RNase A at various states along the enzymatic reaction coordinate including apo, substrate
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45 analog-bound (pTppAp), and product-bound (3'-cytidine monophosphate (3'-CMP))
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47 complexes. [43] The structural changes in the backbone conformation as RNase A proceeds
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49 from the unliganded state to the ES complex are shown crystallographically to be localized to
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51 active site Loop 4 and Loop 1, which is located $\sim 20 \text{ \AA}$ from the active site. [43, 102] A similar set
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53 of structural changes occurs in the product state with the exception of Loops 4 and 1, which
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55 appear to be intermediate between the E and ES forms. The timescale of conformational
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1 motions in these complexes is largely unchanged compared to the apo state and remains
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4 around 1700 s⁻¹ at 20° C. Moreover, these ms motions are confined to similar locations in Loop
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7 1 and Loop 4 in the apo, ES, and EP states.[43] Whereas the kinetics of motion remain
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10 constant, they reflect different processes in each complex. Further, the energetics obtained by
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13 temperature-dependent relaxation dispersion methods are similar in each complex, with an
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16 activation energy of ~5 kcal/mol. In total, these data suggest that motion in RNase A is a two-
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19 site process whereby ligand binding shifts the conformational equilibrium between the two
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22 species. Insight into the structure of the exchanging conformations can be obtained from the $\Delta\omega$
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25 values determined from fits to the relaxation dispersion data.[103] The $\Delta\omega$ values or so-called
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28 dynamic chemical shift difference, when compared between different enzyme complexes, can
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31 inform on the relation between the interconverting structures and those structures induced by
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34 ligand binding. As shown in Figure 5, $\Delta\omega$ values for residues in and near Loop 1 in RNase A
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37 when bound to pTppAp are equivalent to the calculated $\Delta\omega$ values for apo RNase A.
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40 Furthermore, the signs of these $\Delta\omega$ as determined from a comparison of HMQC and HSQC
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43 experiments[104] are opposite. This suggests that Loop 1 in apo RNase A samples the bound
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46 conformation in the absence of pTppAp and when pTppAp is bound to RNase A, this loop
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49 samples a minor conformation that resembles the apo enzyme.[105] Loop 1 is approximately 20
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52 Å from the active site but is linked to Thr45 via a series of hydrogen bonds. Thr45 is located at
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55 the active site and is one of the residues that plays a role in pyrimidine specificity.[106, 107]
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58 This observation raised the question of whether motions at Loop 1 could be propagated to the
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61 active site to facilitate disruption of the Thr45-substrate interaction and thus enable product
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64 dissociation and if so, by what mechanism.
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66 To address this question in more detail, experiments were performed to focus on the
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69 catalytic role of molecular motions that occur distant from the active site. Kinetic solvent isotope
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72 effects (KSIE) are commonly used to elucidate chemical reaction mechanisms. In the case of
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4 RNase A, KSIE were exploited to ascertain the role of hydrogen bonds in the conformational
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6 exchange process. It was demonstrated that the rate of conformational motion in RNase A
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8 decreased with increasing deuterium solvent content (Figure 6).[44] These data indicated that
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10 during the conformational motion of RNase A, one or more solvent exchangeable protons were
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12 involved in the transition state of this process. Further, the linearity of the isotope exchange
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14 studies indicated that there was a single dominant proton transfer step in the conversion
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16 between apo-like and bound-like conformations in RNase A.[44] Additional studies narrowed the
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18 location of the proton transfer site that is crucial to the motion in RNase A to the Loop 1 region
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20 and the surrounding β -sheets (Figure 7).[108] In this work it was noted that ms motions of
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22 residues in this region were sensitive to deuterium content in the solvent, whereas flexible
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24 residues in other parts of RNase A had conformational exchange rate constants that were
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26 unaffected by increasing D₂O concentrations. This indicated that despite the identical k_{ex} values
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28 for the solvent sensitive and insensitive regions in RNase A, the motions in each region must be
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30 distinct processes and their similarity in H₂O was merely coincidental. Moreover, because the
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32 solvent isotope effect ($k_{cat}^H / k_{cat}^D = 2.0 \pm 0.4$) on RNA transphosphorylation is the same as the isotope
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34 effect on conformational dynamics ($k_{ex}^H / k_{ex}^D = 2.2 \pm 0.3$) the motions in these regions were suggested
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36 to be coupled to the rate-limiting step in catalytic turnover.[108] Previous biochemical and
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38 biophysical studies suggested that histidine 48, located in the vicinity of Loop 1, was involved in
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40 a conformational change in RNase A.[109, 110] H48 is located on β -strand 1 and its imidazole
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42 group points into the Loop 1 region. To investigate whether protonation of this residue was
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44 responsible for the isotope sensitive motion in RNase A, H48 was mutated to alanine.[108] The
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46 H48A mutant experiences a 10-fold decrease in catalytic activity despite the imidazole group
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48 being 18 Å from the catalytic site. Interestingly, the H48A variant experiences no deuterium
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50 solvent isotope effect on k_{cat} , unlike the WT, which has a KSIE value of 2. This suggests that the
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52 origin of the isotope effect on enzyme function arises from the H48 residue. NMR relaxation
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4 dispersion studies on the H48A variant indicate the loss of ms motions in all previously flexible
5 residues that also had sensitivity to D₂O (gold spheres in Figure 7, Figure 8). In contrast,
6 residues in WT RNase A that maintained ms motions in H₂O and D₂O were unaffected by
7 mutation of H48 (Figure 8) further confirming the motional independence of these two regions of
8 RNase A. These data further indicate that the site of the exchangeable proton that plays a
9 prominent role in enzyme function and conformational motions is located near H48.
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17 Additional studies were undertaken to attempt to address the details of the interactions
18 between H48 and Loop 1 and their role in RNase A function. Primary sequence analysis and X-
19 ray crystallography studies of RNase homologs indicated that they possessed a shorter Loop 1
20 and substantially lower rates of catalysis. Doucet et al swapped the shorter loop from the RNase
21 A homolog eosinophil cationic protein (ECP) in place of that loop in RNase A, creating
22 RNaseA_{ECP} (Figure 9).[111] This chimeric enzyme had 10x lower catalytic activity as well as a
23 10-fold decrease in the product release rate constant, k_{off} . [111] Importantly, as shown by NMR
24 CPMG relaxation dispersion studies, flexible residues that are involved in product release
25 showed a total loss of ms motions in the chimera. These residues are also those that showed
26 sensitivity to solvent deuterium content. These studies further support the role of the flexible
27 Loop 1 in RNase A activity, as the shorter ECP loop has a limited range of motion, inhibiting
28 product release in RNase A as well as abolishing all motions in the crucial active site β -strands.
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44 In an effort to more accurately define the interactions involved in propagating motion at
45 Loop 1 and H48 to the active site, studies were directed to two threonine residues (T17 and
46 T82) that form hydrogen bonds with H48.[112] T17 is located in Loop 1 and T82 is located on β -
47 sheet 4, which comprises part of the active site RNA recognition region (Figure 11). The results
48 of the T17A and T82A mutations highlight the exquisite and finely tuned nature of the
49 interactions and their impact on propagation of these ms motions. The T82A mutation results in
50 a complete loss of ms motions in a manner similar to that observed in RNaseA_{ECP} as well as a
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10-fold decrease in affinity for the product analog (3'-CMP) of the RNase A reaction (Figure 12). Additional analysis indicated that the motions in the T82A variant are shifted to a faster timescale ($>5000\text{ s}^{-1}$) but not abolished. The effects of the T17A mutation are subtler than those seen with T82A. Residues, A5, Q11, S15, S18, S22, M30, S32, N34, L35, F46, V47 retain ms motions, albeit at an elevated rate constant of 3200 s^{-1} compared to 2000 s^{-1} for WT (Figure 12). Intriguingly, k_{off} for 3'-CMP in T17A is also slightly elevated to 3000 s^{-1} compared to its WT value of 2000 s^{-1} . These results suggested that T17 is involved in dampening, for an unknown reason, ms motions that are directly involved in product release. In contrast, removal of the sidechain of T82 results in elevated ms motions with minimal effect on k_{off} , although T82A reduces k_{on} by 10-fold as observed by NMR lineshape analysis. These studies illuminate how residue-specific participation in protein motions can be deciphered and linked to specific aspects of the catalytic cycle.

Specific focus on H48 provided further insight into its role in functional motions of RNase A. The central involvement of H48 was underscored by its mutation to alanine that resulted in complete loss of ms motions in critical regions of the enzyme as well as a significant loss in catalytic activity.[111] To further analyze the role of H48, an unnatural amino acid, 4-thiamethyl imidazole (RNase A-4MI) was incorporated into uniformly ^{15}N -labeled RNase A.[113] This enzyme was studied by kinetic assay, solution NMR, and computational methods. The additional methylene group and sulfur atom in the 4MI increases the sidechain volume by $35\text{ \AA}^3$ and slightly alters the imidazole pK_a . The 4MI enzyme retained WT catalytic activity and had ms motions similar to those of the WT enzyme; however, the pH dependencies of these motions were not identical to those of WT. This suggested that the altered pK_a of the unnatural imidazole was partly responsible. MD simulations indicate that H-bonds at crucial sites are maintained, but were not identical to those of the WT enzyme. Moreover, the pattern of correlated motions observed during the simulation is different for the two enzymes. These data suggest that, in

part, protein dynamics could be uncoupled from activity, which has interesting implications for the enzyme design field.

Overall, this work demonstrates the involvement of numerous residues in conformational motions of RNase A and highlights the complex physical chemistry at play in modulating these motions. RNase A possesses two flexible regions that move independently (called cluster 1 and cluster 2) yet at room temperature possess the same k_{ex} values (Figure 13). This complexity was recently studied in a series of temperature and pH dependent CPMG relaxation dispersion experiments.[114] Many residues within RNaseA showed evidence of ms motions at all temperatures (10, 15, 20, 25 °C) and pH values >6.4 (Figure 14). In several cases, these motions became too fast for the CPMG experiment at pH values ≤ 5.5 . Residues in dynamic cluster 1 were found to be more sensitive to changes in pH than those of cluster 2, and gradual increases in k_{ex} were observed with increasing temperature ($T = 10 \rightarrow 25$ °C, $k_{\text{ex}} = 980 \rightarrow 1970$ s⁻¹ at pH 7.0) and decreasing pH (pH = 7.0 $\rightarrow$ 4.5, $k_{\text{ex}} = 1970 \rightarrow >3000$ s⁻¹). At a pH of 4.5, no CPMG dispersion was observed in cluster 1 at any of the temperatures tested. However, ¹⁵N R_{1ρ} relaxation dispersion experiments revealed the presence of conformational motions (Figure 14), and global fits to these data yielded $k_{\text{ex}} = 13400$ s⁻¹ for cluster 1. Interestingly, k_{ex} values for cluster 2 residues were insensitive to pH values above 4.5, and low pH k_{ex} values for cluster 2 residues were also determined by R_{1ρ} methods. However, temperature-dependent variations in k_{ex} on the CPMG timescale were found to be greater in cluster 2 when compared with cluster 1.

To develop a stronger understanding of the thermodynamic principles underlying these temperature and pH-dependent motional changes, the enthalpic and entropic contributions to the energy landscape of RNaseA were analyzed for cluster 1 and cluster 2 under various conditions (Figure 15). Lowering the pH from 7.0 to 6.4 increases the motional activation energies of cluster 1 ($E_{\text{a,fwd}}$ and $E_{\text{a,rev}}$) by 3.9 and 1.6 kcal/mol, respectively. Similar changes in $\Delta H^{\ddagger}_{\text{fwd/rev}}$ were observed; however, a net stabilization of $\Delta G^{\ddagger}$ for the motion of cluster 1 resulted from a favorable overcompensation of the activation entropy. $\Delta G^{\ddagger}$ was found to be >10 kcal/mol

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4 for both forward and reverse motions of cluster 1, which is only slightly larger than reported
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6 energies of product dissociation ($\Delta G^{\ddagger}_{\text{diss}} \sim 6.5$ kcal/mol), [115] consistent with cluster 1 being
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8 linked to product release.
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11 Cluster 2, which is pH insensitive over the range of 5.5-7.0, showed little change in its
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13 activation parameters over this range. However, at pH 4.5, when cluster 2 dynamics shift to a
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15 faster timescale, the transition state free energy is stabilized by ~ 1 kcal/mol for both
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17 components of the motion ($\Delta G^{\ddagger}_{\text{fwd}}, \Delta G^{\ddagger}_{\text{rev}}$). Near neutral pH, entropy was the dominating
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19 contributor to the free energy difference ($\Delta\Delta G$) between the major and minor conformational
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21 states of cluster 1. However, as the pH is lowered, the major conformation became more
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23 enthalpically favored, as the minor state was destabilized by ~ 2.3 kcal/mol ($\Delta\Delta H$). In contrast,
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25 the enthalpic and entropic terms governing the conformational states of cluster 2 were
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27 essentially identical over the pH range examined, with small changes in these parameters
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29 arising at low pH.
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33 To build upon these results, values of $\Delta\omega$, the ^{15}N chemical shift difference between the
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35 exchanging conformational states were plotted as a function of pH for clusters 1 and 2 (**Figure**
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37 **16**). All residues in dynamic cluster 1 showed pH dependence to their $\Delta\omega$ values, while cluster 2
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39 residues showed no pH dependence. Although there was good correlation of $\Delta\omega$ for cluster 1 at
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41 various pH values, these data were best approximated with a line of slope 1.3, while $\Delta\omega$ for
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43 cluster 2 fits to a line of slope 1 at all pH values (**Figure 16B**). Both clusters showed similar
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45 distributions of $\Delta\omega$, where cluster 1 ranged from $0.99 \leq \Delta\omega \leq 3.25$ ppm and cluster 2 from 0.86
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47 $\leq \Delta\omega \leq 3.08$ ppm. While several residues within each cluster showed comparatively high (or
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49 low) values of $\Delta\omega$, correlations between a residue's location and the magnitude of $\Delta\omega$ have yet
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51 to be identified. These data for cluster 1 suggest that this region interchanges between a highly
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53 populated conformation and a pH-dependent averaged conformation that exists in rapid
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55 equilibrium between a protonated and deprotonated conformer.
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4 The role of NMR in elucidating dynamic contributions to RNaseA function has been
5 described in detail. CPMG ($R_{1\rho}$) dispersion experiments have been utilized to examine slow
6 (fast) timescale dynamics of residues near the active site and at distant sites, ultimately
7 identifying two independently functioning dynamic clusters. Further, these NMR results have
8 provided additional insight into the role of H48 dynamics in RNaseA function, and established
9 free energy landscapes describing the temperature- and pH-dependence of RNaseA motions.
10 The extensive and detailed nature of these studies demonstrate how intricate protein motions
11 can be and furthermore that their relation to enzyme function is not straightforward. NMR was
12 integral in deciphering the role that these motions play in the catalytic cycle. Nonetheless, other
13 biochemical and biophysical experiments were necessary for a full understanding. We anticipate
14 that this multi-faceted approach will be crucial for studies of other enzymes as well.
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31 **3. Protein Tyrosine Phosphatases (PTP1B and YopH)**

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33 PTP1B and YopH are structurally similar protein tyrosine phosphatases. PTP1B is
34 responsible for regulating insulin and leptin signaling and has been a target of intense drug
35 discovery efforts.[116] YopH is an essential enzyme for *Yersinia* virulence and plays a role in
36 disrupting the human phagocytic process.[117] Despite their different origins, PTP1B and YopH
37 are structurally and mechanistically indistinguishable (Figure 17). PTP1B and YopH each
38 contain essential ten residue active site loops (WPD loop) and conserved eight residue P-loops,
39 which bind a substrate phosphate group and contain nucleophilic residues C215/C403
40 (PTP1B/YopH). The active site WPD loop contains the acidic residue D181/D356
41 (PTP1B/YopH) that promotes protonation of the tyrosine leaving group during catalysis, and a
42 10 Å motion in the position of the WPD loop accompanies the transition from apo (open) to
43 active (closed) enzyme (Figure 17B,C) Both enzymes cleave the phosphate from their tyrosine
44 phosphate (pY) containing substrates in two steps. First, the WPD loop closes on the pY
45 substrate and catalyzes cleavage (k_{cleavage}), generating a phosphoenzyme intermediate and a
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tyrosine peptide. Second, the phosphoenzyme is hydrolyzed ($k_{\text{hydrolysis}}$), releasing inorganic phosphate (P_i) and regenerating the active enzyme (Figure 17D).[116]

PTP1B and YopH have essentially identical mechanisms, and KIE experiments support identical transition states as well.[118-120] However, k_{cat} values for the two enzymes are significantly different, as YopH is over 20-fold more active than PTP1B (*i.e.* 700-1000 s^{-1} vs 15-30 s^{-1} at pH 6.6, respectively).[119, 121, 122] More specifically, k_{cleavage} , which is directly influenced by the rate of active site loop closure, has been shown to vary from 1400-2000 s^{-1} in YopH to 25-80 s^{-1} in PTP1B.[123] The effects of WPD loop motion on the rates of catalysis in PTP1B and YopH have been investigated using solution NMR spectroscopy and have shown that loop closure dynamics plays a large role in the kinetic differences between the enzymes.[124]

3.1. Characterization of the apo enzymes

In a series of ^{15}N CPMG relaxation dispersion experiments, Whittier et al have shown that apo PTP1B and YopH behave quite differently.[124] Of the assigned residues within the WPD loop of PTP1B, W179, F182, and A189 showed dispersion (Figure 18B,C) with individual k_{ex} values each approximating 900 s^{-1} , suggesting concerted loop motion. A subsequent global fit of these data revealed equilibrium exchange populations of $97.5 \pm 1.4\%$ (p_a) and 2.5% (p_b). Using k_{ex} and p_a , the rates of loop opening (k_{open}) and closure (k_{close}) for apo PTP1B were determined to be $890 \pm 190 \text{ s}^{-1}$ and $22 \pm 5 \text{ s}^{-1}$, respectively. Based on these data and numerous X-ray crystallographic studies on apo PTP1B, the major conformer was suggested to contain an open WPD loop, while the minor state ($p_b = 2.5\%$) contains a closed WPD loop. Interestingly, a single X-ray crystal structure of apo PTP1B is shown with a closed WPD loop,[125] suggesting that a closed conformation is accessible in the apo state. This interpretation is consistent with measured differences in the chemical shifts ($\Delta\delta$) of the open and closed states, which are in good agreement with shifts calculated from X-ray crystal structures. Thus, the WPD was found

to convert between open and closed states at a rate of $22 \pm 5 \text{ s}^{-1}$, directly in the regime of k_{cleavage} ($25\text{-}80 \text{ s}^{-1}$), and suggested loop closure may occur simultaneously with leaving group protonation during the first step of catalysis.

Similar ^{15}N CPMG studies of apo YopH showed flat dispersion plots for each loop residue (T358, A359, V360, S361), suggesting that any loop motions occurred outside the μs -ms time regime. Faster motions were then probed with TROSY-enhanced ^1H -R_{1ρ} experiments and dispersion was observed for A359 and S361 (Figure 18D), yielding a global $k_{\text{ex}} = 43000 \pm 6200 \text{ s}^{-1}$. Additional information on the rate of YopH WPD loop motion was obtained from a TROSY Hahn-Echo experiment, where the closure rate (k_{close}) was found to be $1240 \pm 280 \text{ s}^{-1}$. Like PTP1B, k_{close} for YopH occurs in the same regime as k_{cleavage} ($1400\text{-}2000 \text{ s}^{-1}$), consistent with direct involvement of loop closure in pY cleavage. These data were consistent with temperature-jump fluorescence experiments that reported similar rates of loop interconversion. However, data from fluorescence anisotropy and molecular dynamics investigations reported faster loop motions.[126-128]

3.2. Peptide-bound (substrate mimicked) complexes

Complexation of PTP1B with the representative substrate peptide Ac-DADEXLIP-NH₂[116] (where X = difluoromethylphosphono-phenylalanine, a non-cleavable substrate mimic) resulted in significant broadening of several WPD loop residues, namely W179 and F182, indicative of a new motional process. Further, CPMG dispersion curves at 600 and 800 MHz for A189 were different from those of the apo enzyme and showed oscillation at lower pulse frequencies stemming from slow conformational exchange (Figure 19). However, an identical value of $\Delta\omega$ in the apo and peptide-bound states ($\Delta\omega = 3.2 \pm 0.5 \text{ ppm}$) of PTP1B suggested that the WPD loop still sampled the open and closed conformations. A single X-ray crystal structure of substrate-bound PTP1B now shows an open WPD loop, again suggesting that both conformations are accessible.[129] From these data, the rate of loop closure was determined to

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4 be $30 \pm 4 \text{ s}^{-1}$, in line with that of the apo enzyme. Thus, peptide binding had minimal effect on
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6 k_{close} , while loop opening was slowed to $5 \pm 1 \text{ s}^{-1}$, further demonstrating the linkage between
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8 loop closure and substrate cleavage rates.
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11 Loop motions in the peptide-bound YopH complex were slowed considerably, falling
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13 back into the CPMG exchange regime. Global fits of CPMG dispersion data for loop residues
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15 A359 and S361 gave $\Delta\omega$ values of 3.5 and 4.5 ppm, respectively, and were similar to those
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17 determined for the apo enzyme. An overall k_{ex} for the YopH WPD loop was determined to be
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19 $1790 \pm 240 \text{ s}^{-1}$ with a population of closed conformer above 99%. The resulting k_{close} value was
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21 found to be $1770 \pm 240 \text{ s}^{-1}$, similar to the value of k_{cleavage} , again suggesting a close connection
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23 between loop motion and the first catalytic step. Interestingly, even with differing substrate
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25 cleavage rates, the product (EP complex) mimic tungstate was found to have identical
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27 dissociation rates from both PTP1B and YopH and suggests that the rate of product release is
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29 similar in both enzymes.
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33 Studies of these related protein tyrosine phosphatases, particularly those of Whittier et
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35 al, have shown that loop motions in two members of the tyrosine phosphatase enzyme family
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37 are closely correlated with a key step in the catalytic cycle, namely substrate cleavage and
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39 protonation of the leaving group. These data have also shown that ligand binding at the active
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41 site modulates loop kinetics, especially the rate of opening, and highlighted a dynamic energy
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43 profile describing tyrosine phosphatase function.
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49 **4. Concluding remarks**

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51 Solution NMR spectroscopy remains at the forefront of techniques designed to
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53 characterize structural and dynamic properties of biomolecules. Advances in the experimental
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55 library of NMR methods have only enhanced the possibility for atomic level information
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57 regarding processes such as ligand binding, catalysis, and allostery. The case studies outlined
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59 in this review provide examples of the power of NMR in monitoring molecular motions that are
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4 directly involved in an enzyme's catalytic cycle and the propagation of those motions throughout
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6 the protein architecture. Coupled with other biophysical and computational methods, solution
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8 NMR promises to play a pivotal role in establishing new insight into biomolecular processes for
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10 years to come.
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15 **Acknowledgements:**

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17 JPL acknowledges support from NSF grant MCB1121372 and NIH grant GM112781.
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22 **Abbreviations:**

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24 NMR, nuclear magnetic resonance; TROSY, transverse relaxation-optimized spectroscopy;
25 CPMG, Carr-Purcell-Meiboom-Gill pulse sequence; RF, radio-frequency; SQ, single quantum;
26 MQ, multiple quantum; RNase A, ribonuclease A; pTppAp, 5'-phosphothymidine(3',5')
27 pyrophosphate adenosine 3'-phosphate; ES, enzyme-substrate complex; EP, enzyme-product
28 complex; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single
29 quantum coherence; KSIE, kinetic solvent-isotope effect; ECP, Eosinophil Cationic Protein;
30 3'cMP, 3'-cytidine monophosphate; 4MI, 4-thiamethyl imidazole; PTP1B, protein tyrosine
31 phosphatase 1B; YopH, protein tyrosine phosphatase YopH from *Yersinia*
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Figure 1. Timescale depicting enzyme motions. (Top) A cartoon representation of the types and variety of enzyme motions. (Bottom) A list of the types of solution NMR experiments and the timescale of motions to which they are sensitive.

Figure 2. Temperature-dependent S^2 values for RNase A for apo (open circles) and pTppAp bound (filled circles). Breaks in the lines indicate residues in which quantitation of dynamical parameters was not possible. Error bars have been omitted to aid in visualization of the data trends. Uncertainties in S^2 values for the data shown, in all cases, is $\sim 1\text{--}2\%$. Adapted with permission from [100]. Copyright 2003 American Chemical Society.

Figure 3. Temperature dependence of the order parameters for RNase A. The values of $1-S$ for glutamine 11, lysine 41, and threonine 99 are shown as a function of temperature for apo RNase A (open circles/solid lines) and RNase A/pTppAp (filled circles/dashed lines). The slopes of the fitted lines are Gln11: $7.5 \pm 0.4 \cdot 10^{-4} \text{ K}^{-1}$, $2.9 \pm 2.6 \cdot 10^{-4} \text{ K}^{-1}$; Lys41: $2.0 \pm 0.7 \cdot 10^{-4} \text{ K}^{-1}$, $1.6 \pm 0.2 \cdot 10^{-3} \text{ K}^{-1}$; and Thr99: $0.2 \pm 0.1 \cdot 10^{-3} \text{ K}^{-1}$, $1.5 \pm 0.1 \cdot 10^{-3} \text{ K}^{-1}$ for apo and RNase A/pTppAp, respectively. Reprinted with permission from reference [100]. Copyright 2003 American Chemical Society.

Figure 4. Distribution of $d(1-S)/dT$ across 2° structure. The variation in $d(1-S)/dT$ within each secondary structure element in RNase A is depicted in the box plots for (A) apo RNase A and (B) RNase A/pTppAp. Elements of 2° structure are arranged in order of occurrence in the RNase A primary structure. The bar inside the box indicates the median value of $d(1-S)/dT$, while the ends of the box mark the boundaries with the upper and lower quartile of the data and represent the $\pm 25\%$ variation of the population, the bars extending from the box indicate the minimum and maximum data value. The circles represent outliers in the data set, calculated as points whose value is either: greater than upper quartile + $1.5 \cdot \text{IQD}$ or less than lower quartile - $1.5 \cdot \text{IQD}$ in which IQD (inter quartile distance) is defined as the upper quartile - lower quartile. The ratio of the number of residues from each 2° structure element used in the box plot relative to the total number of non-proline residues in that segment of 2° structure for apo RNase A (RNase A/pTppAp) is: α -1, 9(9)/9; loop-1, 8(6)/12; α -2, 7(8)/8; loop-2, 8(8)/9; β -1, 5(0)/5; loop-3, 2(2)/3; α -3, 9(9)/9; β -2, 4(4)/4; loop-4, 8(7)/8; β -3, 3(3)/3; loop-5, 4(4)/4; β -4, 7(5)/8; loop-6, 7(7)/9; β -5, 13(13)/15; loop-7, 3(3)/3; β -6, 7(6)/7. The $d(1-S)/dT$ values presented in this plot are for all residues in which S^2 could be quantitated at all three temperatures. Reprinted with permission from reference [100]. Copyright 2003 American Chemical Society.

Figure 5. Chemical shift differences in RNase A complexes. Correlation of $\Delta\omega$ values determined by global fitting with equation (14) for residues with quantifiable data in both the apo and pTppAp forms of RNase A. The red line represents a linear fit to the data points for Q11, S15, S16, T17, S22, and Q101. Data from H119 and K66, shown in blue, are excluded from this fit. Reprinted with permission from reference [105]. Copyright 2005 American Chemical Society.

Figure 6. Relaxation dispersion data for the C ϵ position of Met29 at 18.8 T for 4.8% (orange), 52% (blue), and 98% (black) D₂O. The fitted lines to the data points are the result of a global two-field fit to all residues using equation (15). Reprinted with permission from [44]. Copyright 2006 American Chemical Society.

Figure 7. Location of flexible residues. Residues involved in chemical exchange are shown as spheres with the amino acid residue number indicated. Gold spheres indicate residues in which a normal ²H solvent isotope effect of 2 is observed. Spheres colored green are atoms that are flexible and do not exhibit a solvent isotope effect. The four histidine residues are depicted as red sticks. Selected secondary structure elements are indicated. Reprinted from reference [108] with permission. Copyright 2007 National Academy of Sciences, USA.

Figure 8. Solvent isotope and mutation effects. ¹⁵N and ¹³C ϵ rcCPMG relaxation for 5% (black), 10% (aqua), 52% (blue), and 100% (green) ²H₂O. WT data are indicated by circles, and triangles are used to indicate H48A data. NMR CPMG dispersion data were collected at 18.8T (open symbols) and 14.1T (closed symbols) at 298 K. Reprinted from reference [108] with permission. Copyright 2007 National Academy of Sciences, USA.

Figure 9. Structural comparison of bovine ribonuclease A (RNase A) and human Eosinophil Cationic Protein (ECP). A) Superposition of RNase A (blue structure, PDB code 1FS3 [130]) and ECP (red structure, PDB code 1DYT [131]). H12 highlights the position of the active site relative to the position of His48 and loop 1 in RNase A. B) Zoomed view of a few atomic interactions between His48 and loop 1 in RNase A. Note the longer loop 1 in RNase A (D₁₄SSTSAASSNY₂₅) relative to the shorter loop 1 of ECP (S₁₇LNPPR₂₂, ECP numbering). C) Structural alignment of RNase A, ECP and EDN. Active site positions Q11, H12, K41, H119 and D121 are marked with stars while cysteine residues forming the strictly conserved four disulfide bridges are represented by arrows. Loop 1 and position 48 are boxed. Alignment was performed with Toffee Expresso [132] using PDB coordinates 7RSA [133], 1DYT [131] and 1GQV [134]. Reprinted from [111] with permission. Copyright 2009 American Chemical Society.

Figure 10. Conformational dynamics in RNase A. (A) Residues in RNase A_{ECP} undergoing conformational exchange with a ΔR_2 ($1/\tau_{cp}$) greater than 1.5 s^{-1} are plotted on the structure of RNase A (PDB code 7RSA [133]). Red spheres = similar rate constants (k_{ex}) in WT and RNase A_{ECP}; Blue spheres = Decreased k_{ex} values in RNase A_{ECP} relative to WT. H12 and H48 are shown as yellow sticks and loop 1 is shown in cyan. B) CPMG dispersion curves for WT RNase A (black circles), RNase A_{ECP} (red squares) and mutant H48A (blue diamonds). ¹⁵N relaxation dispersion curves are shown for two residues displaying similar conformational exchange in the three enzymes (Gln69 and Asn71), and three residues displaying significantly affected k_{ex} in both RNase A_{ECP} and mutant H48A (Thr82, Asp83 and Gln101). Y115 is represented as a motionless control. Fitted lines to data points are from single-field fits at 14.1 T using equation (15). Reprinted from [111] with permission. Copyright 2009 American Chemical Society.

Figure 11. Hydrogen bonding interactions between His48 and its surrounding environment in RNase A. Hydrogen bonds are shown as dashed lines and atoms are displayed according to a

standard coloring scheme: green for carbon, red for oxygen, and blue for nitrogen. Reprinted from [112] with permission. Copyright 2011 American Chemical Society.

Figure 12. Conserved short-range conformational dynamics in mutant T17A. 14.1 T ¹⁵N-CPMG dispersion curves of residues (A) F46, (B) V47, and (C) T82 for WT RNase A (black circles), RNase A-T17A (red squares), RNase A-T82A (orange triangles), RNase A-H48A (green diamonds) [108, 111], and RNase A_{ECP} (blue inverted triangles) [111]. Insets show zoomed views of the RNase A-T17A data (same legend and axis labels). Reprinted from [112] with permission. Copyright 2011 American Chemical Society.

Figure 13. Crystal structure of apo WT RNase A (PDBID: 7RSA). Active site residues H12, K41, and H119, as well as dynamically important residues H48 and D121 are depicted as sticks and are labeled, along with the elements of secondary structure. Dynamic clusters 1 and 2 are enclosed within the blue boxes. Reprinted from [114] with permission. Copyright 2015 American Chemical Society.

Figure 14. Representative global fits of relaxation dispersion data for clusters 1 and 2 of RNase A. Data collected at 500 and 600 MHz are represented with ▼ and ▲, respectively. Global 2-field CPMG fits of dynamic cluster 1 at 10°C, pH 7.0 and dynamic cluster 2 at 25°C, pH 5.5 and 20°C, pH 6.4 are shown, as well as lack of CPMG-detected dispersion for Cluster 1 at 600 MHz at 25°C, pH 4.5. 600 MHz *R*_{1ρ} data at 25°C and pH 4.5 of both clusters are shown in the bottom panels. The backbone nitrogen atoms of the residues involved in each global fit are mapped onto the structure in each panel and are color matched to the color of the respective dispersion data. Dynamic cluster 1 residues S15, S16, T17, F46, V47, H48, T82, D83, T100 and Q101 are shown in gray, black, blue, purple, green, dark teal, teal, orange, brown, and dark red, respectively. Dynamic cluster 2 residues A64, C65, K66, T70, and N71 at 25°C and pH 5.5 are shown in fuchsia, yellow, red, cyan, and green, respectively. Reprinted from [114] with permission. Copyright 2015 American Chemical Society.

Figure 15. Energy landscape surfaces for conformational changes (*A* ↔ *TS*[‡] ↔ *B*) in RNase A. pH-dependent changes in enthalpy (left) and entropy (right) are depicted for Cluster 1 (top), 2 (middle), and R33 (bottom). Data were obtained from temperature dependent NMR relaxation experiments. In all cases conformation A is set at a reference value of 0 kcal/mol. Reprinted from [114] with permission. Copyright 2015 American Chemical Society.

Figure 16. The dependence of Δω on pH for clusters 1 and 2 of RNase A. (Top) Correlation plot of Δω at pH 7.0 vs. Δω pH 6.4 of dynamic cluster 1 residues, S16, T17, F46, T82, D83, and Q101. The dashed line of unit slope shows a weak correlation (*R*² = 0.46) with the data at the two values of pH, and is depicted for reference. The data is best described by a line of slope 1.29 ± 0.16 (weighted *R*² = 0.97), with near-zero y-intercept, suggesting that the global motion may not be adequately described by a simple two-state model. (Bottom) Correlation plot of Δω at pH 5.5 vs. Δω at pH 7.0 (green), 6.4 (red) and 4.5 (blue) of loop 4 residues A64, K66, T70, and N71. Δω values determined at each of four pH values are well-described by a line of unit slope passing through the origin (*R*² > 0.98), suggesting that the two chemical environments being sampled by cluster 2 remain unchanged over the pH range. Reprinted from [114] with permission. Copyright 2015 American Chemical Society.

Figure 17. Structural and mechanistic comparison of PTP1B and YopH. (A) Structural overlay of PTP1B (grey, PDB 3I80) and YopH (cyan, PDB 2I42) with a phosphate analog vanadate shown at the active site in red spheres. (B) Open (grey) and closed (blue) WPD loops of PTP1B. Vanadate (spheres) binds in the P-loop (orange) with C215 and R221 shown as sticks. Movement of D181 is indicated by the dashed lines. (C) Superposition of closed loops of PTP1B (grey) and YopH (cyan) with the three catalytic residues shown as sticks. (D) PTP catalytic reaction illustrating cleavage and hydrolysis. Reprinted with permission from reference [124]. Copyright 2013 American Association for the Advancement of Science.

Figure 18. Loop motions in PTP1B and YopH detected by NMR. (A) Open and closed PTP1B and YopH WPD loops. (B,C) ^{15}N CPMG dispersion curves for W179, F182, and A189. (D) TROSY-detected off-resonance ^1H - $\text{R}_{1\rho}$ dispersion curves for A359 (blue) and S361 (red). Error bars indicate standard errors determined from duplicate measurements. Reprinted with permission from reference [124]. Copyright 2013 American Association for the Advancement of Science.

Figure 19. (A) Overlaid ^1H - ^{15}N -TROSY HSQC spectra of apo PTP1B (red) in the presence of saturating DADEXILP peptide (X = difluoromethylphosphono-phenylalanine, blue) expanded to show the shift of WPD loop residue A189, indicated by the arrow. (B,C) Shifts of YopH WPD loop residues A359 and S361 compared in the apo enzyme (red) and in the presence of saturating DADEXILP peptide (blue). (D) ^{15}N CPMG dispersion curves for peptide-bound PTP1B (Top) and YopH (Bottom) showing global fits at 600 (red) and 800 (blue) MHz for A189 (PTP1B) and A359 and S361 (YopH). Reprinted with permission from reference [124]. Copyright 2013 American Association for the Advancement of Science.

*Highlights

NMR methods for characterizing ps-ns and μ s-ms motions are reviewed.

Applications of these methods to Ribonuclease A (RNase A) is reviewed with emphasis on the role of motions in product release

Mechanism of motions in catalytic cycle of RNase A is covered.

Application of NMR methods to study active site loop dynamics in two protein tyrosine phosphatases is reviewed.

Figure1

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