Published in final edited form as:

Anal Chem. 2017 September 05; 89(17): 9154–9158. doi:10.1021/acs.analchem.7b01896.

Characterization of Chemical Exchange Using Relaxation Dispersion of Hyperpolarized Nuclear Spins

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Abstract

Chemical exchange phenomena are ubiquitous in macromolecules, which undergo conformational change or ligand complexation. NMR relaxation dispersion (RD) spectroscopy based on a Carr-Purcell-Meiboom-Gill pulse sequence is widely applied to identify the exchange and measure the life time of intermediate states on the millisecond time scale. Advances in hyperpolarization methods improve the applicability of NMR spectroscopy when rapid acquisitions or low concentrations are required, through an increase in signal strength by several orders of magnitude. Here, we demonstrate the measurement of chemical exchange from a single aliquot of a ligand hyperpolarized by dissolution dynamic nuclear polarization (D-DNP). Transverse relaxation rates are measured simultaneously at different pulsing delays by dual-channel ^{19}F NMR spectroscopy. This two-point measurement is shown to allow the determination of the exchange term in the relaxation rate expression. For the ligand 4-(trifluoromethyl)benzene-1-carboximidamide binding to the protein trypsin, the exchange term is found to be equal within error limits in neutral and acidic environments from D-DNP NMR spectroscopy, corresponding to a pre-equilibrium of trypsin deprotonation. This finding illustrates the capability for determination of binding mechanisms using D-DNP RD. Taking advantage of hyperpolarization, the ligand concentration in the exchange measurements can reach on the order of tens of μM and protein concentration can be below 1 μM , *i.e.* conditions typically accessible in drug discovery.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental details are given in the Supporting Information and include: sample injection, NMR experiments, R_2 relaxation analysis and pK_a of TFBC.

Notes

The authors declare no competing financial interest.

Macromolecular functions, including ligand binding, signal transduction, and protein-protein interactions, often rely on dynamic properties of the molecules.^{1–3} For ligand binding, the conformational selection or induced fit model, for example, accounts for changes that need to take place prior to or during binding. Nuclear magnetic resonance (NMR) spectroscopy is used extensively to characterize these and other dynamic processes involving both large and small molecules in solution. Owing to the long nuclear spin decoherence time, characteristic time constants such as those governing chemical exchange can be determined under equilibrium up to the second time scale.^{1,4,5} In an intermediate exchange regime, where the exchange rate is approximately equal to the difference in resonance frequencies of the two exchanged states ($k_{ex} \approx \nu$), a measurement of relaxation dispersion (RD) using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences yields information on minor molecular conformations that are not directly observable.^{6–8} Kay and co-workers have applied the RD measurements to study a series of invisible or excited protein conformations.^{9–14} In addition to the conformational change of one macromolecule, binding interactions can also be quantitatively analyzed through the exchange between bound and free states using the RD method.^{5,15,16} Binding mechanisms can then be distinguished by comparing intrinsic dissociation constants $K_{D,i} = k_{off}/k_{on}$, which can be determined from RD, with apparent K_D measured by other techniques.^{5,16–19} In general, simple two-state, conformational selection and induced fit mechanisms follow $K_{D,i} = K_D$, $K_{D,i} < K_D$, and $K_{D,i} > K_D$ respectively.⁵ In contrast to protein-detected RD measurements, recently developed ligand-detected RD experiments^{17,20–22} do not require protein labeling, and protein concentrations can be low compared to ligand concentrations. ¹³C ligand-detected RD at natural abundance has been demonstrated to be viable.²⁰ Such RD measurements have been proposed for use in drug discovery, because the obtained exchange rates directly reflect the life time of the drug – target complex.¹⁷ Drug discovery applications in particular are subject to stringent limitations on the sample amounts and time available, which are not always compatible with the requirements for NMR experiments. Hyperpolarization methods can in principle be used to increase the NMR signal, and thus would reduce both the needed sample concentration and averaging time. Dissolution dynamic nuclear polarization (D-DNP) offers signal gains of up to four orders of magnitude in a single NMR scan.²³ Meanwhile, the inherent single

scan nature of this technique combined with the time required to hyperpolarize each sample presents its own challenges to obtain multiple measurements at different pulsing delays required for RD.

Here, we describe a method for a two-point RD measurement using a single sample of a ligand hyperpolarized by D-DNP in parallel NMR detection channels. Applied to the protein trypsin and a ligand, we illustrate that this experiment allows the measurement of the exchange contribution to the transverse relaxation rate R_2 of the ligand, leading to determination of the binding mechanism.

EXPERIMENTAL SECTION

For hyperpolarization, 10 μL aliquots containing 7 mM 4-(trifluoromethyl)benzene-1-carboximidamide hydrochloride hydrate (TFBC $\cdot\text{HCl}$; Maybridge, U.K.) were prepared in D_2O /dimethyl sulfoxide (DMSO)- d_6 ($v/v = 1:1$) solvent, which is glass forming when frozen, with 15 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO; Sigma-Aldrich, St. Louis, MO). These aliquots were hyperpolarized on ^{19}F in a HyperSense DNP polarizer (Oxford Instruments, Abingdon, U.K.), by irradiation with 100 mW of 94.055 GHz microwaves for 20 min, at a temperature of 1.4 K. 300 μL of trypsin (AMERSCO, Road Solon, OH) solutions of 2 and 4 μM in 50 mM tris(hydroxy-methyl)aminomethane buffer at pH 8, or of 4 and 10 μM in 50 mM acetate buffer at pH 5 were pre-loaded into a sample loop of a stopped-flow injection device.^{24,25} Subsequently, hyperpolarized ligand was dissolved in 4 mL buffer that had been pre-heated until reaching a vapor pressure of 5 bar. This dissolution buffer was of the same composition as that used for preparation of trypsin samples, at pH 5 or pH 8. Dilution factors during sample injection were measured prior to DNP experiments, using 500 mM trifluoroethanol and 15 mM trifluoroacetic acid as reference compounds, with the same trypsin concentration and dissolution buffer for each experimental condition. These dilution factors were used to calculate the final concentrations $[\text{TFBC}]_0$ and $[\text{trypsin}]_0$ in the flow cell in the DNP experiments. After dissolution and injection, TFBC and trypsin were diluted by a factor of around 200 and 7–8 respectively (see Supporting Information (SI) part 1).

NMR experiments were measured using a homebuilt two-channel NMR probe²⁵ and spectrometer (see SI part 2). A pulse sequence to measure R_2 simultaneously at two different CPMG pulsing delays consisted of $p1_a - [d_a - p2_a - d_a] \times q$ in the first channel and $p1_b - [d_b - p2_b - d_b] \times m$ in the second channel. $p1_a = p1_b = 11.5 \mu\text{s}$, as well as $p2_a = p2_b = 23 \mu\text{s}$ are $\pi/2$ and π pulses applied with phases x and y respectively. Signal acquisition occurred during the delays d_a and d_b . Delays were chosen such that pulses in the two channels were aligned, and $m/q=19$. Hence, the pulsing delay was $\tau_{cp} = 2d_a + p2_a = 5700 \mu\text{s}$ in the long pulsing Channel 1, and $\tau_{cp} = 2d_b + p2_b = 300 \mu\text{s}$ in the short pulsing Channel 2.

Full relaxation dispersion curves were obtained from non-hyperpolarized samples at higher concentrations (3.5 – 31 μM trypsin containing 10 mM of TFBC), by applying an identical CPMG pulse sequence to both channels. R_2 values were measured at τ_{cp} of 300, 600, 1200, 2400, 4800 and 9600 μs . For all CPMG experiments, data retrieved from the spectrometer was analyzed with Matlab (MathWorks, Natick, MA). After a phase correction was applied

to maximize the real part and minimize the imaginary part, a single exponential function was fit to the real part to obtain R_2 (see SI part 3).

Dissociation constants of trypsin and TFBC were determined by ^{19}F NMR titration experiments without hyperpolarization, using concentrations $[\text{TFBC}]_0 = 16, 8, 4, 2, 1$ and 0.5 mM and $[\text{trypsin}]_0 = 50$ μM at pH 8 or $[\text{trypsin}]_0 = 100$ μM at pH 5. Changes in line width and resonance frequency were fitted to $LW = X_b \cdot LW_{\text{max}}$ and $\nu_{\text{obs}} = X_b \cdot \nu_{\text{max}}$, with fit parameters K_D to calculate X_b (bound fraction of the ligand), as well as LW_{max} (line width change extrapolated to $X_b = 1$) and ν_{max} (resonance frequency change extrapolated to $X_b = 1$).²⁶ In all titration experiments, trifluoroacetic acid in D_2O was used as an external resonance frequency reference. Since the resonance frequency of free TFBC varies at different concentrations, free TFBC of the same concentration was measured as a second reference.

The full relaxation curves at each pH, parametrized by R_{2b} , ν , and τ_b , were fitted to Equation 1 in the Results section by constrained minimization in Matlab. X_b and X_f in Equation 1 were calculated from K_D and known concentrations. Constraints included two additional equations relating the three fit parameters based on the titration experiments.

RESULTS

R_2 measurements using the hyperpolarized ligand

The R_2 relaxation rates of the hyperpolarized ligand TFBC in the presence of the target protein trypsin were measured simultaneously in the flow cells of the two-channel NMR probe, at a short and a long CPMG pulse interval. Figure 1a shows the intensities obtained at the center of every echo from channels 1 and 2 in the experiment carried out at pH 8. Since a single hyperpolarized aliquot of the ligand was split and injected symmetrically into the corresponding flow cells, a difference in decay rates is expected primarily because of the different pulse intervals. The more rapidly decaying signal from Channel 1 corresponds to the slower refocusing rate (longer τ_{cp}), giving rise to more coherence loss during the chemical exchange between free and bound ligands. The experiment was further conducted at pH 5 using the same ligand and protein concentrations loaded into the instrument as in the case of pH 8 (Figure 1b). Comparing these data, a pH dependence of R_2 relaxation is clearly seen. At pH 8, a larger change in R_2 is observed, while at pH 5 the two decay curves are almost overlapped and decaying more slowly. A priori, the smaller R_2 at lower pH can be explained by either a smaller fraction of the bound ligand or a reduced exchange contribution to the relaxation rate (see below). Results from three repetitions of the experiments are shown in the insets in Figure 1, as well as in Table S1. On average, $R_2 = R_2(\text{Channel 1}) - R_2(\text{Channel 2})$ obtained from the three nominally identical repetitions is $2.42 \pm 0.14 \text{ s}^{-1}$ and $0.22 \pm 0.05 \text{ s}^{-1}$ at pH 8 and 5, respectively (compare colored 'O' in the insets in Figure 1a and b). A second set of measurements at a different trypsin concentration is shown with uncolored symbols in the insets. The final concentrations of $[\text{TFBC}]_0$ and $[\text{trypsin}]_0$ indicated in the figure were calculated using pre-determined dilution factors for each sample condition (see Experimental section). These dilution factors were approximately 200 for the ligand component and 7–8 for the protein component, and varied by less than 10 % due to experimental errors (see Table S1).

As seen in Figure 1, the signal-to-noise ratio (SNR) is lower in Channel 1 compared to Channel 2 because fewer echoes are acquired during the fixed experimental time. In principle, the SNR in Channel 1 could be improved by approximately three-fold, if multiple data points per echo would be used for this slower echo train (Figure S4). However, this would require different methods for analyzing the echo trains from the two channels, which was not preferred here for reasons of consistency.

Chemical exchange between free and bound ligand at different pH

In the intermediate exchange regime, the R_2 dependence on the pulsing delay τ_{cp} in the CPMG pulse sequence can be explicitly described by Equation 1, when the concentration of the bound ligand is much smaller than the free ligand.¹⁵

$$R_2 = X_f R_{2f} + X_b / (1 / R_{2b} + \tau_b) + X_b X_f \tau_b (2\pi \Delta\nu)^2 [1 - (2\tau_b / \tau_{cp}) \tanh(\tau_{cp} / 2\tau_b)] \quad (1)$$

R_{2f} and R_{2b} are the R_2 relaxation rates of the free and bound ligand, respectively. X_f and $X_b = 1 - X_f$ represent the free and bound fractions of the ligand. τ_b is the life time of the protein-bound ligand complex. $\Delta\nu$ is the resonance frequency difference of the free and bound ligands. At the slow pulsing limit ($\tau_{cp} \rightarrow \infty$), $R_2 \rightarrow X_f R_{2f} + X_b / (1/R_{2b} + \tau_b) + X_b X_f \tau_b (2\pi \Delta\nu)^2$, while at the fast pulsing limit ($\tau_{cp} \rightarrow 0$), $R_2 \rightarrow X_f R_{2f} + X_b / (1/R_{2b} + \tau_b)$. The difference in R_2 from the two limits is equal to $X_b X_f \tau_b (2\pi \Delta\nu)^2$ which includes the exchange term $\tau_b (2\pi \Delta\nu)^2$.

The τ_{cp} values of 300 and 5700 μ s in the two-point RD measurement using D-DNP were chosen to approximate the two pulsing limits, and therefore $R_2 \approx \tau_b X_b X_f (\delta\omega)^2$. In this case, X_b and X_f can be determined based on K_D , which is often known for a protein of interest. Here, $K_D = 0.17$ and 1.7 mM at pH 8 and 5, respectively, were determined from non-hyperpolarized titration experiments using a single $\pi/2$ pulse (Figure 2). Based on these K_D values and the measured R_2 , $\tau_b (2\pi \Delta\nu)^2$ were calculated. Despite more than a 10-fold difference between the R_2 values measured at pH 8 and 5, the values of $\tau_b (2\pi \Delta\nu)^2$ are equal within the error limits of all four conditions measured (Table 1). Since $\Delta\nu_{max}$ is the same within error limits at pH 8 and 5 (Figure 2), the life time of the bound complex τ_b , *i.e.* the chemical exchange dynamics, is found to be the same within measurement uncertainty at the two pH.

Binding equilibrium of TFBC and pK_a determination

Upon changing pH by 3 units, the binding affinity for trypsin and TFBC varies 10-fold (Figure 2), while the chemical exchange dynamics remains the same (Table 1). These characteristics can be rationalized by a pre-equilibrium of protonated and deprotonated forms of trypsin, where only the deprotonated form interacts with TFBC (Scheme 1). This scheme is supported by the fact that the binding of benzamidine and trypsin occurs through the formation of a salt bridge between the amidinium cation of benzamidine and the negatively charged carboxyl group of Asp 189 which is the substrate binding site in trypsin.^{27,28} A similar binding interaction is expected for the TFBC used here, since it is a para-

substituted benzamidine with a trifluoromethyl group and its amidinium cation is intact. Because the pK_a of TFBC is 11.0 (Figure S5), more than 99 % of TFBC is positively charged at both pH 8 and 5, *i.e.* $[L^+] \approx [L]$. Therefore, protonation of the ligand does not need to be considered. The pK_a of the pre-equilibrium stage can thus be estimated based on the binding equilibrium determined from the two-point RD measurement. From Scheme 1, $pK_a = \text{pH} + \log_{10}([PrH]/[Pr])$ and the term $[PrH]/[Pr]$ can be expressed in a relationship between the intrinsic $K_{D,i}$ and K_D . The difference between these values is that the concentrations of both protonated and deprotonated free trypsin are considered in $K_D = [L^+]/([Pr] + [PrH])/[PrL]$, whereas $K_{D,i} = [L^+]/[PrL]$ only includes the active deprotonated form of free trypsin. From these equations, $pK_a = \text{pH} + \log_{10}(K_D/K_{D,i} - 1)$. When K_D values from the titrations are substituted into the prior equation, $pK_a = 6.0 \pm 0.3$ and $K_{D,i} = 0.17 \pm 0.02$ mM are found. At pH 8, nearly 100% of trypsin is negatively charged, and $K_{D,i}$ is close to K_D . For comparison, the pK_a value for Asp 102, which is at the catalytic site of trypsin near Asp 189, was previously reported as 6.8.²⁸

Supporting measurements without hyperpolarization

To validate the results obtained from the hyperpolarized two-point RD measurements, additional RD experiments were performed with non-hyperpolarized samples. The concentration of TFBC was increased by ~280-fold and the concentration of trypsin was increased by ~60- and 7-fold at pH 8 and 5, respectively. These sample concentrations were chosen to have similar bound complex fractions and SNR as the DNP experiments, while allowing measurement by 8 averaging scans. The experiments were performed at six different τ_{cp} values, applied simultaneously to both channels. The R_2 values measured from four different samples as a function of τ_{cp} are shown in Figure 3a and b. Using Equation 1, the data points from the same pH were fitted to a single set of parameters R_{2b} , τ_b , and ν . Constraints from the titration experiments (Figure 2) were included in the fitting, since $\pi \cdot LW_{max} = R_{2b} + \tau_b(2\pi \nu)^2$ and $\nu_{max} = \nu / [(1 + R_{2b}\tau_b)^2 + \tau_b^2(2\pi \nu)^2]$ when $X_b \sim 0$.²⁹ From the fitted τ_b value, $k_{on} = 1/(\tau_b K_{D,i}) = 1.9 \cdot 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, can further be calculated, which is within the range of the diffusion limit for protein-ligand binding.¹⁸

Based on these fitted parameters, R_2 dispersion curves were calculated for the sample conditions in the DNP experiments, and overlaid with the measured data points in Figure 3c and d. These curves are in good agreement with the R_2 values measured by DNP. The fitted exchange terms $\tau_b(2\pi \nu)^2 = 798$ and 794 s^{-1} at pH 8 and 5, respectively, are also comparable to those obtained from DNP (Table 1). Additionally, it can be seen that the τ_{cp} values chosen in the DNP experiments indeed approximate the long and short pulsing limits of the CPMG sequence.

DISCUSSION

Using D-DNP, it was possible to reduce the final protein concentrations in the relaxation dispersion experiments into the sub-micromolar range, and ligand concentration was approximately 35 μM . These concentrations should alleviate solubility constraints in screening applications. Due to dilution during the liquid driven injection, the concentrations of samples are higher when loaded into the polarizer. This requirement can typically be met

by preparing aliquots for hyperpolarization in DMSO or DMSO/H₂O based glassing solvents, which increase the solubility of ligands. The dissolution into aqueous buffer after hyperpolarization then sufficiently reduces the concentration to achieve aqueous solubility, even for ligands that may be difficult to dissolve in water at concentrations that would be required for non-hyperpolarized experiments. DNP of the ¹⁹F nuclei provided a polarization enhancement of ~1500 fold compared to thermal polarization in a 400 MHz NMR magnet. In order to obtain the same SNR as in the DNP experiment but without hyperpolarization, a sample made in a standard 5 mm NMR tube from the amount loaded into the DNP polarizer (70 nmol) would require 26,000 averages, and a sample at the final concentration after dilution (35 μM) in the flow cell would require 650,000 averages.

Dual-channel NMR spectroscopy allows a two-point RD measurement using DNP hyperpolarized samples, which was shown here to be sufficient for the determination of an exchange term. The RD measurements of TFBC-trypsin binding illustrate the ability to determine a binding mechanism based on the measured exchange dynamics. Specifically, they support a conformational selection mechanism with the deprotonated trypsin in pre-equilibrium, which is also reflected in that the dissociation constants $K_D > K_{D,i}$ at acidic pH. The DNP RD measurements were performed at two different pH, which changes the populations of protonated and deprotonated forms. In general, determination of a binding mechanism that does not depend on pH can be achieved by instead measuring RD at several different τ_{cp} values to access full dispersion curves under a single condition, from which on- and off rates for the ligand can be calculated.¹⁷ Here, the relaxation decay was measured without spectral resolution since TFBC only shows one fluorine signal, although NMR spectra with low spectral resolution could be obtained in the long pulsing channel from Fourier transform of each echo (Figure S4). It would be possible to obtain full spectral resolution with a constant-time CPMG experiment, which is also compatible with the D-DNP experiment. This experiment gives access to the signal intensity after a constant CPMG time, without measuring each echo.^{6,30} With the same dual-channel instrumentation used here, data for four or more τ_{cp} values can be measured from two or more hyperpolarized aliquots. Further, it would be possible to increase the number of simultaneous measurements from a single hyperpolarized sample through the development of detection hardware with additional channels.

CONCLUSION

A hyperpolarized relaxation dispersion experiment was shown to enable the determination of chemical exchange dynamics. To this end, two-point CPMG RD measurements were performed from a single hyperpolarized aliquot, using dual-channel ¹⁹F NMR spectroscopy. For binding of the ligand TFBC to the protein trypsin, the exchange term contributing to transverse relaxation was found to be the same at pH 8 and pH 5. This result is explained by a pre-equilibrium of protein deprotonation, and illustrates the ability to evaluate protein–ligand binding mechanisms by this method. Based on the binding equilibrium, the overall pK_a of the binding pocket was further determined using apparent dissociation constants. While the fluorine nucleus used here is abundant in artificial drugs, the same method is applicable to the detection of other nuclei. The reduction in sample amount or averaging

time more generally permits the determination of binding mechanisms by hyperpolarized RD under typical experimental conditions used in drug discovery experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

Financial support from the National Institutes of Health (Grant *R21-GM107927*) is gratefully acknowledged.

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Figure 1. Simultaneous measurements of R_2 relaxation at (a) pH 8 and (b) pH 5 using long and short pulse delays. Blue and red colored 'O' represent the real parts of the data points after phasing obtained from Channel 1 ($\tau_{cp} = 5700 \mu s$) and Channel 2 ($\tau_{cp} = 300 \mu s$) respectively. Shown data points are averages within 0.03 s intervals. R_2 relaxation rates were obtained from a fit to a single exponential; (a) blue curve: $R_2 = 3.88 s^{-1}$, red curve: $R_2 = 1.46 s^{-1}$, (b) blue curve: $R_2 = 1.04 s^{-1}$, red curve: $R_2 = 0.82 s^{-1}$. The insets display the fitted R_2 values from three repetitions at pH 8 in (a) and pH 5 in (b), using two different protein concentrations. The colored data points represent the results from the shown relaxation data. The uncolored open 'O' and 'x' represent the results from the measurements using a different trypsin concentration. The concentrations $[TFBC]_0 / [trypsin]_0$ are indicated for each set of points.

Figure 2.

Non-hyperpolarized titration experiments to determine K_D of TFBC-trypsin. a) Observed changes in line width (LW) at pH 8. b) Changes in line width at pH 5. c) Resonance frequency change (ν_{obs}) of the ^{19}F peak of TFBC from the same experiment as in (a), at pH 8. d) Resonance frequency change at pH 5. Fit values with 90 % confidence intervals are: $K_D = (168 \pm 33) \mu\text{M}$, $LW_{\text{max}} = (401 \pm 18) \text{ Hz}$ and $\nu_{\text{max}} = (160 \pm 21) \text{ Hz}$ at pH 8; $K_D = (1730 \pm 92) \mu\text{M}$, $LW_{\text{max}} = (403 \pm 14) \text{ Hz}$ and $\nu_{\text{max}} = (168 \pm 14) \text{ Hz}$ at pH 5.

Figure 3.

Relaxation dispersion measurements at pH 8 (a,c) and 5 (b,d). ‘O’, data points from Channel 1; ‘x’, data points from Channel 2. a) and b), five repetitions of non-hyperpolarized measurements with 8 scans at different τ_{cp} with fitted dispersion curves. c) and d), three repetitions of simultaneous two-point measurement from DNP measurements with calculated dispersion curves (---, Channel 1; ---, Channel 2). Non-hyperpolarized sample concentrations: $[\text{TFBC}]_0 = 10 \text{ mM}$, $[\text{trypsin}]_0 = 15 / 31 \text{ }\mu\text{M}$ at pH 8, $[\text{trypsin}]_0 = 3.5 / 8.8 \text{ }\mu\text{M}$ at pH 5. Parameters obtained from fitting are $R_{2b} = 461 \text{ s}^{-1}$, $\tau_b = 3.2 \cdot 10^{-4} \text{ s}$, and $\nu = 251 \text{ Hz}$ at pH 8; $R_{2b} = 472 \text{ s}^{-1}$, $\tau_b = 3.0 \cdot 10^{-4} \text{ s}$, and $\nu = 259 \text{ Hz}$ at pH 5.

Scheme 1.

Binding equilibrium of TFBC and trypsin. *Pr*, *PrH*, *L*⁺ and *PrL* represent negatively charged trypsin, protonated trypsin, positively charged TFBC and TFBC-trypsin complex respectively. *Pr*⁻ is the active form that binds with TFBC. *K_a* is the acid dissociation constant of *PrH* and *K_{D,i}* = *k_{off}*/*k_{on}* is the intrinsic dissociation constant of *PrL*.

Table 1.

Exchange terms $\tau_b(2\pi \nu)^2$ determined from simultaneous two-point RD measurements using D-DNP. X_b is calculated with known values of $[\text{TFBC}]_0$, $[\text{trypsin}]_0$ and K_D . Standard deviations are calculated from three repetitions.

pH	X_b	$\tau_b(2\pi \nu)^2 / \text{s}^{-1}$
8	$1.31 \cdot 10^{-3}$	875 ± 43
	$2.67 \cdot 10^{-3}$	912 ± 53
5	$2.84 \cdot 10^{-4}$	788 ± 154
	$7.47 \cdot 10^{-4}$	881 ± 31