Hydrogen exchange (HX) analysis is a powerful tool for studying macromolecular dynamics, stability, and folding.\(^1\) High-resolution NMR methods\(^2\) have provided a wealth of data on amide hydrogen exchange in proteins, with fewer studies of faster exchanging side-chain hydrogens.\(^3\) Whereas slowly exchanging hydrogens are exploited in saturation-transfer MRI experiments,\(^4\)\(^5\) fast HX between labile hydrogens and four internal water molecules in BPTI,\(^6\)\(^8\) the dramatic increase of \(R_2\) at high frequencies is due to the water CSM by HX with the labile protein hydrogens, whereby each hydrogen contributes to \(R_2\) (estimated from the water line width or measured by the spin–echo) according to\(^9\)\(^10\):

\[
\Delta R^2_j = f_k [R^2_j (R^2_j + k_j) + \Delta \omega^2_j]/[(R^2_j + k_j)^2 + \Delta \omega^2_j] \tag{1}
\]

where \(2f\) is the protein/water mole ratio, \(R^2_j\) and \(k_j\) are intrinsic transverse relaxation and exchange rates of a labile hydrogen, and \(\Delta \omega_j = 2 \pi \nu_{\text{lab}} (\delta_j - \delta_{\text{bulk}})\) is the chemical shift difference between the hydrogen and the bulk solvent, the total line broadening being \(R^2_x = \sum (\Delta R^2_j)\), with the sum going over all labile protein hydrogens. Due to aqueous acid/base catalysis of the HX rates, \(k_j = k_{\text{eq}} + k_i[H^+] + k_j[OH^-]\), the individual hydrogen contributions in eq 1 are strongly pH-dependent (cf. Figure 1A,B), reaching their maximum values,

\[
(\Delta R^2_j)_{\text{max}} = f(\Delta \omega^2_j + R^2_j)/(2|\Delta \omega_j|) \tag{2}
\]

at \(k_j = (\Delta \omega^2_j + R^2_j)/(2|\Delta \omega_j| - R^2_j)\), provided that \(|\Delta \omega_j| > R^2_j\). If the exchange rates \(k_j\) are all fast with respect to both \(R^2_j\) and \(\Delta \omega_j\), eq 1 predicts a quadratic frequency scaling of \(R^2_x\) (via the chemical shifts), with the scaling parameter\(^11\) \(\alpha = (\partial R^2_x/\partial \nu_{\text{lab}})/(\partial \nu_{\text{lab}}/\nu_0) = 2\). The data in Figure 1 exhibit weaker frequency dependence of \(R^2_x\), with an apparent \(\alpha\) approaching 0.5 (pH 3.9) and 1.5 (pH 7.5) at high frequencies. This reflects mixing of CSM contributions from several labile groups in BPTI with different values of \(k_j\) and \(\Delta \omega_j\).

To separate the contributions, we measured \(R_2\) at \(\nu_0 = 360\) MHz, and using the CPMG pulse sequence\(^12\) in the pH range of ca. 1–11, exploiting the extreme stability of BPTI.\(^13\) The resulting pH variation (Figure 2) exhibits several unresolved peaks from different labile groups (except carboxyls, which exchange rapidly at all pH values). Further separation may be achieved by measuring the CPMG dispersion alone, i.e., by varying the \(\pi\)-pulse spacing, \(r_{\text{ex}}\), or by measuring at different Larmor frequencies or temperatures. On the other hand, using the CPMG dispersion alone (at fixed pH) is not sufficient to separate the contributions.\(^14\)

To analyze the data, the total exchange line broadening was calculated analytically using the Carver–Richards formulas\(^14\)\(^15\) and numerically by standard matrix techniques\(^16\) starting from the extended Bloch equations for multisite exchange. The intrinsic relaxation rates \(R^2_j\) were calculated on the basis of the BPTI crystal structure and using the protein tumbling time of 5 ns. We used published data for the chemical shifts,\(^17\) exchange rate constants,\(^19\) and ionization constants.\(^13\) Since HX from the N-terminus NH\(_2\) may be catalyzed by salt-bridging to the C-terminal COO\(^-\) (p\(K_a = 2.9\)),\(^24\) its rate was modeled as a weighted average (assuming fast exchange on the N-terminal NH time scale) of the bridged and unbridged conformations at each pH.

A least-squares fit of the analytical expressions to the combined pH and CPMG data was made with the Levenberg–Marquardt algorithm (Figure 2). The following 10 parameters were adjusted in the fit: \(R_2\) offset (from the bulk water and internal water molecules in BPTI), \(k_1\) and \(k_3\) for Tyr OH (except Tyr 35), \(k_2\) for Tyr-35 OH, \(k_5\) for the Arg \(\epsilon\)-NH, Arg \(\eta\)-NH\(_2\), and amino protons,
Figure 2. 1H R2 in 10 mM solution of (extensively dialyzed) BPTI, measured at 360 MHz, 27 °C, using the CPMG pulse sequence (τex = 2 ms). The inset shows the CPMG dispersion at two pH values (vertical dashed lines). The black dashed curves represent an analytical fit to the combined pH and CPMG data. The (nearly coincident) black solid curves are calculated numerically with the same parameters as obtained from the fit. The colored curves show contributions from Tyr OH (orange, solid), Ser and Thr OH (orange, dashed), Lys NH3 (green, solid), N-term NH3 (green, dashed), Arg η-NH2 (blue, solid), Arg ε-NH (blue, dash), peptide amides (magenta, solid), and primary amides of Gln and Asp (magenta, dash). The dash–dot curve shows R2 in the absence of CSM (i.e., with all Δνf set to zero).

The present approach allows HX rates to be determined in protein solutions and biomaterials. It can also be used to study HX catalysis by cosolutes and buffers,7 of relevance for magnetization transfer in biological MRI,31 metabolite imaging,5 and development of HX-based contrast agents.52

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References