Structure, dynamics, and ionization equilibria of the tyrosine residues in *Bacillus circulans* xylanase

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Abstract We have developed NMR spectroscopic methods to investigate the tyrosines within *Bacillus circulans* xylanase (BcX). Four slowly exchanging buried tyrosine hydroxyl protons with chemical shifts between 7.5 and 12.5 ppm were found using a long-range \(^{13}\text{C}-\text{HSQC}\) experiment that exploits the \(^{3}\text{J}_{\text{CH}}\) coupling between the ring \(^{1}\text{H}\) and \(^{13}\text{C}\) nuclei. The NMR signals from these protons were assigned via \(^{13}\text{C}\)-tyrosine selective labelling and a suite of scalar and \(^{13}\text{C},^{15}\text{N}\)-filtered/edited NOE correlation spectra. Of the fifteen tyrosines in BcX, only the buried Tyr79 and Tyr105 showed four distinct, rather than two averaged, signals from ring \(^{13}\text{C}–^{1}\text{H}\) pairs, indicative of slow flipping on the chemical shift timescale. Ring flipping rate constants of \(\sim 10^5\) and \(\sim 0.2\ \text{s}^{-1}\) were measured for the two residues, respectively, using a \(^{13}\text{C}\) longitudinal exchange experiment. The hydrogen bonding properties of the Tyr79 and Tyr105 hydroxyls were also defined by complementary NOE and J-coupling measurements. The \(^{1}\text{H}\) hydrogen–deuterium exchange rate constants of the buried tyrosines were determined from \(^{13}\text{C}/^{15}\text{N}\)-filtered spectra recorded as a function of pH. These exchange rate constants correspond to estimated protection factors of \(\sim 10^8\) relative to a random coil tyrosine. The phenolic sidechain \(\text{pK}_a\) values were also measured by monitoring their pH-dependent \(^{13}\text{C}\) chemical shifts via \(^{1}\text{H}–^{13}\text{C}\) correlation spectra. Exposed tyrosines had unperturbed \(\text{pK}_a\) values of \(\sim 10.2\), whereas buried residues remained predominantly neutral at or even above pH 11. Combined with selective isotope labelling, these NMR experiments should prove useful for investigating the structural and electrostatic properties of tyrosines in many interesting proteins.

Keywords Glycoside hydrolase · Tyrosine hydroxyl · Protein dynamics · Protein electrostatics · NMR spectroscopy

Introduction

Tyrosine residues play critical structural and functional roles in proteins (Creighton 2010). With both an aromatic ring and an ionizable hydroxyl group, the amphiphatic sidechain of this amino acid can partake in a range of hydrophobic and polar interactions necessary to stabilize the native, folded conformation of a protein or protein complex (Pace et al. 2001). In addition to ligand and substrate binding, the tyrosine phenolic group can also contribute directly to enzymatic catalysis (Holliday et al. 2009), serving as a nucleophile (Watts et al. 2003; Yang 2010), general acid/base (Li et al. 1993; Sun and Toney 1999; Lunin et al. 2004), or redox active centre (Barry and Einarsdottir 2005). Key to all of these roles is the ionization state and hydrogen bonding properties of a given tyrosine. However, unless neutron (Kossiakoff et al. 1990) or very high resolution X-ray diffraction data (Ho and Agard 2008) are available, electron density from a hydroxyl \(^{1}\text{H}\) is typically not observed in a crystallographically-determined protein structure. Similarly, although tyrosines have been well studied since the earliest days of protein NMR spectroscopy, the hydroxyl proton is rarely detected.
via this technique due to rapid hydrogen exchange with solvent water (Liepinsh et al. 1992; Liepinsh and Otting 1996). Thus, the functional properties of tyrosine sidechain hydroxyls are more often inferred from physicochemical arguments than from direct experimental measurements.

In this study, we report the detailed characterization of the tyrosines in Bacillus circulans xylanase (BcX) using a suite of both established and new NMR methods. This well characterized family 11 glycoside hydrolase (Cantarel et al. 2009) exploits two catalytic glutamic acid residues to hydrolyze xylan via a double displacement retaining mechanism (Miao et al. 1994). Although composed of only 185 amino acids, BcX contains fifteen tyrosines that both contribute to its β-jellyroll fold and line its active site cleft (Wakarchuk et al. 1994; Sidhu et al. 1999). Indeed, carbohydrate-protein interactions are often mediated by the tyrosine hydrogen/deuterium exchange (HX) kinetics, we found that the hydroxyls from these residues are protected against exchange by ~10^2–10^5 fold relative to a random coil tyrosine due to their burial within the well structured core of BcX. Two of these tyrosines also undergo slow ring flipping on the seconds timescale, thus allowing their 1H nuclei to be positioned within the crystal structure of BcX using complementary NOE and J-coupling restraints. This both unambiguously defines their neutral ionization states and hydrogen bonding properties. Finally, the pK_a values of the tyrosines in BcX were determined from their pH-dependent 13C chemical shifts. Three exposed tyrosines have relatively unperturbed pK_a values of ~10.2, whereas the remainder remain predominantly neutral at or even above pH 11. Collectively, these results both shed new insights into the structure and stability of BcX, and illustrate routes for the characterization of tyrosines in proteins by NMR spectroscopy.

Materials and methods

Protein expression and purification

The expression and purification of BcX has been described previously (Joshi et al. 2001). Briefly, E. coli BL21(λDE3) cells were transformed with a pET22b(+) vector encoding for BcX and grown at 37°C in LB media or in M9 medium with 1 g/L 15NH4Cl or 1 g/L 15NH4Cl and 3 g/L 13C6-glucose (Spectra Stable Isotope) to produce unlabelled or uniformly labelled protein, respectively. BcX, selectively labelled with 13C-tyrosine, was obtained from BL21 cells grown in M9 media containing per litre 25 mg 13C6-L-tyrosine (Isotec), 100 mg unlabelled tryptophan, 100 mg unlabelled phenylalanine, and 1 g N-(phosphonomethyl)glycine (Sigma-Aldrich). The latter, also known as glyphosate, inhibits the biosynthesis of aromatic amino acids (Kim et al. 1990). The cells were initially incubated at 37°C, then transferred to 30°C when an OD_600 of ~0.4 was achieved. In all cases, at OD_600 = 0.6, protein expression was induced with 1 mM IPTG. The cells were harvested after ~20 h growth at 37°C (or overnight at 30°C for 13C-tyrosine selective labelling), and lysed with a French press in the presence of a protease inhibitor cocktail (Roche Diagnostics). BcX was eluted with a 0–1 M NaCl gradient (10 mM sodium phosphate, pH 6.0) from a 20 mL Hi-Trap SP-Sepharose (GE Healthcare) ion exchange chromatography column. The resulting protein samples were purified further using a HiPrep 16/60 Sephacryl S-100 (GE Healthcare) size-exclusion column equilibrated with 150 mM NaCl, 10 mM sodium phosphate, pH 6.0, and then concentrated with buffer adjustment by ultrafiltration. The final sample concentrations were determined by absorbance spectroscopy using a predicted ε_280 value of 81,790 M^-1 cm^-1.

NMR spectroscopy

NMR spectra were recorded with Varian Unity 500 MHz and cryoprobe-equipped Inova 600 MHz spectrometers. Data were processed with NMRpipe (Delaglio et al. 1995) and analyzed using Sparky (Goddard and Kneeler 1999). Unless stated otherwise, protein samples were in 10 mM sodium phosphate, pH 6.5, with 5% D_2O lock solvent and 15N/13C-HSQC, HNCACB, CBCA-CO, HNCO, HN(CA)CO, and 15N-editted TOCSY- and NOESY-HSQC spectra (Sattler et al. 1999), combined with previously reported data for the wild type protein (Plesniak et al. 1996b; Connelly et al. 2000). The signals from tyrosine 1H and 13C nuclei in 13C-Tyr labelled BcX were assigned using a variety of single and multiple bond correlation experiments, presented in the Results section. The pulse sequences for these experiments are given as Supplemental Fig. S1–S8 and are available upon request.

Hydrogen exchange

Tyrosine hydrogen–deuterium exchange rates were measured at 25°C using one-dimensional 13C/15N-filtered experiments (Supplemental Fig. S1). 13C/15N-BcX samples (~500 µL) in 10 mM sodium phosphate, H_2O, were adjusted to a desired pH value, lyophilized, reconstituted in an equivalent volume of D_2O, and immediately placed in the spectrometer. The decay of the tyrosine 1H peak
intensity, $I_i$, was monitored as a function of time. Data were fit to an exponential decay with GraphPad Prism 5, according to the equation:

$$I_i = I_{0e}^{-k_{ext}t} + I_{in}$$

where $I_{0e}$ and $I_{in}$ are the initial and final peak intensities, respectively. The sample pH* (i.e., the pH meter reading without correction for the deuterium isotope effect) was measured after completion of the experiment.

Tyrosine ring flipping

The ring flipping rates of Tyr79 and Tyr105 in $^{13}$C-tyrosine selectively labelled BcX were measured using the pulse sequence of Farrow et al. (Farrow et al. 1994), modified for an aromatic $^{13}$C-$^1$H spin system (Supplemental Fig. S4). The experiment provides both the $^{13}$C longitudinal decay ($R_{1c}$) and conformational exchange rate constants ($k$) of the non-degenerate $^{13}$C-$^1$C-$^1$C-$^1$C or $^{13}$C-$^1$C-$^1$C-$^2$ of a given tyrosine ring, detected in the form of a one-bond $^{13}$C-HSQC spectrum. These values were extracted by simultaneously fitting time-dependent “auto” ($IC_{1/H}(t)$) and “exchange” ($IC_{2/H}(t)$) peak intensities to the following equations, using GraphPad Prism 5:

$$IC_{1/H}(t) = IC_{1/H}(0) \left(\frac{e^{-\lambda_- t} + (\lambda_+ - \lambda_a) e^{-\frac{\lambda_-}{2} t} - \lambda_-}{(\lambda_+ - \lambda_-)}\right)$$

$$IC_{2/H}(t) = IC_{2/H}(0) \left(\frac{-k e^{-\frac{\lambda_-}{2} t} + k e^{-\lambda_- t} - \lambda_-}{(\lambda_+ - \lambda_-)}\right)$$

where

$$\lambda_+ = \frac{1}{2} \left\{(a_1 + a_2) \pm \left[(a_1 - a_2)^2 + 4k^2\right]^{1/2}\right\}$$

and $a_1 = (R_{1C1} + k)$ and $a_2 = (R_{1C1} + k)$. These expressions follow from those of Farrow et al. (1994) with the simplification of a single rate constant for flipping of the symmetrical ring.

$pK_a$ measurements

Two-dimensional proton-detected $^1$H-$^{13}$C-$^{13}$C and $^1$H-$^{13}$C-$^{12}$N-$^{13}$C experiments were developed to measure the $pK_a$ of tyrosine residues by monitoring the ionization-sensitive $^{13}$C shifts as a function of sample pH value (Supplemental Fig. S5 and S7). Three-dimensional versions of these pulse sequences were also used to facilitate resonance assignments (Supplemental Fig. S6 and S8). Experiments were carried out with $^{13}$C-tyrosine selectively labelled BcX in 10 mM sodium phosphate, 5% D$_2$O at 35°C. The sample pH value was adjusted by the addition of small aliquots of 0.1 mM NaOH. Site-specific $pK_a$ values were obtained by fitting with MatLab the pH-dependent $^{13}$C chemical shifts to the equation describing a single titration,

$$\delta_{obs} = \delta_a 10^{\text{p}K_a} + \delta_b 10^{\text{p}K_a}$$

where $\delta_a$ and $\delta_b$ are the limiting chemical shift values for the conjugate acid and base forms of the tyrosine, respectively. In the case of a partial titration, ($\delta_b - \delta_a$) was constrained to 9.7 ppm. This value is based on the pH-dependent $^{13}$C shifts of free tyrosine in 10 mM sodium phosphate buffer and 5% D$_2$O, measured from $^{13}$C-HMBC spectra (Supplemental Fig. S9) (Bax and Summers 1986).

Results

Detection of tyrosine $^1$H signals

The $^1$H-NMR spectrum of BcX contains three signals downfield of 11 ppm (Fig. 1a). One of these has been assigned to the nitrogen-bonded $^1$H$^2$ of the buried, neutral His149 (Plesniak et al. 1996a; Connelly and McIntosh 1998). The other two must arise from slowly exchanging oxygen- or less likely, sulfur-bonded protons because they

![Fig. 1](image-url)
remain as singlets when BcX is uniformly $^{13}$C/$^{15}$N-labelled. To confirm this, we recorded the 1D spectrum of $^{13}$C/$^{15}$N-BcX with filtering against protons bonded directly to $^{13}$C and $^{15}$N nuclei (Fig. 1b and Supplemental Fig. S1). In addition to the peaks at 12.53 and 11.50 ppm, at least seven more signals were detected downfield of 6 ppm. Based on their chemical shifts, it was reasonable to attribute at least some of these to the tyrosine hydroxyls in BcX. As tabulated in the BioMagResBank (Ulrich et al. 2008), the average tyrosine $^1$H$^g$ chemical shift in diamagnetic proteins is 9.33 ppm, compared to 5.34 ppm for serine $^1$H$^c$, 4.96 ppm for threonine $^1$H$^e$, 1.94 for cysteine $^1$H (BcX is thiol-free), and 9–12 ppm for the rarely observed carboxyl protons of aspartic and glutamic acids.

Four of the signals from -OH groups were unambiguously assigned to tyrosine hydroxyls using a multiple bond $^{13}$C-HSQC experiment recorded on a sample of BcX selectively labelled with $^{13}$C-Tyr (Fig. 1c and Supplemental Fig. S2). The experiment exploits a modest $^3$J$^{HC}$ to correlate the $^1$H$^g$ and $^{13}$C$^e$ of a tyrosine (Werner et al. 1997). Although this scalar coupling is conformationally dependent, the $^1$H$^g$ signal of $^{13}$C-tyrosine in DMSO is split into a double doublet due to an averaged $^3$J$^{HC}$ $\sim 5$ Hz with two equivalent $^{13}$C$^e$ nuclei. Note that the multiple-bond assignment was recorded using an INEPT 1/4 J transfer delay of n/(2$\times$160 Hz) to minimize signals from tyrosine one-bond $^1$H-$^{13}$C couplings. Due to a balance of transfer versus relaxation, delays of 6.2, 9.4 or 15.6 ms gave similar quality spectra with BcX, with the latter two being optimal at 30 and 37°C, respectively. Weak $^1$H$^g$–$^{13}$C$^f$ correlation were also observed due to a small $^2$J$^{HC}$ (not shown), aiding in the specific assignments of the $^1$H$^g$ signals, as outlined below. Although the long-range $^{13}$C-HSQC experiment can be recorded using a uniformly $^{13}$C-labelled protein sample, identification of the tyrosine signals will be complicated by the presence of numerous 2- and 3-bond correlations between $^{13}$C nuclei and various aromatic, indole, imidazole, and amide protons.

Assignment of tyrosine $^1$H$^g$ signals

The signals from tyrosine $^1$H$^g$ nuclei were assigned using a set of scalar and NOE correlation experiments (Fig. 2 and Supplemental Table S1). Briefly, the chemical shifts of the main chain nuclei of BcX were identified previously using standard methods (Plesniak et al. 1996b; Connelly et al. 2000). The ring $^1$H$^d$–$^{13}$C$^d$ and $^1$H$^e$–$^{13}$C$^e$, which give well resolved peaks in a constant-time one-bond $^{13}$C-HSQC spectrum, from which $\alpha_1$ (horizontal)—$\alpha_3$ (vertical) strips at the indicated $^{13}$C chemical shifts are shown. Spectra a–d were recorded with $^{13}$C-Tyr BcX and e–h with the uniformly labelled $^{13}$C-BcX, all at 35°C. For clarity, only peaks from the four tyrosines with detectable $^1$H$^g$ are annotated. Complete assignments are given in Supplemental Table S1.

Fig. 2 Signals from the tyrosine $^1$H$^g$ nuclei were assigned using a a long-range $^{13}$C-HSQC spectrum to provide correlations to the $^{13}$C$^e$, which were then aligned to a b one-bond constant time $^{13}$C-HSQC spectrum. The $^1$H$^d$–$^{13}$C$^d$ and $^1$H$^e$–$^{13}$C$^e$ peaks in this spectrum were assigned using c $^3$C$^d$(C(C-C-TOSCY))$^1$H$^d$ and d $^3$C$^e$(C(C-C))$^1$H$^e$ spectra, which correlate the ring $^1$H$^d$ or $^1$H$^e$, respectively, to the $^{13}$C$^d$. The assignments were confirmed using a e–h $^{13}$C-edited NOESY-HSQC spectrum, from which $\alpha_1$ (horizontal)—$\alpha_3$ (vertical) strips at the indicated $^{13}$C chemical shifts are shown. Spectra a–d were recorded with $^{13}$C-Tyr BcX and e–h with the uniformly labelled $^{13}$C-BcX, all at 35°C. For clarity, only peaks from the four tyrosines with detectable $^1$H$^g$ are annotated. Complete assignments are given in Supplemental Table S1.
NOEs from the 1H of this protein (Fig. 3; Table 1). In addition, each is hydrogen bonded to neighbouring protein atoms and/or bound waters. This of course is expected, since a tyrosine 1H must be protected from rapid HX in order to be detected under the conditions used for these measurements. The four detected 1H signals arise from Tyr26, Tyr53, Tyr79, and Tyr105 (Figs. 1, 2). Of the fifteen tyrosines in BcX, these are the most buried with O areas <1 Å2 in the static X-ray crystallographic structure of this protein (Fig. 3; Table 1). In addition, each is hydrogen bonded to neighbouring protein atoms and/or bound waters. This of course is expected, since a tyrosine 1H must be protected from rapid HX in order to be detected under the conditions used for these measurements.

Tyrosine 1H hydrogen exchange kinetics

The HX kinetics of the protected tyrosines in BcX were measured by recording one-dimensional 13C/15N-filtered spectra immediately after transfer of the 13C/15N-labelled protein into D2O buffer (Fig. 4a,b). Fitting of the time-dependent decay of the signals from Tyr26, Tyr53, and Tyr79 yielded the exchange rate constants, kex, summarized in Fig. 4c. Interestingly, the exchange kinetics of Tyr53 showed little dependence on the pH conditions examined. This is suggestive of an EX1 or open-limited mechanism (Hvidt and Nielsen 1966; Englander and Kallenbach 1983). In contrast, Tyr26 and Tyr79 exhibited pH-dependent HX, indicative of exchange via an EX2 or pre-equilibrium mechanism. Furthermore, both exchanged most slowly around pH5–6, which is similar to the pHmin ~ 5.5 reported for an unperturbed tyrosine (Liepinsh et al. 1992; Liepinsh and Otting 1996). Based on an estimated pseudo-first order k predict ~ 103 s−1 for the exchange of a free tyrosine hydroxyl under comparable conditions (Liepinsh et al. 1992; Liepinsh and Otting 1996), the protection factors (k predict/kex) of Tyr53, Tyr26, and Tyr79 at pH* 4.3 and 25°C are ~ 106, 107, and 108, respectively. The effects of possible general base catalysis have been neglected in these approximations.

Although the 1H of Tyr105 is detectable in H2O, its signal was absent immediately after transfer to D2O under all pH* conditions examined (Fig. 4b). Assuming a dead time of ~180 s between rehydration of the lyophilized sample and recording the first NMR spectrum, this indicates that its kex > 0.01 s−1. In contrast, no transfer of magnetization from H2O to Tyr105 (or to the other three observable tyrosines) was detected in a one-dimensional CLEANEX experiment (Hwang et al. 1997) with a 13C/15N-filtered readout (pH 4.5–6.5; not shown). This sets an approximate upper limit of kex < 0.1 s−1 for Tyr105, and hence a protection factor within the range of ~104–106. This limit is also consistent with the requirement that kex < 3JCH in order to detect the 1H in a long range 13C-HSQC spectrum without significant loss of polarization transfer (Henry and Sykes 1990).

Tyrosine ring flipping

As typically observed with proteins, eleven of the thirteen assigned tyrosines in BcX exhibited only two signals in a one-bond 13C-HSQC spectrum (Fig. 2 and Supplemental Table S1). This results from protein fluctuations allowing rapid ring flipping to yield averaged chemical shifts for the symmetrically-related 1H and 13C nuclei (Snyder et al. 1975; Wüthrich and Wagner 1975). In contrast, Tyr79 and Tyr105 both yielded four signals, indicative of slow two-state flipping on the chemical shift time scale. To probe this further, we used a 13C-HSQC-detected exchange experiment to measure the time-dependant intrareside transfer of longitudinal magnetization between tyrosine 13C/13C nuclei at 25 and 35°C (Supplemental Fig. S4) (Farrow et al. 1994). As shown in Fig. 5, the
presence of exchange cross peaks demonstrated that the rings of Tyr79 and Tyr105 do flip, but on the seconds timescale. Fitting of these data for the $^1$H$^\alpha$–$^{13}$C$^\alpha$ signals yielded the interconversion rate constants summarized in Table 1. The $^1$H$^\alpha$–$^{13}$C$^\alpha$ correlations were not analyzed due to spectral crowding. Parenthetically, we speculate that the absence of assignable signals from Tyr80 may result from conformational exchange broadening due to partially hindered ring flipping on the intermediate (msec–l sec) timescale.

Previously, the kinetics of tyrosine and phenylalanine ring flipping have been determined from one- and two-dimensional $^1$H lineshape (Wagner et al. 1976; Hattori et al. 2004) or magnetization transfer measurements (Campbell et al. 1976; Nall and Zuniga 1990; Fejzo et al. 1990; Skalicky et al. 2001; Rao and Bhuyan 2007). The advantages of the $^{13}$C exchange experiment presented herein include the added dispersion of a $^{13}$C-HSQC spectrum, particularly when combined with amino acid selective labelling, as well as the lack of complications from interproton NOEs and the possibility to use long transfer times (relative to $^{13}$C T$_1$) to measure slow flipping rates.

### Table 1

Summary of BcX tyrosine properties

<table>
<thead>
<tr>
<th>Tyr</th>
<th>Hydrogen bonding$^a$</th>
<th>Accessible surface area: ring/O$^\alpha$ (Å$^2$)$^a$</th>
<th>$^\alpha$/C$^\alpha$/C$^\alpha$/C$^\alpha$ B-value (Å$^2$)$^a$</th>
<th>HX rate constant$^b$</th>
<th>Ring flipping rate constant$^d$</th>
<th>pK$^\alpha_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y5</td>
<td>M169 O (2.9 Å)</td>
<td>56/21</td>
<td>16.0</td>
<td>(1.6 ± 0.01) $\times$ 10$^{-4}$ s$^{-1}$</td>
<td>&gt;12</td>
<td>11.5 ± 0.1$^f$</td>
</tr>
<tr>
<td>Y26</td>
<td>H2O (2.8 Å)</td>
<td>0/0</td>
<td>8.8</td>
<td>(9.9 ± 0.1) $\times$ 10$^{-4}$ s$^{-1}$</td>
<td>&gt;12</td>
<td>10.1 ± 0.1$^f$</td>
</tr>
<tr>
<td>Y53</td>
<td>D83 O$^\gamma$ (2.6 Å)</td>
<td>4/1</td>
<td>7.5</td>
<td>(3.8 ± 0.4) $\times$ 10$^{-6}$ s$^{-1}$</td>
<td>2.4 ± 0.1 s$^{-1}$/9.3 ± 0.5 s$^{-1}$</td>
<td>&gt;12</td>
</tr>
<tr>
<td>Y65</td>
<td>H2O (2.9 Å)</td>
<td>22/12</td>
<td>9.3</td>
<td>0.1 s$^{-1}$ &gt; k$_{cs}$ &gt; 0.01 s$^{-1}$c</td>
<td>Slow/0.17 ± 0.02 s$^{-1}$</td>
<td>11.9 ± 0.1$^f$</td>
</tr>
<tr>
<td>Y69</td>
<td>E78 O$^\gamma$ (2.6 Å)</td>
<td>7/7</td>
<td>8.9</td>
<td>10.1 ± 0.1$^f$</td>
<td>10.1 ± 0.1$^f$</td>
<td></td>
</tr>
<tr>
<td>Y79</td>
<td>Q167 N$^\gamma$ (3.1 Å)</td>
<td>0.1/0.1</td>
<td>6.9</td>
<td>0.14 s$^{-1}$ &gt; k$_{cs}$ &gt; 0.01 s$^{-1}$c</td>
<td>Slow/0.17 ± 0.02 s$^{-1}$</td>
<td>10.1 ± 0.1$^f$</td>
</tr>
<tr>
<td>Y80</td>
<td>E172 O$^\gamma$ (2.7 Å)</td>
<td>5/5</td>
<td>7.9</td>
<td>8.8</td>
<td>8.8</td>
<td>11.9 ± 0.1$^f$</td>
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<tr>
<td>Y88</td>
<td></td>
<td>93/23</td>
<td>17.5</td>
<td>11.4 ± 0.1$^f$</td>
<td>11.4 ± 0.1$^f$</td>
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<tr>
<td>Y94</td>
<td></td>
<td>83/19</td>
<td>12.8</td>
<td>12.8</td>
<td>12.8</td>
<td>10.1 ± 0.1$^f$</td>
</tr>
<tr>
<td>Y105</td>
<td>D83 O$^\gamma$ (2.6 Å)</td>
<td>0.2/0.2</td>
<td>7.3</td>
<td>0.1 s$^{-1}$ &gt; k$_{cs}$ &gt; 0.01 s$^{-1}$c</td>
<td>Slow/0.17 ± 0.02 s$^{-1}$</td>
<td>11.9 ± 0.1$^f$</td>
</tr>
<tr>
<td>Y108</td>
<td>R89 N$^\gamma$ (3.1 Å)</td>
<td>16/10</td>
<td>8.8</td>
<td>10.1 ± 0.1$^f$</td>
<td>10.1 ± 0.1$^f$</td>
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<tr>
<td>Y113</td>
<td></td>
<td>115/32</td>
<td>14.0</td>
<td>8.7</td>
<td>8.7</td>
<td>11.0 ± 0.2$^f$</td>
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<tr>
<td>Y128</td>
<td>N159 O (2.9 Å)</td>
<td>17/13</td>
<td>Not assigned</td>
<td>11.9 ± 0.2$^f$</td>
<td>11.9 ± 0.2$^f$</td>
<td></td>
</tr>
<tr>
<td>Y166</td>
<td>H2O (2.8 Å)</td>
<td>19/7</td>
<td>11.0</td>
<td>8.7</td>
<td>8.7</td>
<td>10.3 ± 0.1$^f$</td>
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<tr>
<td>Y174</td>
<td></td>
<td>102/34</td>
<td>19.3</td>
<td>8.7</td>
<td>8.7</td>
<td>10.3 ± 0.1$^f$</td>
</tr>
</tbody>
</table>

$a$ Based on 1XNB.pdb. Hydrogen bond distances are between O and/or N atoms. Accessible surface areas were determined with VADAR using a 1.4 Å radius sphere probe (Willard et al. 2003). B-values are average of $^\alpha$/C$^\alpha$/C$^\alpha$/C$^\alpha$

$b$ Values at pH* 4.3 and 25°C. With the exception of the unassigned Tyr80 and Tyr128, a blank entry indicates that the $^1$H$^\alpha$ was not detected, presumably due to fast HX

$c$ Detected in H$_2$O buffer but not immediately after transfer to D$_2$O buffer

$d$ Except for Tyr80 and Tyr128, a blank entry indicates that the ring undergoes rapid flipping to yield averaged chemical shifts for the $^{13}$C$^\alpha$, $^{13}$C$^\beta$, $^1$H$^\alpha$, and $^1$H$^\beta$ nuclei. Tyr79 and Tyr105 values listed for 25/35°C

$e$ pK$_a$ values determined from the pH-dependent $^{13}$C$^\alpha$ chemical shifts. Residues with pK$_a > 12$ showed no significant shift change (i.e. <1 ppm) over the pH range examined

$f$ Fit with a fixed pH-dependent chemical shift change of 9.7 ppm

$g$ Fit with floating end-point chemical shifts to yield the tabulated $D$ = $D_b - D_a$
The pKₐ values of the tyrosine residues in BcX were measured by monitoring their pH-dependent ¹³C chemical shifts (Figs. 6, 7). The ¹³C chemical shifts of a random coil tyrosine are *₁₁₅ and ¹₆₈ ppm when its hydroxyl is neutral and deprotonated, respectively, and thus serve as a clear indicator of the ionization state of this residue (Supplemental Fig. S9) (Norton and Bradbury 1974; Oldfield et al. 1975b; Richarz and Wüthrich 1978; Boswell et al. 1983). For increased sensitivity and spectral dispersion, these shifts were obtained indirectly via two-dimensional ¹³C(¹³C)¹H experiments, which correlate the ¹³C with the ¹H or ¹H exchanges.
via strong one-bond couplings (Supplemental Fig. S5 and S7). The assignments of the $^{13}$C signals were facilitated by three-dimensional versions of these experiments, which also provide the $^{13}$C shifts of each aromatic ring (Fig. 6 and Supplemental Fig. S6 and S8).

Over the pH range of 6.5-10.9, three tyrosines (Tyr88, Tyr113, and Tyr174) exhibited clear $^{13}$C-monitored titrations corresponding to fit $pK_a$ values of 10.1–10.3 (Fig. 7; Table 1). These relatively unperturbed values are similar to that of 9.8 reported for a tyrosine in a random coil polypeptide (Thurlkill et al. 2006; Grimslie et al. 2009). As expected, the three are the most solvent exposed tyrosines in BcX and their hydroxyls are not involved in intramolecular hydrogen bonding. Four additional partially buried tyrosines showed small pH-dependent $^{13}$C chemical shift changes with increasing sample pH, yielding estimated $pK_a$ values >12. As also expected, this latter group includes the well buried Tyr26, Tyr26, Tyr79, and Tyr105. The above conclusions are supported by a parallel analysis of the pH-dependent $^1H$ chemical shifts of these residues (Supplemental Fig. S10). It should be mentioned that all tyrosine hydroxyls in BcX are exchangeable with the solvent, and thus the lack of detectable pH-titrations is unlikely a kinetic issue. Also, BcX remains folded under alkaline conditions since its tyrosines still give well resolved, native state $^{13}$C- and $^1H$-NMR signals at even pH 10.9.

Discussion

Detection of tyrosine $^1H$ by NMR spectroscopy

Currently, chemical shifts for only 145 tyrosine $^1H$ nuclei from diamagnetic proteins have been deposited in the BioMagResBank (Ulrich et al. 2008). Most of these $^1H$ signals appear to have been identified and assigned based on interproton NOEs. This low number, which corresponds to ~1% of the reported tyrosine amide shifts, certainly results in large part from the facile HX of sidechain hydroxyl groups under conditions typically used for protein NMR spectroscopy. However, the assigned $^1H$ signals range from 5.99 to 13.75 ppm and cluster near an average value of 9.31 ppm with a standard deviation 1.37 ppm (Ulrich et al. 2008). Thus, it is likely that many HX-protected tyrosine hydroxyls are undetected simply because their signals are hidden under those of the amide and aromatic protons in 1D $^1H$-NMR spectra, and because they are not observable in “work horse” one-bond $^{15}N$- or $^{13}C$-HSQC spectra. Weakly protected $^1H$ nuclei may also be unnoticed when employing pre-saturation, rather than selective pulses, for water suppression.

Using BcX as a model system, we have found that signals from tyrosine $^1H$ nuclei protected from rapid HX can be readily detected in a simple $^{13}C/^{15}N$-filtered $^1H$-NMR spectrum measured with a uniformly $^{13}C/^{15}N$-labeled protein sample. Residue specific assignments can then be obtained from a long range $^{13}C$-HSQC, best recorded with
a 13C-tyrosine selectively labeled sample. Indeed, a 1D difference spin echo version of this approach, which exploits the weak \(^3J_{CH}\) coupling between the \(^1H\) and \(^{13}C\) nuclei, was first used to identify a tyrosine hydroxyl buried at the interface between the DNA-binding Ets-1 ETS domain and a consensus oligonucleotide (Werner et al. 1997). Alternatively, a \(^{13}C/^{15}N\)-filtered/edited NOESY-HSQC spectrum recorded with a protein highly enriched in \(^{13}C\) and \(^{15}N\) can also be exploited to detect and assign these signals, albeit with the inherent ambiguity of a through space interaction. Although this latter experiment is typically used to detect intermolecular NOEs within a complex of isotopically labeled and unlabeled species (Ikura and Bax 1992; Zwahlen et al. 1997), intramolecular NOEs involving oxygen- or sulfur-bonded protons will also be observed for an isolated \(^{13}C/^{15}N\)-protein.

Structural characterization of Tyr79 and Tyr105

The observation of both slow ring flipping and slow HX for Tyr79 and Tyr105 presented an interesting opportunity to characterize the structural features of these two tyrosine residues (Fig. 8). By way of background, recall that phenol is a planar molecule with its \(\text{O}^\circ\) sp\(^2\)-hybridized (Carey and Giuliano 2011). Furthermore, neutron and very high resolution X-ray crystallographic studies confirm that tyrosines in proteins preferentially adopt planar rotameric states with the \(\chi_3\) dihedral angle (\(C^\text{c}_1-C^\text{c}_5-O^\circ-H^\circ\)) near 0° or 180° (Kossiakoff et al. 1990; Ho and Agard 2008). Inspection of the long-range \(^{13}C\)-HSQC spectrum of BcX revealed crosspeaks between the \(^1H\) of Tyr79 and Tyr105 and only one of their two non-degenerate \(^{13}C\) nuclei (Figs. 2, 8). Based on the Karplus relationship for a \(^3J_{CH}\) coupling (Karplus 1959), the detected \(^{13}C\) must be oriented in a "trans" conformation relative to the \(^1H\). This conclusion is supported by the observation of a strong intra-residue NOE between the \(^1H\) and the adjacent \(^1H\) directly bonded to the "cis" \(^{13}C\) in each ring. Together, these data indicate that the hydroxyl groups of Tyr79 and Tyr105 are well ordered with respect to their aromatic rings and do not undergo facile rotation about the \(C^\text{f}-O^\circ\) bond.

Having defined the positions of the \(^1H\) relative to the rings of Tyr79 and Tyr105, we then used inter-residue NOEs to orient these rings in the X-ray crystallographic structure of BcX. The aromatic ring of Tyr79 is surrounded by hydrophobic sidechains, and its \(O^\circ\) is within hydrogen bonding distance of a buried water (2.7 Å) and the amide \(N^\text{C_2}\) of Gln167 (3.1 Å) (Fig. 8g; Table 1). Given the NMR-defined position of its \(^1H\), Tyr79 must donate a hydrogen bond to the water and accept one from the glutamine.

![Fig. 7](attachment:image.png)

**Fig. 7** The pH-dependent \(^{13}C^\text{ζ}\) chemical shifts of the tyrosine residues in BcX (35°C) measured from the \(^{13}C(^{13}C_{\text{d}})/^{1H}_{\text{h}}\) experiment (lower panels). The lines represent the best fits to a single \(pK_a\) value with a fixed pH dependent chemical shift change (\(\delta_b-\delta_a\)) of 9.7 ppm, except for Tyr88, Tyr113, and Tyr174 for which floating end-points were allowed. The fit \(pK_a\) values and chemical shift changes are summarized in Table 1. Also shown in the upper panel are the mean \(^{13}C\) and \(^1H\) chemical shifts of tyrosine ring nuclei in diamagnetic proteins, as reported in the BioMagResBank (Ulrich et al. 2008), with solid and open boxes indicating a range of one and two standard deviations, respectively. The arrows indicate the directions and magnitudes of the chemical shift changes expected upon deprotonation (Norton and Bradbury 1974; Richarz and Wüthrich 1978; Bundi and Wüthrich 1979). See also Supplemental Fig. S9 and S10.
is consistent with the O\textsuperscript{1} of Gln167 accepting a hydrogen bond from Arg49 and argues against flipping the amide group of this residue to reverse the donor/acceptor behaviour of Tyr79. Intriguingly, these data indicate that Tyr79 shows significant protection against HX despite donating a hydrogen bond to a buried water. A similar situation has been documented for the protected N\textsuperscript{2}H of neutral His149 in BcX (Connelly and McIntosh 1998).

Tyr105 participates in a fascinating set of polar interactions within the interior of BcX (Fig. 8m; Table 1). In light of our current NMR results, this tyrosine donates a strong hydrogen bond to the O\textsuperscript{61} of Asp83 (2.6 Å). This may account for its highly downfield shifted \textsuperscript{1}H\textsuperscript{a} signal at 12.53 ppm. Previous studies have shown that Asp83 has a pK\textsubscript{a} < 2 and thus is negatively-charge within the folded structure of BcX, forming a buried salt bridge with Arg136 (Joshi et al. 1997; McIntosh et al. 2011). Parenthetically, Asp83 O\textsuperscript{2} is also positioned to accept a hydrogen bond from the detectable \textsuperscript{1}H\textsuperscript{b} of Tyr53. Having defined the relative location of Tyr105 \textsuperscript{1}H\textsuperscript{b} by NMR methods, we conclude that two crystallographically-identified buried waters must be donating hydrogen bonds to the O\textsuperscript{2} of this aromatic sidechain (2.8 and 2.9 Å). One of these waters also accepts hydrogen bonds from Arg132 and the other is tightly positioned by negatively-charged Asp101 [pK\textsubscript{a} < 2 (Joshi et al. 1997)], the above mentioned neutral His149 [pK\textsubscript{a} < 2.3 (Plesniak et al. 1996a)], and Ser100. Surprisingly, despite these extensive interactions and its slow rate of ring flipping, Tyr105 exhibits less protection from HX than does Tyr79.

Tyrosine dynamics and hydroxyl HX

Complementary insights into the dynamic properties of the BcX tyrosines were obtained using NMR spectroscopy to measure their ring flipping and HX kinetics. In particular, the four residues with detectable \textsuperscript{1}H\textsuperscript{a} signals had k\textsubscript{ex} < 0.1 s\textsuperscript{-1} over the pH range examined and hence estimated HX protection factors of \sim 10\textsuperscript{-4}–10\textsuperscript{8}. As expected, each of these sidechain hydroxyls is sequestered from bulk solvent due to burial and hydrogen bonding within the core of BcX. Given that \textsuperscript{1}H\textsuperscript{a} resonances from the remaining tyrosines were not detected in spectra recorded at pH 6.5 in H\textsubscript{2}O buffer, these residues likely undergo rapid HX with k\textsubscript{ex} greater than 10–100 s\textsuperscript{-1} and thus have protection factors less than 1,000–100 under these conditions (Liepinsh et al. 1992; Liepinsh and Otting 1996). The first of these two order-of-magnitude limits reflect the fact that if k\textsubscript{ex} > 3\textsubscript{JCH} \sim 5 Hz, then loss of polarization transfer will preclude detection of the \textsuperscript{1}H\textsuperscript{a} signal in a long range \textsuperscript{13}C-HSQC spectrum (Henry and Sykes 1990). The second assumes that line broadening of more than about 30 Hz due to exchange (\Delta\text{linewidth} = k\textsubscript{ex}/\pi at half-height) will preclude detection in a 1D \textsuperscript{1}H-NMR spectrum, particularly if requiring an echo delay for \textsuperscript{13}C/\textsuperscript{15}N-filtering. More accurate rates or rate limits could be obtained by the elegant approach of Kainosho and co-workers, which relies upon the interplay of exchange kinetics and an \sim 0.13 ppm deuterium isotope shift experienced by the phenolic \textsuperscript{13}C in D\textsubscript{2}O versus H\textsubscript{2}O solutions (Takeda et al. 2009). Of course, this also assumes that none of these residues has a protected \textsuperscript{1}H\textsuperscript{a} with a chemical shift upfield of \sim 7 ppm, as such a signal might be obscured by the various multiple-bond correlations between non-labile \textsuperscript{1}H and \textsuperscript{13}C nuclei in the spectrum of Fig. 1c. Consistent with this conclusion, each of these apparently rapidly exchanging tyrosines has a hydroxyl O\textsuperscript{b} that is at least partially solvent exposed in the crystal structure of BcX (Table 1).

Classically, HX kinetics are interpreted in terms of local or global conformational fluctuations between a closed and an open state (k\textsubscript{eq} \equiv k\textsubscript{open}/k\textsubscript{close}). Exchange occurs from the unprotonated open state with the pseudo-first order rate constant (k\textsubscript{open}) predicted for the mainchain or sidechain moiety in a reference random coil polypeptide under similar experimental conditions (Hvidt and Nielsen 1966; Englander and Kallenbach 1983). In the case of Tyr53, exchange appeared to approach the pH-independent EX1 limit. If so, the measured k\textsubscript{ex} \sim 10\textsuperscript{-3} s\textsuperscript{-1} likely corresponds to the rate constant k\textsubscript{open} for conformational fluctuations leading to an exchange competent open state. This also implies that the closing rate constant k\textsubscript{close} for those
fluctuations is slower than the \( k_{\text{pred}} \sim 10^3 \, \text{s}^{-1} \) estimated for an unprotected tyrosine at pH \( \sim 4.3 \) such that exchange occurs with every opening event (Liepinsh et al. 1992; Liepinsh and Otting 1996). Accordingly, \( K_{\text{eq}} > 10^{-6} \) for the conformational fluctuations leading to HX of the Tyr53 hydroxyl. As mentioned above in reference to the crystal structure of \( \text{BeX} \), this protected tyrosine most likely donates a hydrogen bond to the negatively-charged sidechain of buried Asp83 \( [pK_a < 2 \text{ (Joshi et al. 1997)}] \) and accepts one from a bound water (Table 1).

In contrast to Tyr53, both Tyr26 and Tyr79 exhibited pH-dependent HX indicative of a pre-equilibrium or EX2 mechanism. In this case, \( k_{\text{close}} > k_{\text{pred}} \) and \( k_{\text{ex}} = K_{\text{eq}} \, k_{\text{pred}} \). Thus, the protection factor \( k_{\text{pred}} / k_{\text{ex}} \) is the inverse of the equilibrium constant for the conformational fluctuations from the closed to the open state. Converting to a free energy scale, the protection factors of \( 10^7 \) and \( 10^8 \) estimated for Tyr26 and Tyr79 correspond to \( \Delta G_{\text{HX}} \) values of 9.6 and 10.9 kcal/mol, respectively. Although substantial, the free energy change for global unfolding of \( \text{BeX} \) could still be larger than these values, as suggested by its highly ordered main chain (Connelly et al. 2000) and its irreversible denaturation at a midpoint temperature \( T_m \sim 60^\circ \text{C} \) (Davoodi et al. 1995) and \( [\text{urea}]_m \sim 5.5 \, \text{M} \) (pH 5.5 and 20°C; unpublished results). In the crystal structure of \( \text{BeX} \), Tyr26 appears to donate a hydrogen bond to the carbonyl oxygen of Met169, whereas NMR data demonstrates that Tyr79 accepts a hydrogen bond from the sidechain nitrogen of Gln167 and donates one to a bound water (Fig. 8g). Although readily detectable, the \( ^1\text{H}^\gamma \) of Tyr105 exchanged with water on a timescale too fast for measurement after transfer to \( \text{D}_2\text{O} \) buffer, yet too slow for analysis by magnetization transfer transfer approaches. Thus, insights into the kinetic mechanism of Tyr105 HX were not obtained. As shown in Fig. 8m, this latter residue also donates a hydrogen bond to the carbonylate of buried Asp83 and accepts hydrogen bonds from two bound waters.

Tyrosine dynamics and ring flipping

The observation that the stereochemically equivalent nuclei of aromatic rings located within the anisotropic environment of a protein typically show averaged chemical shifts provided some of the earliest and clearest evidence for the dynamic nature of these biopolymers (Snyder et al. 1975; Wüthrich and Wagner 1975). Indeed, based on an analysis of assignments reported in the BioMagResBank, Skalicky et al. (Skalicky et al. 2001) estimated that \( >95\% \) of all tyrosine and phenylaniline rings in proteins undergo fast flipping \( (>10^3 \, \text{s}^{-1}) \) on the chemical shift time scale at ambient temperatures, thereby yielding such averaged signals. Conversely, distinct signals from \( \delta_1 / \delta_2 \) to \( \varepsilon_1 / \varepsilon_2 \) nuclei (indicative of hindered ring flipping on this timescale) have been detected in a surprisingly limited number of examples. This behaviour presumably results from slower conformational fluctuations yielding transient cavities in which an aromatic sidechain can undergo a rapid two-site jump between symmetrically equivalent positions differing by only a \( 180^\circ \) rotation about the \( \text{C}^\alpha - \text{C}^\gamma \) bond. Through temperature- and pressure-dependent \( ^1\text{H} \) lineshape or magnetization transfer measurements, the rate constants and activation \( \Delta H^\prime, \Delta S^\prime, \) and \( \Delta V^\prime \) parameters for these fluctuations have been characterized in a few proteins (Wagner et al. 1976; Wagner 1980; Nall and Zuniga 1990; Skalicky et al. 2001; Hattori et al. 2004; Rao and Bhuyan 2007).

Consistent with these general observations, only two of the fifteen tyrosines in \( \text{BeX} \) showed four distinct, rather than two averaged, signals from ring \( ^{13}\text{C} - ^1\text{H} \) pairs diagnostic of slow ring flipping. Using a \( ^{13}\text{C} \) longitudinal exchange experiment, flipping rate constants of \( \sim 10 \) and \( \sim 0.2 \, \text{s}^{-1} \) were measured for Tyr79 and Tyr105, respectively. The remaining assigned tyrosines must rotate at least 100-fold faster in order to yield averaged NMR signals. Although it is expected that surface tyrosines such as Tyr88, Tyr113, and Tyr174 show relatively unimpeded motions, several of these fast flipping residues are extensively buried. Skalicky et al. (Skalicky et al. 2001) also reported that solvent accessibility alone is not a key parameter for identifying slowly rotating rings. This prompts the question as to why only Tyr79 and Tyr105 flip slowly. Interestingly, in the X-ray crystallographic structure of \( \text{BeX} \) (1XNB.pdb), these two residues have the lowest average \( \text{C}^\alpha \) thermal factors (B values) of all tyrosines, indicating that they are relatively well ordered within the protein core. Although a similar trend was reported for the fastest flipping tyrosines in yeast iso-2-cytochrome c (Nall and Zuniga 1990), other faster flipping buried residues have only marginally higher average B values. It is also noteworthy that all four tyrosines in \( \text{BeX} \) with detectable \( ^1\text{H}^\gamma \) signals exhibited faster ring flipping than hydroxyl HX. For example, the flipping rate constant of Tyr53 must be \( >10^3 \, \text{s}^{-1} \) to yield averaged NMR signals, whereas the opening rate constant, \( k_{\text{open}} \sim 10^{-3} \, \text{s}^{-1} \), for its apparent EX1-regime HX is at least \( 10^6 \)-fold slower. Furthermore, Tyr105 showed more hindered rotation, yet faster HX, than Tyr79. Thus, conformational fluctuations allowing a two-site jump about the \( \text{C}^\alpha - \text{C}^\gamma - \text{C}^\delta - \text{O}^\gamma \) axis of an aromatic sidechain are not correlated with those disrupting hydrogen bonding interactions with its hydroxyl OH, as required for HX with water. A similar lack of correlation between ring flipping and protein stability/amide HX was also reported for a series of BPTI homologs (Wagner and Wüthrich 1978).

Collectively, the current studies of tyrosine ring flipping and hydroxyl HX, along with previous amide \( ^1\text{N} \) relaxation measurements (Connelly et al. 2000), highlight the complex dynamics exhibited by the backbone and
sidechain residues of BcX. These NMR data should also serve as a benchmark for the development of theoretical approaches to better describe the amplitudes and timescales of the conformational fluctuations of a protein in its native state ensemble.

Tyrosine pKₐ measurements

The pKₐ value of a tyrosine in a random coil polypeptide is ~10. (Thurkil et al. 2006; Grimsley et al. 2009). Thus, within the context of a folded protein under physiological conditions, the vast majority of these sidechains are expected to exist in the neutral state. Of course, functionally critical tyrosines, such as those serving as a catalytic nucleophile or general acid/base, should be charged in their active form and thus will have significantly perturbed pKₐ values (Harris and Turner 2002). For example, the general base Tyr149 in UDP-galactose 4-epimerase is reported to be less than or greater than the lowest or highest sample pH value examined, respectively. Given that the ¹³C chemical shift of this active site tyrosine remained constant over all pH conditions examined, this apparent titration is certainly due to the ionization of the adjacent catalytic general acid Glu172, rather than Tyr69 itself. Third, in the absence of any measurable titration, the pKₐ value of an ionizable residue could be less than or greater than the lowest or highest sample pH value examined, respectively. Given that the ¹³C chemical shifts of a neutral and charged tyrosine are ~158 and 168 ppm, it is safe to say that all of these residues in BcX are protonated under neutral conditions (Fig. 7). In contrast, such a conclusion cannot be drawn unambiguously from ¹³C or ¹H shifts alone. Thus, alternative approaches for determining the protonation state of an ionizable sidechain, such as direct observation of the titratable proton, as well as the measurement of deuterium isotope shifts (Ladner et al. 1975; Yamazaki et al. 1994; Joshi et al. 1997; Takeda et al. 2009) or J-coupling patterns (Pelton et al. 1993; McIntosh et al. 2006; Iwahara et al. 2008), would be required. Although not investigated in this study, the ¹³C of a tyrosine also shows a substantial chemical shift change (~0.5 ppm) upon deprotonation, and thus the above advantages should also apply for determining pKₐ values by monitoring this nucleus. Fortunately, pulse sequences for measuring tyrosine ¹³C chemical shifts via their sidechain ¹H, ¹H, and/or ¹H nuclei have also been published (Prompers et al. 1998).

Summary

In closing, we have used a combination of established and new NMR spectroscopic methods to characterize the structural (J-coupling, NOE), dynamic (HX, ring flipping), and thermodynamic (pKₐ values) properties of the tyrosine residues in BcX. In addition to providing specific insights into the roles played by these aromatic sidechains in this model glycoside hydrolase, we hope this work will stimulate further studies of these functionally and structurally
important residues in other interesting proteins and protein complexes.

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References


Harriss KD, Turner DJ (2002) Structural basis of perturbed pKa values of catalytic groups in enzyme active sites. IUBMB Life 53:85–98


Joshi MD, Hedberg A, McIntosh LP (1997) Complete measurement of the pKa values of the carboxyl and imidazole groups in Bacillus circulans xylanase. Prot Sci 6:2667–2670


Snyder GH, Rowan R, Karplus S, Sykes BD (1975) Complete tyrosine assignments in high field $^{1}H$ nuclear magnetic resonance spectrum of bovine pancreatic trypsin inhibitor. Biochemistry 14:3765–3777


Wagner G, Demarco A, Wüthrich K (1976) Dynamics of aromatic amino-acid residues in globular conformations of basic pancreatic