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A ^{15}N-filtered 2D ^{1}H TOCSY experiment for assignment of aromatic ring resonances and selective identification of tyrosine ring resonances in proteins: Description and application to Photoactive Yellow Protein

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Summary

A new method to selectively detect the ring resonances of the aromatic residues in ^{15}N-labelled proteins is presented. The experiment consists of a 2D ^{1}H TOCSY sequence with removal of the amide signals via ^{15}N-filtering. Experiments are acquired in the absence and presence of water inversion; combining the two spectra allows selective observation of the tyrosine ring resonances and enables the identification of their δ and ε ring protons. The experiment is demonstrated on a ^{15}N-labelled sample of Photoactive Yellow Protein and is shown to give good selectivity for tyrosine ring resonances under a wide range of temperatures and pH values.

Assignment of the aromatic resonances in proteins is an essential step for structure determination, since the aromatic amino acids often play an important role in the formation of the hydrophobic core of proteins and give rise to important long-range NOEs. Generally, the assignment of aromatic resonances is achieved in two distinct stages. First, the resonances within the rings are linked by means of scalar connectivities; then the ring resonances are linked to the aliphatic protein side-chain resonances by means of NOEs between the ring protons and the side-chain α or β protons (Wüthrich, 1986).

In unlabelled samples, the linking of the aromatic ring resonances is performed by means of 2D TOCSY spectra. However, for proteins containing large numbers of aromatic residues, the overlap present in normal 2D spectra makes complete assignment of the aromatic resonances difficult or impossible. For ^{13}C-labelled samples, a variety of 3D experiments for the assignment of aromatic resonances have been proposed and utilised (e.g. Kay et al., 1993; Yamazaki et al., 1993; Zerbe et al., 1996). Unfortunately, the advantages gained by frequency labelling in a third dimension are largely offset by the limited ^{13}C dispersion, the presence of strong ^{13}C-^{13}C coupling, and the rapid dipolar relaxation of the ^{1}H resonances induced by

the ^{13}C nuclei. Reverse labelling of the phenylalanine resonances has been used to overcome these difficulties (Vuister et al., 1994). Despite these advances, the assignment of aromatic resonances in proteins often remains a challenging problem.

In this communication, we present a new method to selectively detect the ring resonances of the aromatic residues in ^{15}N-labelled proteins. The experiment consists of a 2D ^{1}H TOCSY sequence with removal of the amide signals via ^{15}N-filtering. Experiments are acquired in the absence and presence of water inversion; combining the two spectra allows selective observation of the tyrosine ring resonances and enables the identification of their δ and ε ring protons.

The proposed pulse scheme is shown in Fig. 1 and is a modified sensitivity-enhanced TOCSY experiment with pulsed-field gradient coherence-pathway selection (Cavanagh and Rance, 1990; Adell et al., 1995). The indirect ^{1}H chemical shift measurement is performed concurrently with ^{15}N-filtering in a semi-CT manner (Grzesiek et al., 1993), allowing the assignment to be performed on a ^{15}N-labelled sample in H$_2$O. The H$_2$O magnetization is retained along the z-axis by replacement of all ^{1}H hard pulses with jump–return pulses (Plateau and Gueron,
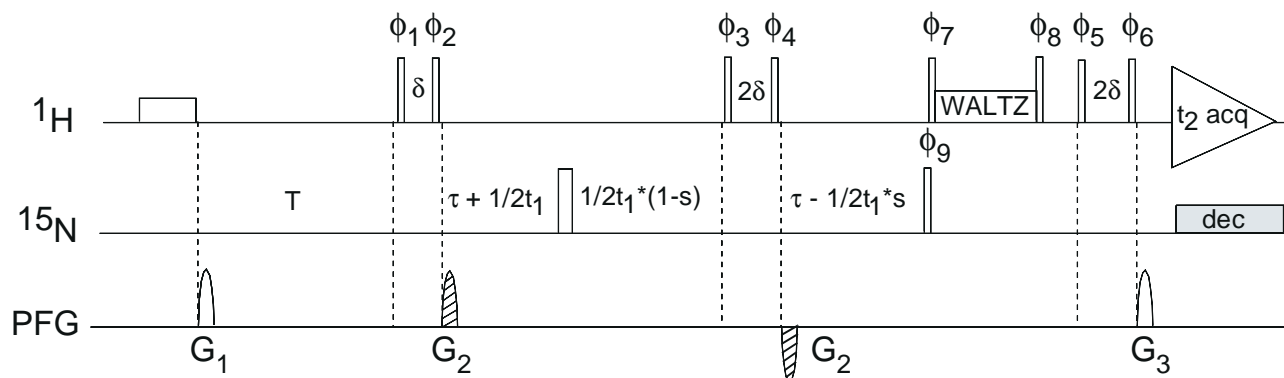
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Fig. 1. Pulse scheme for the gradient-enhanced TOCSY experiment used in this study. Narrow and wide pulses correspond to flip angles of 90° and 180°, respectively. Experiments with and without water saturation are acquired in an interleaved manner; water inversion is achieved by means of an 8 ms square pulse applied on the water resonance. Delay durations: $T = 100$ ms; $\delta = 242$ μs (yielding the first null at 2.07 kHz); $\tau = 2.75$ ms. The semi-constant time factor, s, is set to $2(\tau - G_2)/t_{1,max}$, yielding 0.06. The phase of all pulses is assumed to be x, unless indicated otherwise. Phase cycling: $\phi_1 = x,-x$; $\phi_2 = -x,x$; $\phi_3 = 2x,2(-x),2y,2(-y)$; $\phi_4 = 2(-x),2x,2(-y),2y$; $\phi_5 = 2y,2(-x),2(-y),2x$; $\phi_6 = 2(-y),2x,2y,2(-x)$; $\phi_7 = x$; $\phi_8 = -x$; $\phi_9 = 2x,2(-x)$; Receiver $= x,2(-x),x,-x,2x,-x$. For each t_1 value, two FIDs are recorded and stored separately, with phases ϕ_2 and ϕ_7 incremented by 180° and the amplitude of the hatched gradient pulses inverted for the second FID. For processing, the signals for each t_1 value are added and subtracted, a 90° zero-order phase correction is applied to every second FID, and the resultant data set is processed according to the STATES recipe. For each increment of t_1, the phases of ϕ_1, ϕ_2, and the receiver are inverted. Isotropic mixing for the TOCSY sequence was achieved with WALTZ-16 (Shaka et al., 1983), applied for 25 ms at an 8 kHz field strength. A relaxation delay of 1 s was allowed between transients. All gradients were applied for a duration of 1 ms at strengths of 20, 15, and 30 G/cm for G_1, G_2, and G_3, respectively.

1982). Additionally, the coherence terms of interest are selected by the gradients applied before and after the TOCSY mixing period.

Experiments with and without inversion of the water resonance are acquired in an interleaved manner; these experiments will be referred to as 'on' and 'off', respectively. Water inversion is achieved by means of a selective square π pulse on the water resonance, followed by a 100 ms delay before the first pulse of the TOCSY sequence. During this delay, saturation transfer from the water via solvent exchange leads to saturation of exchangeable protons, e.g. the hydroxyls of tyrosine rings. This saturation is also transferred via the NOE to protons that are spatially close to the exchangeable protons, leading to attenuation of e.g. tyrosine δ and ε resonances. Subtraction of the 'off' and 'on' spectra will therefore yield a difference spectrum in which the tyrosine ring resonances are much stronger in intensity than the other aromatic ring resonances. A differential intensity of the two cross peaks corresponding to each tyrosine ring is expected, with the most intense cross peak at $(\omega_1, \omega_2) = (\Omega_\varepsilon, \Omega_\delta)$. Potentially, non-tyrosine signals in the difference spectrum could arise from other exchangeable protons, e.g. rapidly exchanging amide protons, in close proximity to non-tyrosine aromatic ring protons.

The sum of the on and off spectra is simply the complete TOCSY spectrum of the aromatic resonances of the protein without the overlapping amide signals. Since the tyrosine resonances are readily recognised from the difference spectrum, the phenylalanine and tryptophan ring resonances can be more easily identified in the sum spectrum.

The experiment is demonstrated on a 2 mM ^{15}N-labelled sample of Photoactive Yellow Protein (PYP), a photochemically active receptor protein thought to be involved in the negative phototaxis of *Ectothiorhodospira halophilia* (Sprenger et al., 1993). PYP contains a *p*-coumaric acid chromophore attached via a thioester link to residue Cys[69] (Baca et al., 1994; Hoff et al., 1994; Borgstahl et al., 1995). The chemical structure of the chromophore is indicated in Fig. 2. The protein contains nine phenylalanines, five tyrosines, one tryptophan, and two histidines; in addition, the aromatic and α/β protons from the chromophore resonate in the aromatic region of the ^{1}H spectrum. All experiments were acquired on a Varian UNITYplus 500 spectrometer equipped with a triple-resonance probe with a shielded z-gradient coil, operating at a ^{1}H frequency of 499.91 MHz.
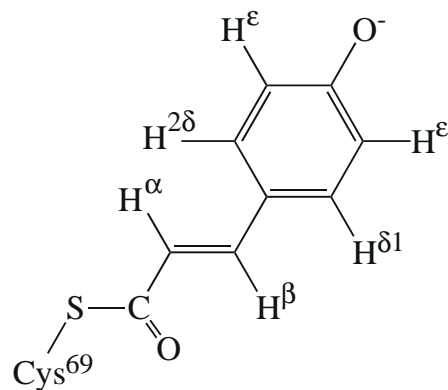

Fig. 2. Chemical structure of the *p*-coumaric acid chromophore in PYP.
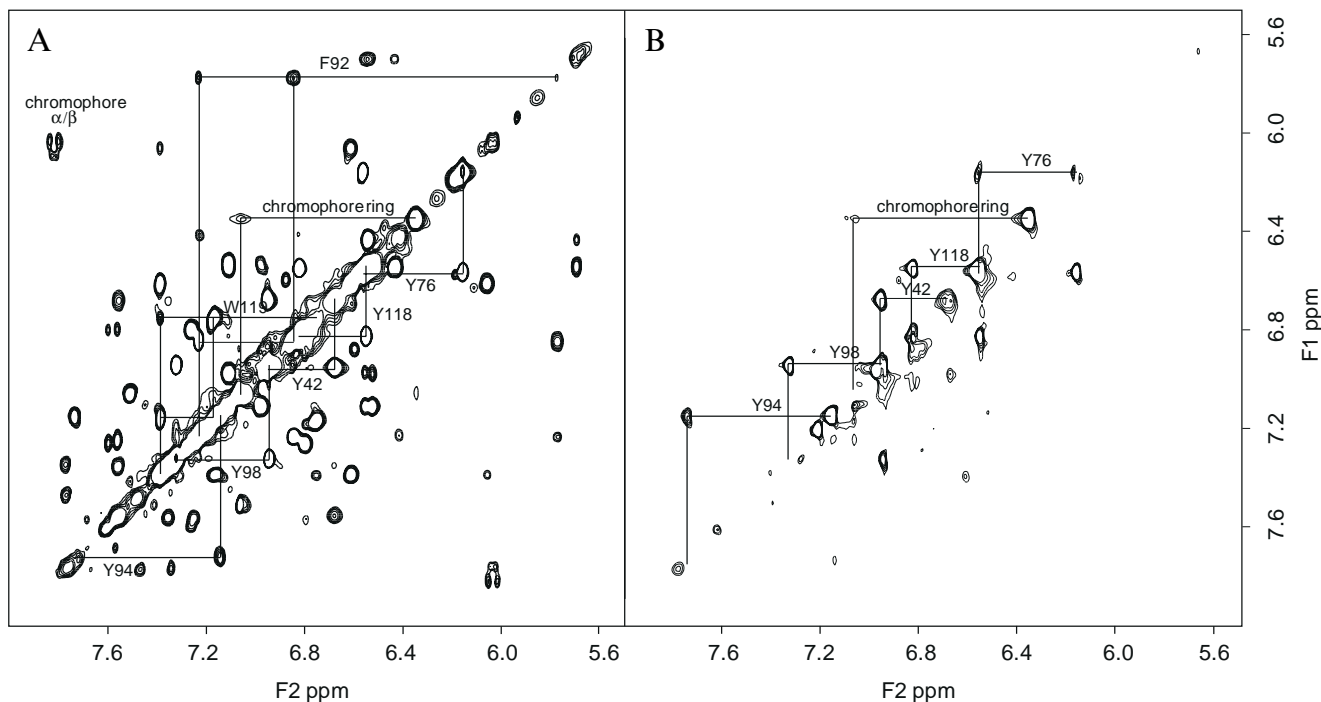
Fig. 3. Aromatic regions of the sum (A) and difference (B) spectra acquired on the PYP sample at pH 5.7 and 311 K. The spectra were acquired with the pulse sequence of Fig. 1 using 32 transients per complex increment with the following spectral widths and acquisition times: F1 (^{1}H) 5000 Hz, 51 ms; F2 (^{1}H) 6666 Hz, 154 ms.

The aromatic region of the sum spectrum of PYP at pH 5.7 and 311 K is shown in Fig. 3A. The high quality of the ^{15}N-filtering is apparent. All the protein aromatic spin systems could be discerned and examples of these are indicated in Fig. 3A. The aromatic and α/β protons of the chromophore are also labelled in the figure.

Figure 3B shows the difference spectrum for PYP at pH 5.7 and 311 K. The high intensity of the tyrosine ring resonances in comparison to other ring resonances is clear. Additionally, the two cross peaks corresponding to each tyrosine ring are of unequal intensities, with the strongest cross peaks at $(\omega_1, \omega_2) = (\Omega_\varepsilon, \Omega_\delta)$. The spectrum is plotted such that the tyrosine ring resonances are at similar intensities in the sum/difference spectra, in order to quantitate the selectiveness of this technique. In the difference spectrum, all the tyrosine ring $(\Omega_\varepsilon, \Omega_\delta)$ cross peaks are at least four times as intense as the most intense cross peak corresponding to a phenylalanine ring. Therefore, identification of the tyrosine ring δ and ε resonances is straightforward and unambiguous.

In order to demonstrate the general applicability of this technique, spectra were also acquired on a sample of 2 mM PYP in H$_2$O solution at pH 5.7 and 280 K, and on a sample of 2 mM PYP in H$_2$O solution at pH 8.7 and 311 K. In both cases (data not shown), the selectivity of the experiment for tyrosine ring resonances was good; again intensity ratios of tyrosine:phenylalanine cross peaks of 4:1 were obtained. In the case of the PYP sample at pH 5.7 and 280 K, only three tyrosine rings were

identified in both spectra, owing to exchange broadening caused by ring flipping on an intermediate timescale at this low temperature.

Information about the chromophore ring of PYP is also obtained from this experiment. Under all conditions studied, a resonance corresponding to the chromophore ring is observed in the difference spectrum. Since in a 7 Å radius of the chromophore no α-protons resonating at the H$_2$O chemical shift are found, the observed signal should be the result from an NOE with an exchangeable proton. Earlier studies of PYP showed that the chromophore ring is deprotonated (Kim et al., 1995), and completely buried in the major hydrophobic core of the protein (Borgstahl et al., 1995; P. Düx, unpublished results). Residue Glu[46] is in close proximity to the chromophore ring and is presumably protonated, with its hydroxylic proton involved in a hydrogen bond to the chromophore. Other exchangeable protons in close proximity include the hydroxylic protons of Tyr[42], Thr[50], and the guanidinium group of Arg[52]. In the ^{1}H NMR spectrum of PYP recorded at 280 K, two low-field resonances are observed at 13.6 and 15.1 ppm, corresponding to two hydrogen-bonded hydroxyl protons, one or both of which is likely to be hydrogen-bonded to the chromophore phenolic oxygen and thus could be the required, spatially close exchangeable proton.

In summary, we have presented a useful pulse scheme for selectively recording a 2D TOCSY spectrum of the aromatic resonances in proteins. Utilisation of the rapid

solvent exchange properties of the tyrosine hydroxyl protons allows an unambiguous identification of the tyrosine ring resonances, thereby aiding the assignment of aromatic resonances. The method is simple to implement, highly robust, and could be easily utilised as a building block in a 3D experiment.

Acknowledgements

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