New Multidimensional Editing Experiments for Measurement of Amide Deuterium Isotope Effects on C^β Chemical Shifts in ^{13}C, ^{15}N-Labeled Proteins

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Novel multidimensional NMR pulse sequences for measurement of the three- and four-bond amide deuterium isotope effect on the chemical shifts of ^{13}C$^\beta$ in proteins are presented. The sequences result in editing into two subspectra of a heteronuclear triple resonance spectrum $\{\omega(\text{N}), \omega(\text{C}^\beta), \omega(\text{H}^\alpha)\}$ according to there being a deuterium or a proton attached to ^{15}N for the pertinent correlations. The new experiments are demonstrated by an application to the first module of the ^{13}C,^{15}N-labeled protein RAP 18-112 (N-terminal module of α_2-macroglobulin receptor associated protein). © 1998 Academic Press

It has been known for many years that isotope effects on chemical shifts reflect structural information (*1–7*) but isotope effects have so far not found widespread use in structure determination of proteins by NMR. Excellent structures have been determined based on the nuclear Overhauser effect (NOE) and *J* coupling constants; nevertheless the independent constraints of particularly deuterium isotope effects hold the potential of paving the way for even more accurate structures. Partial deuteration necessary for study of deuterium isotope effects is not only useful for this purpose but also serves to prolong transverse relaxation times which provides most welcome sensitivity enhancement in particular for larger proteins.

Contributing to the little use of deuterium isotope effects in protein structure determination has undoubtedly been the overlap of signals from different isotopomers that has made it difficult to quantify the individual effects. Ottiger and Bax (*8*) have proposed an elegant solution to this problem, namely to relate the well-resolved one-bond deuterium isotope effects to longer-range ones in a two-dimensional (2D) way very similar to E.COSY (*9–11*). As a small long-range *J* coupling constant can be measured easily if it can be related to a large one-bond *J* in an E.COSY-type way, the same holds true when long-range isotope effects are related to one-bond effects.

In analogy to S^{3}E (*12, 13*) and S^{3}CT (*14*) editing of E.COSY-type spectra which greatly ease measurement of *J* coupling constants, we recently proposed triple resonance editing experiments for measurement of the amide deuterium

isotope effect on the chemical shifts of the ^{13}C$^\alpha$ and ^{13}CO chemical shifts in the backbone of ^{13}C,^{15}N-labeled proteins (*15*). The result is two subspectra, one from molecules with a deuterium nucleus at pertinent amide positions and one from molecules with an amide proton. In this Communication we extend these ideas to measurement of the amide deuterium isotope effect on the chemical shifts of C^β in the side chains, which requires a different type of pulse sequence.

An appropriate isotope-selective-polarization (ISP) three-dimensional (3D) pulse sequence dubbed ISP H(CA)CB,N is outlined in Fig. 1a. Starting from the magnetization of H$^\alpha$ protons there is an initial INEPT-type transfer to C$^\alpha$ carbons where antiphase character with respect to N and C$^\beta$ is built up during the delay τ_{CA}. Then follows coherence transfer to C$^\beta$ that evolves during t_2 and a bit later also transfer to N evolving during t_1. Appended to this period is the editing element distinguishing between coherences with a proton and a deuterium nucleus at the amide position in the product operators. The editing element consists of a two-step phase cycle $\phi = -x, x$, the data sets of which are stored separately in order to be added (^{2}D subspectrum) and subtracted (^{1}H subspectrum) from each other. For $\phi = -x$ or x the first or second dashed proton pulse, respectively, is applied. The rest of the experiment is the reverse of the beginning, i.e., via C$^\alpha$ transferring magnetization back to H$^\alpha$ for detection. The spectrum will exhibit three- and four-bond amide deuterium isotope effects on the C$^\beta$ chemical shifts that can be distinguished based on the ^{15}N chemical shifts in the F_1 dimension.

The C$^\beta$ evolution in t_2 occurs in a constant time delay (*16*) equal to multiples of $(^1J_{C^\alpha C^\beta})^{-1}$, which ensures efficient coherence transfer C$^\alpha \to$ C$^\beta \to$ C$^\alpha$ and no multiplet structure due to ^{13}C-^{13}C couplings. During ^{15}N chemical shift evolution in t_1 all heteronuclear couplings are suppressed either by ^{1}H decoupling or by π pulses on the ^{13}C channels. The dashed ^{1}H π pulses serve to refocus ^{13}C-^{1}H couplings during the window without ^{1}H decoupling in both steps of the editing cycle. With this pulse sequence construction there are two limitations on the resolution in the t_1 dimension, namely $t_1^{\text{max}} < T - 8\tau_{\text{N}}$ and
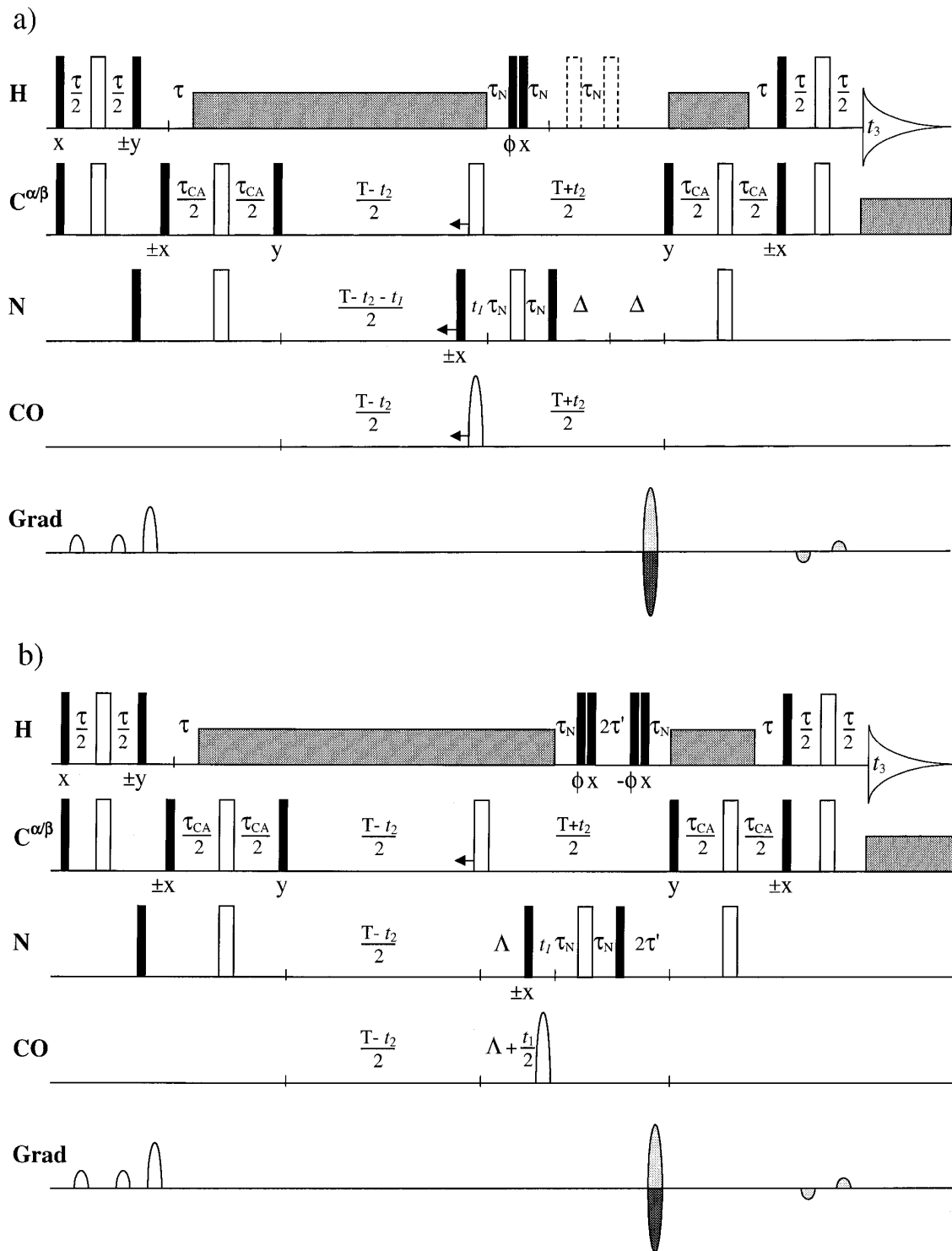
FIG. 1. H^N/D^N-edited ISP H(CA)CB,N pulse sequences for determination of the amide deuterium isotope effect on the chemical shifts of C^β in protein side chains. $\tau = (2^1 J_{C^\alpha H})^{-1}$, $\tau' = (2^1 J_{C^\beta H})^{-1}$, $\tau_N = (2^1 J_{NH})^{-1}$, (a) $\Delta = (T + t_2 - t_1)/4 - \tau_N$, (b) $\Lambda = (T + t_2)/2 - t_1 - 2(\tau_N + \tau')$, where T is a constant time delay equal to twice or higher multiples of $(^1 J_{C^\alpha C^\beta})^{-1}$. Filled and open bars indicate $\pi/2$ and π pulses, respectively, and phases are included below the pulses. Pulse phases with the prefix $\pm$ indicate independent two-step phase cycles with alternating receiver phase. Each of the two $C^{\alpha/\beta}$ $\pi/2$ pulses of phase y can be phase shifted by π at constant receiver phase. The shaded pulsed field gradients are used for echo antiecho selection. The editing cycle consists of two steps $\phi = -x$ and $\phi = x$. The resulting two data sets are stored separately and subsequently added and subtracted to yield the two subspectra. In the pulse sequence in (a) the phase $\phi = -x$ is combined with the first dashed π^H pulse and the phase $\phi = x$ with the second dashed π^H pulse.
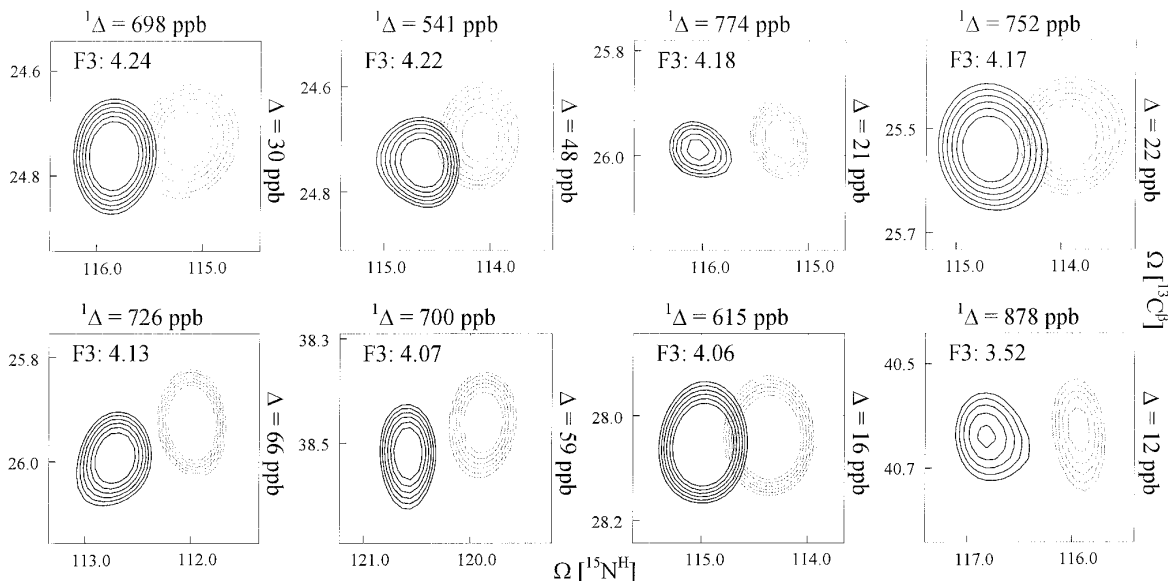
FIG. 2. Representative cross peaks from H^N/D^N edited ISP H(CA)CB,N spectra of the first module of $^{13}C,^{15}N$-labeled RAP 18-112 (50% H_2O/50% D_2O, pH 6.4) recorded with the sequence in Fig. 1a at 25°C on a Varian Unity Inova 750-MHz spectrometer. The two edited subspectra corresponding to protonated (black contours) and deuterated (gray contours) amide groups, respectively, have been overlaid using the software package PRONTO [18]. During the relaxation delay a BIRD [19] rotation is applied for selective inversion of ^1H spins not bound to ^{13}C. The delay after the BIRD rotation is adjusted for minimal longitudinal water magnetization at the beginning of the sequence and a weak gradient during this delay is applied to reduce radiation damping. Parameters: Relaxation delay 0.80 s before and 0.62 s after BIRD, $t_1(max) = 8.55$ ms; $t_2(max) = 22.66$ ms; 16 scans; $\tau = 3.57$ ms; $\tau_{CA} = 17.50$ ms; $\tau_N = 5.56$ ms; $T = 57.14$ ms. WALTZ-16 [20] was used for proton decoupling while GARP [21] was used for ^{13}C decoupling in t_3 and a SINC shape for the selective CO π pulse with duration 132.6 μs for CO decoupling in t_1. A data matrix of $28 \times 280 \times 2048$ points covering $1520 \times 7545 \times 10000$ Hz was zero-filled to $256 \times 2048 \times 2048$ prior to Fourier transformation. Cosine square in t_1 and t_2 and 12 Hz exponential line broadening in t_3 was applied. States–TPPI mode was employed in t_1 and echo–antiecho mode in t_2/t_3. The isotope effects were estimated from F1/F2 2D sections with a precision of about ± 3 ppb. The determined $^1\Delta(N)$ and $\Delta(C^\beta)$ isotope effects are indicated next to the boxes, while the chemical shifts of H^α in F3 are given on top of the peaks. The spectra of RAP 18-112 have not been assigned as yet.

$t_1^{max} < T - t_2^{max}$. In practice, this requires a delay T on the order of $2(^1J_{C^\alpha C^\beta})^{-1}$ with transverse C^β magnetization, which represents a limiting factor for larger proteins. That only a coarse resolution is feasible in the t_1 dimension is not a problem since it is only required to resolve the ^{15}N chemical shifts in that dimension. The one-bond isotope effects can be conveniently measured as a by-product of the ISP methods presented in Ref. [15] measuring amide deuterium isotope effects on carbonyl and alpha carbon chemical shifts.

An interesting variant of the pulse sequence in Fig. 1a is to shift both dashed ^1H π pulses by $\tau' = (2^1J_{C^\beta H})^{-1}$ to the right. That causes an evolution of $^1J_{C^\beta H}$ during $(^1J_{C^\beta H})^{-1}$, which inverts the signals associated with an odd number of H^β protons. This variant also allows a larger t_1^{max} as there is a new inequality, $t_1^{max} < T - 4(\tau_N + \tau') - \delta_G$ where δ_G is the delay required for the gradient at the end of the second Δ delay. In practice, the increase in t_1^{max} is from 12.7 to 18.5 ms. However, any increase of the resolution in the t_1 dimension occurs at the expense of the resolution in the t_2 dimension as for both experiments $t_1^{max} + t_2^{max} < T$.

When it is desirable to maximize the resolution in the t_2 dimension where the isotope effects on the C^β chemical shifts are measured the pulse sequence in Fig. 1b is recommended. In this one $t_2^{max} = T$ and signals associated with odd and even numbers of H^β protons have opposite phase as the $^{13}C^\beta$-^1H couplings evolve during $(^1J_{C^\beta H})^{-1}$. The price to pay for the improved resolution in t_2 is a shorter $t_1^{max} < T/2 - 2(\tau_N + \tau')$ (typically $t_1^{max} < 9.8$ ms) and no suppression of heteronuclear couplings between ^{15}N and $^{13}C^\alpha$ during ^{15}N evolution. However, for the coarse resolution achievable the latter point is not of any concern.

In Fig. 2 is shown the result of applying the ISP H(CA)CB, N pulse sequence in Fig. 1a to the 95-residue $^{13}C,^{15}N$-labeled protein RAP 18-112 (N-terminal module of α_2-macroglobulin receptor associated protein) [18]. The editing into two spectra, that have been overlaid, offers an easy recognition of which peaks represent amide protons (black contours) and which represent amide deuterium nuclei (gray contours) for the pertinent correlations. Hence it is straightforward to measure the amide deuterium isotope effects; in the horizontal dimension it is the one-bond effect while it is the long-range one in the vertical dimension.

In conclusion, we have introduced novel 3D editing pulse sequences for efficient measurement of the amide deuterium isotope effect on C^β chemical shifts in protein side chains. The structural implications of these isotope effects still remain to be

investigated in detail and they could prove just as valuable as the amide deuterium isotope effects on the C^α (8) and carbonyl chemical shifts.

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