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Time-shared experiments for efficient assignment of triple-selectively labeled proteins

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Abstract

Combinatorial triple-selective labeling facilitates the NMR assignment process for proteins that are subject to signal overlap and insufficient signal-to-noise in standard triple-resonance experiments. Aiming at maximum amino-acid type and sequence-specific information, the method represents a trade-off between the number of selectively labeled samples that have to be prepared and the number of spectra to be recorded per sample. In order to address the demand of long measurement times, we here propose pulse sequences in which individual phase-shifted transients are stored separately and recombined later to produce several 2D HN(CX) type spectra that are usually acquired sequentially. Sign encoding by the phases of ^{13}C 90° pulses allows to either select or discriminate against $^{13}\text{C}'$ or $^{13}\text{C}^{\alpha}$ spins coupled to ^{15}N . As a result, ^1H - ^{15}N correlation maps of the various isotopomeric species present in triple-selectively labeled proteins are deconvoluted which in turn reduces problems due to spectral overlap. The new methods are demonstrated with four different membrane proteins with rotational correlation times ranging from 18 to 52 ns.

Keywords

BEST-TROSY; Cell-free expression; Deuteration; Hadamard decoding; Isotope labeling; Membrane proteins; Triple-resonance NMR

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1. Introduction

Obtaining complete backbone resonance assignment is desirable for detailed NMR studies of the structure of proteins, their dynamics, and interactions with other molecules. However, in cases where severe degeneracy of peak positions meets large linewidths due to slow tumbling and/or conformational exchange, well-established assignment strategies in solution NMR exclusively based on matching intraresidual and sequential correlations of amide groups with other nuclei may fail. Typical examples include α -helical membrane proteins [1–5].

For uniformly labeled proteins considerable simplification of ^1H - ^{15}N correlation spectra as well as valuable residue-type information can be gained from amino-acid type selective experiments [6–10]. Unfortunately, an increased number of coherence transfer steps or editing elements in the corresponding pulse sequences may render this approach inefficient for larger systems. Alternatively, amino-acid specific isotope labeling methods are available for which molecular size restrictions apply to a much lesser extent and which may take advantage of cell-free expression systems. The latter offer reduced isotope scrambling and requires relatively low amounts of labeled amino acids [11–17]. While amino-acid type information is obtained via selective ^{15}N labeling [12, 14, 18–20], dual selective labeling with ^{15}N and ^{13}C isotopes additionally permits selective detection of specific amino acid pairs based on interresidual $^1J(^{15}\text{N}, ^{13}\text{C}')$ scalar interactions [11, 13, 21–26]. The number of samples required to obtain sufficient anchor points for sequence-specific assignments can be minimized by designing labeling patterns in a combinatorial fashion [27–31].

Recently, we have introduced combinatorial triple-selective labeling [32] to further increase residue-type and sequence-specific information that can be collected with a given number of samples. The disadvantage to this strategy is that a greater number of different 2D ^1H - ^{15}N detected triple-resonance experiments must be acquired including less sensitive ones such as HN(COCA), (CO)HN(CA) and DQ-HN(CA). Additionally, and common to all combinatorial labeling protocols, is the problem that some amide signals can remain undiscovered in the case of chemical shift degeneracy in the two dimensions. Here we present modified pulse sequences that allow recording several experiments in a time-shared (ts) manner to address both prolonged measurement times and spectral overlap.

Time-shared schemes, originally proposed by Sørensen [33], have been extensively used to correlate nuclei involved in independent coherence transfer pathways in a single experiment, resulting in appreciable time savings [34–49]. Although different experiment types required to distinguish individual sites in triple-selectively labeled proteins are recorded simultaneously, identical nuclei (^1H and ^{15}N) are correlated in the implementations described here. Measurement times are dictated by the least sensitive of the combined experiments, while the remaining experiments are obtained “for free”. This is achieved by separate storage of transients acquired with different pulse phases followed by appropriate data recombination [50–57] in order to select amide signals involved in different scalar coupling networks. As a desired side-product of the new methods isotope filtering against either $^{13}\text{C}'$ or $^{13}\text{C}^\alpha$ simplifies $[^{15}\text{N}, ^1\text{H}]$ -HSQC type spectra [58–60], enabling the identification of otherwise overlapping signals.

2. Materials and methods

2.1. Sample preparations

Production of isotopically labeled proteins was carried out using a continuous-exchange cell-free expression system based on an *E. coli* S30 extract [61]. Samples of TMD0 [62] as well as KvAP VSD and proteorhodopsin [32] were prepared as described previously. Lipoprotein signal peptidase II (LspA) was cloned into pET28b with an N-terminal streptactin, 6x histag and thrombin cleavage site. Continuous-exchange cell-free expression in the presence of preformed MSP1D1 H5-DMPC nanodiscs was performed using the standard protocol as described elsewhere [63, 64]. Empty nanodiscs were prepared before use in cell-free expression system as previously published [65]. LspA - MSP1D1 H5-DMPC nanodisc NMR samples were purified by metal affinity chromatography. Empty nanodiscs were removed from the samples by streptactin purification. On overview of the isotopic labeling patterns employed for the four proteins is provided in Table S1 of the supplementary material.

Final sample conditions were as follows: 0.55 mM TMD0 in 1% dihexanoyl-phosphatidylcholine (diC6PC), 25 mM sodium acetate (pH 5), 100 mM NaCl; 0.3 mM KvAP VSD in a detergent mixture of 5% n-dodecylphosphocholine (DPC) and 1.7% n-dodecyl-N,N-dimethylamine-N-oxide (LDAO), 25 mM sodium acetate (pH 5), 2 mM dithiothreitol (DTT); 0.5 mM proteorhodopsin in 4% diheptanoyl-phosphatidylcholine (diC7PC), 25 mM sodium acetate (pH 5), 2 mM DTT; 0.25 mM LspA in MSP1D1 H5/DMPC nanodiscs, 20 mM sodium acetate (pH 4). The samples of KvAP VSD, proteorhodopsin and LspA had a volume of 320 μ l including 5% D₂O and 0.15 mM 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as internal standard and were placed in 5 mm susceptibility-matched Shigemitsu tubes. The sample volume of TMD0 was 190 μ l, contained in a 4 mm Shigemitsu tube that was inserted into a standard 5 mm tube filled with a 0.15 mM DSS solution in D₂O.

2.2. NMR spectroscopy

All NMR experiments were carried out at a Bruker Avance 600 MHz spectrometer equipped with a cryogenic ¹H[¹³C/¹⁵N] triple-resonance probe with sample temperatures set to either 318 K (proteorhodopsin and LspA) or 323 K (TMD0 and KvAP VSD). Pulse sequences were of the [¹⁵N, ¹H]-TROSY [66, 67] type including a sensitivity-enhanced gradient coherence selection scheme [68–70]. Shaped proton pulses centered at 8.5 ppm were employed in all time-shared and separately recorded reference experiments to accelerate longitudinal relaxation between scans according to the Bandselective-Excitation Short-Transient (BEST) [71, 72] protocol. Proton magnetization longitudinally relaxed during the pulse sequences is transferred to ¹⁵N by the final single-transition-to-single-transition (ST2-PT) element. In order to constructively add the resulting ¹⁵N polarization in the subsequent scan a ¹⁵N 180° pulse was applied immediately following ¹H acquisition [73]. Pulse schemes to obtain ¹³C-edited 2D ¹H-¹⁵N correlations were based on magnetization transfer pathways followed in HNCO [74], HNCA [74], HN(CO)CA [75], COHNCA [76] and double-quantum (DQ) HNCA [77] triple-resonance experiments. Carbon-13 evolution periods were replaced by fixed 3- μ s delays and, where applicable, ¹H 180° decoupling

pulses required in the corresponding higher dimensional sequences were omitted [32]. Reference 2D HN(CO), HN(CA), (CO)HN(CA), HN(COCA) and DQ-HN(CA) spectra were recorded using INEPT [78] type ^{15}N - ^{13}C polarization transfer steps and standard phase cycling. Modified pulse sequences employed in the current study (Fig. 1) are based on HMQC-like [79, 80] ^{15}N - ^{13}C transfer rather and involve individual storage of FIDs recorded with different phase settings of ^{13}C 90° pulses. Details are given in the figure legend.

The time between the end of acquisition and the beginning of the next scan of triple-resonance pulse sequences was set to 0.3 s in experiments on TMD0, KvAP VSD, and proteorhodopsin and to 0.7 s in experiments on LspA. Recycle delays in HSQC experiments were 0.3 s for TMD0 and 0.7 s for the remaining proteins. All spectra were acquired with spectral widths of 13 ppm in the ^1H dimension and 50 ppm in the ^{15}N dimension, with carrier frequencies placed on the water resonance and in the center of the amide region, respectively. The ^1H acquisition time was uniformly set to 41 ms (320 complex data points) whereas acquisition times in the ^{15}N acquisition was varied between 42.1 and 73.6 ms for different experiments and samples according to the number of expected signals. The exact number of t_1 data points and corresponding acquisition times employed in the individual experiments are summarized in the supplementary material (Table S1) along with delay durations for ^{15}N - ^{13}C and ^{13}C - ^{13}C magnetization transfer and measurement times.

Spectra processing and analysis was performed with TopSpin 3.2 (Bruker). Cosine-squared window functions were applied for apodization in both dimensions. Contour levels were drawn on an exponential scale using a factor of $2^{1/2}$ in all plots shown in the following.

3. Results and discussion

As shown in a previous publication [32], recording two-dimensional ^{13}C -edited proton-nitrogen correlation spectra on a set of combinatorial ^{15}N , 1- ^{13}C and fully $^{13}\text{C}/^{15}\text{N}$ -labeled protein samples can significantly aid the backbone assignment in situations where high-quality three-dimensional spectra on uniformly labeled samples are difficult to obtain. The method termed combinatorial triple-selective labeling involves acquisition of a total of six experiments, i.e. [^{15}N , ^1H]-TROSY-HSQC and TROSY-based HN(CO), HN(CA), (CO)HN(CA), HN(COCA), and DQ-HN(CA) to unambiguously distinguish ^{15}N - ^1H cross peaks from the six possible combinations of ^{15}N - or $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids in position i and either 1- ^{13}C -labeled, $^{13}\text{C}/^{15}\text{N}$ -labeled or non- ^{13}C -labeled amino acids in position $i-1$. Compared to combinatorial dual-selective labeling which employs only ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ - [13, 28] or ^{15}N - and 1- ^{13}C -labeled [29, 31] amino acids, the percentage of amino acid types as well as sequential pairs that can be identified from a limited number of samples is increased. As a drawback, considerably longer measurement times per sample are required to acquire the supplementary 2D HN(CA), (CO)HN(CA), HN(COCA) and DQ-HN(CA) data sets. On the other hand, dispensing with the ^{13}C evolution periods of the triple-resonance experiments opens up the possibility for time-shared acquisition of different experiment types.

For instance, the (CO)HN(CA) pulse sequence [76] involves a bifurcated magnetization transfer pathway, where phase cycling selects those amides that are coupled to both carbonyl

and α -carbon spins. In triple-selectively labeled proteins backbone ^{15}N nuclei may be coupled to only one ^{13}C species or neither of the two. These are normally detected in separate HNCO, HNCA and HSQC experiments. In the following, it will be shown that simple modifications allow spectral editing of amides with different coupling patterns in a single experiment.

3.1. Description of time-shared 2D triple-resonance pulse sequences

A prerequisite for retaining signals of amide sites from different $^{12}\text{C}/^{13}\text{C}$ isotopomers is a change of ^{15}N - ^{13}C transfer schemes. Out-and-back type HNCX triple resonance pulse sequences usually employ an INEPT module. For those nitrogens that have a suitable coupling partner a pair of 90° $^{13}\text{C}/^{15}\text{N}$ pulses following the delay for J evolution creates antiphase ^{13}C magnetization, whereas in-phase magnetization of isolated nitrogens remains unaffected. The latter is typically dephased by gradients and/or chemical shift evolution and is not converted into detectable coherence at the end of the pulse sequence. In contrast, the spin-echo difference element employed here, which is equivalent to an HMQC-type correlation in 3D versions of the experiments, leaves ^{15}N magnetization transverse irrespective of whether it is coupled to ^{13}C spins or not, samples their chemical shifts as a function of t_1 and converts it to observable proton coherence. More detailed descriptions of the individual experiments are given below.

3.1.1. ts- HN(CO)/HN(CA)—In the pulse sequence of Fig. 1a scalar couplings between ^{15}N and $^{13}\text{C}'$ and between ^{15}N and $^{13}\text{C}^\alpha$ are active during periods δ and ϵ , respectively, allowing for the simultaneous build-up of antiphase magnetization with respect to the two carbon types. Conventional phase cycling applied to 90° ^{13}C pulses exclusively selects for those amide nitrogens coupled to both ^{13}C and $^{13}\text{C}^\alpha$. Separate storage of transients acquired with $\varphi_2 = x, -x$ and with $\varphi_3 = x, -x$ in an interleaved manner results in a sign encoding of signals from the different $^{12}\text{C}'/^{13}\text{C}'/^{12}\text{C}^\alpha/^{13}\text{C}^\alpha$ isotopomers within a dipeptide. Linear combinations of spectra resulting from the four possible phase combinations enable their spectroscopic separation. For transients acquired with (A) $\varphi_2 = x, \varphi_3 = x$, (B) $\varphi_2 = -x, \varphi_3 = x$, (C) $\varphi_2 = x, \varphi_3 = -x$, and (D) $\varphi_2 = -x, \varphi_3 = -x$, the combined spectra contain signals of the following moieties: (A + B + C + D) amides not coupled to either $^{13}\text{C}'$ or $^{13}\text{C}^\alpha$ spins, (A + B - C - D) amides coupled to $^{13}\text{C}^\alpha$ but not to $^{13}\text{C}'$ spins, (A - B + C - D) amides coupled to $^{13}\text{C}'$ but not to $^{13}\text{C}^\alpha$ spins, and (A - B - C + D) amides coupled to both $^{13}\text{C}'$ and $^{13}\text{C}^\alpha$. As a result, rather than individually recording HSQC, HN(CO), HN(CA) and (CO)HN(CA) spectra the desired information can be reconstructed from a single experiment recorded in the same measurement time as the (CO)HN(CA), which is the least sensitive.

A pictorial representation of this Hadamard decoding scheme [56, 87, 88] applied to the ts-HN(CO)/HN(CA) experiment is provided in Fig. 2a. In a triple-selectively labeled sample containing ^{15}N -labeled amino acids preceded by an unlabeled or by a $1\text{-}^{13}\text{C}$ -labeled amino acid type and $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids also preceded by an unlabeled or by a $1\text{-}^{13}\text{C}$ -labeled amino acid type, the four separately stored ^1H - ^{15}N correlation spectra (Fig. 2a panels A-D) show cross peaks for each, but their sign depends on the pulse phases φ_2 and φ_3 employed in the individual transients. Building different linear combinations separates the

cross peaks of the four isotopomeric moieties of the current mixture of carbon labels present in the same molecule into different subspectra (Fig. 2a panels E-H). It should be noted that, while the subspectrum shown in panel H is identical to what is obtained with a conventional (CO)HN(CA) experiment, neither of the other subspectra is identical with a conventional HN(CO) or HN(CA) experiment, which would each contain two cross peaks. However, if desired, the latter can be retrieved by subtracting the spectra of panels B and C, respectively, from the spectrum in panel A.

The described procedure resembles well-known X half filter [89] and double half filter [90] methods that afford editing of intra- and intermolecular NOEs into different subspectra of biomolecular complexes in which only one of the two components is isotope labeled. Conceptually, it is also closely related to isotopically discriminated (IDIS) NMR [59, 91] as well as carbonyl carbon label selective (CCLS) [58] and especially dual carbon label selective (DCLS) [60, 92] schemes. In the former, 2D HN(CO) based pulse sequences with and without evolution of $^1J(^{15}\text{N}, ^{13}\text{C}')$ for a period equaling the inverse coupling constant are applied to mixtures of uniformly ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labeled proteins. When the coupling is active, the two species will acquire a 180° phase difference. Addition and subtraction of the two spectra therefore separates the signals of the individual components. The DCLS approach allows deconvolution of $[^{15}\text{N}, ^1\text{H}]$ -HSQC spectra from ternary mixtures of ^{15}N -, $1\text{-}^{13}\text{C}/^{15}\text{N}$ and $^{13}\text{C}/^{15}\text{N}$ -labeled proteins. Suppression of amide signals attached to either $^{13}\text{C}'$ or $^{13}\text{C}^\alpha$ is achieved by independent $^1J(^{15}\text{N}, ^{13}\text{C})$ filtering during a ^{15}N constant-time evolution period. The advantage over the IDIS method and the time-shared HN(CO)/HN(CA) sequence proposed here is that signal attenuation due to relaxation is reduced because the required period of transverse ^{15}N magnetization is only half as long (i.e. $1/(2\ ^1J(^{15}\text{N}, ^{13}\text{C}))$). On the other hand, forming linear combinations of filtered and reference spectra to obtain the subspectra of the $1\text{-}^{13}\text{C}/^{15}\text{N}$ and $^{13}\text{C}/^{15}\text{N}$ -labeled species, is accompanied by a reduction of signal-to-noise ratios by a factor of $2^{1/2}$. In contrast, as all signals are present in each of the separately stored time-shared HN(CX) spectra recorded with different ^{13}C pulse phases, the signal-to-noise increases upon building linear combinations. Furthermore, due to the HMQC-type transfer in the latter both the $J(^{15}\text{N}, ^{13}\text{C})$ de- and rephasing periods can be used for ^{15}N chemical shift evolution in a full-sweep constant-time manner, resulting in enhanced resolution [76, 93, 94].

3.1.2. ts-HN(CO)/HN(COCA)—The two-dimensional HN(COCA) experiment is indispensable to distinguish $[^{13}\text{C}/^{15}\text{N}]_{i-1} - [^{15}\text{N}]_i$ from $[1\text{-}^{13}\text{C}]_{i-1} - [^{13}\text{C}/^{15}\text{N}]_i$ sequential pairs in triple-selectively labeled proteins, since both give rise to cross peaks in HSQC, HN(CO) and HN(CA) spectra and lack signals in the DQ-HN(CA). In the HN(COCA) pulse sequence magnetization is relayed from amide nitrogens via carbonyls to the α -carbon of the preceding amino acid. It therefore lends itself for time-sharing with an HN(CO) experiment without compromising sensitivity. A suitable pulse scheme is shown in Fig 1b. It employs an HMQC-type module not only for the $^{15}\text{N}\text{-}^{13}\text{C}'$ but also for the $^{13}\text{C}'\text{-}^{13}\text{C}^\alpha$ transfer [75, 95] such that detectable magnetization is retained for $^1\text{H}_i\text{-}^{15}\text{N}_i$ groups not coupled to $^{13}\text{C}'_{i-1}$ and for $^1\text{H}_i\text{-}^{15}\text{N}_i\text{-}^{13}\text{C}'_{i-1}$ moieties not coupled to $^{13}\text{C}^\alpha_{i-1}$. Alternating phases φ_2 and φ_3 between x and $-x$ again entails sign encoding of the different isotopomers, as visualized in Fig. 2b. Addition of the experiments with all four phase combinations (A + B + C + D) yields a

subspectrum with amides not preceded by a ^{13}C -labeled amino acid, the combination (A – B + C – D) a subspectrum with amides preceded by a ^{13}C -labeled amino acid, and the combination (A – B – C + D) a subspectrum with amides preceded by a $^{13}\text{C}/^{15}\text{N}$ -labeled amino acid. In the current implementation both the periods for $^1J(^{15}\text{N}, ^{13}\text{C}')$ de- and rephasing (2δ) and for $^1J(^{13}\text{C}', ^{13}\text{C}^\alpha)$ de- and rephasing (2ϵ) can be exploited for constant-time ^{15}N chemical shift evolution. It should however be noted that decoupling of one-bond and two-bond ^{15}N - $^{13}\text{C}^\alpha$ interactions is only achieved when $\phi_3 = -x$ (i.e. when the 90° $^{13}\text{C}^\alpha$ pulses combine to a 180° pulse), causing some line-broadening for amide nitrogens within or preceded by $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids.

3.1.3. ts-SQ-HN(CA)/DQ-HN(CA)—Sequential pairs of $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids are uniquely identified by their appearance in DQ-HN(CA) spectra. The corresponding pulse sequence is identical to that of a conventional single quantum (SQ) HN(CA) experiment, but the phase cycle differs. To select the DQ state with respect to α -carbons, a four-step phase cycle is usually applied, where the phase of the ^{13}C pulse labeled ϕ_2 in Fig. 1c is incremented in 90° steps with inversion of the receiver phase in every other scan. Separate storage of the four scans, however, allows to retrieve the SQ-HN(CA) spectrum, too, by subtracting the first and third scan, recorded with $\phi_2 = x$ and $\phi_2 = -x$, respectively. Again, an HMQC ^{15}N - $^{13}\text{C}^\alpha$ transfer is required since either the single-quantum or the double-quantum state would be rejected when an INEPT module is used. Addition of all four scans provides a $^{13}\text{C}^\alpha$ -filtered HSQC subspectrum containing the signals of ^{15}N -labeled amino acids and lacking those of $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids.

Of note, all three pulse sequences can be applied to deuterated samples without modification (i.e. ^2H decoupling, because the periods of transverse $^{13}\text{C}^\alpha$ magnetization are negligibly short. For both deuterated and non-deuterated proteins, the success of the spectral editing procedure applied here depends on the match between ^{15}N - ^{13}C and ^{13}C - ^{13}C coupling constants and the duration of periods for the respective J evolution. For those dipeptides containing $^{13}\text{C}^\alpha$ and/or $^{13}\text{C}'$ labels, incomplete conversion of $^{15}\text{N}_y$ in-phase into $^{15}\text{N}_x$ $^{13}\text{C}_z$ antiphase or $^{13}\text{C}'_y$ in-phase into $^{13}\text{C}'_x$ $^{13}\text{C}_z^\alpha$ antiphase magnetization entails unequal intensities in transients recorded with different settings of phases ϕ_2 or ϕ_3 . As a result of imperfect cancellation, subspectra resulting from linear combinations that discriminate against these isotopomers may contain spurious breakthrough signals. Whereas $^1J(^{15}\text{N}, ^{13}\text{C}')$ and $^1J(^{13}\text{C}', ^{13}\text{C}^\alpha)$ coupling constants are comparatively uniform, $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ exhibits a pronounced dependence on backbone conformation [96, 97] and values can range from about 8 to 13 Hz [98]. Of relevance for the majority of membrane proteins, $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ coupling constants tend to be small in α -helical conformations and exact J matching would make the corresponding delay in the pulse sequences of the time-shared HN(CO)/HN(CA) (Fig. 1a) and SQ-HN(CA)/DQ-HN(CA) (Fig. 1c) prohibitively long. Moreover, two-bond ^{15}N - $^{13}\text{C}^\alpha$ couplings neglected in the above description occur for a ^{15}N -labeled amino acid preceded by a $^{13}\text{C}/^{15}\text{N}$ -labeled one. Their magnitude is almost invariably smaller than that of $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ such that simultaneous tuning of to both couplings is impossible. Consequently $^2J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ correlations are likely to show up in both $^{13}\text{C}^\alpha$ edited and filtered subspectra.

3.2. Efficacy of time-sharing in 2D triple-resonance experiments

The novel time-shared pulse sequences allow a more efficient use of measurement time as spectra normally recorded separately are obtained simultaneously while, at the same time, they contain more information. Their viability shall first be verified by application to the N-terminal transmembrane domain of the lysosomal peptide transporter TAPL, named TMD0. This 142-residue protein is predicted to consist of four helices and exists as a homodimer in diC6PC micelles. Backbone resonance assignments have been obtained with the help of combinatorial triple-selective labeling and will be reported elsewhere. A sample containing $^{13}\text{C}/^{15}\text{N}$ -labeled methionine and tryptophan, ^{15}N -labeled alanine, isoleucine, lysine, and threonine, and 1- ^{13}C -labeled phenylalanine and valine was employed in the current study.

A full set of 46 expected backbone correlations is observed in the regular ^{15}N -HSQC (Fig. 3a). Application of the ts-HN(CO)/HN(CA) pulse sequence with delay settings of $\tau = 44$ ms and $\delta = 33$ ms and subsequent building of linear combinations yields four spectra (Fig. 3b–e), each containing a subset of signals: The HSQC subspectrum (b) exclusively shows cross peaks from isolated ^{15}N -labeled amino acids, the HN(CO) subspectrum (c) ideally shows only cross peaks from ^{15}N -labeled amino acids preceded by 1- ^{13}C -labeled ones, the HN(CA) subspectrum (d) shows exclusively cross peaks from isolated $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids, and the (CO)HN(CA) subspectrum (e) shows cross peaks from $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids preceded by 1- ^{13}C -labeled ones, but also from ^{15}N - or $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids preceded by $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids. To distinguish the three possible isotopomeric species in the (CO)HN(CA) subspectrum, additional recording of HN(COCA) and DQ-HN(CA) spectra is required. Overall, a clean separation of the signals from different dipeptide types into the four subspectra is observed, the only exceptions being K2 and W135, both preceded by a $^{13}\text{C}/^{15}\text{N}$ -labeled amino acid type, that are detected in both HN(CO) and (CO)HN(CA) subspectra. The reason for the undesired detection of the K2 signal in the HN(CO) is the above mentioned J -mismatch between $^2J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ and the duration of period τ which was adjusted to the usually larger $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ interaction. In the case of W135 the amide nitrogen is involved in both one-bond and two-bond couplings with α -carbons allowing for a build-up of $^{15}\text{N}_y \text{ } ^{13}\text{C}^{\alpha,i}_z \text{ } ^{13}\text{C}^{\alpha,i-1}_z$ double antiphase coherences during τ that are not cancelled by cycling of ϕ_3 (i.e. the phase of the first $90^\circ \text{ } ^{13}\text{C}^\alpha$ pulse). Whether or not such breakthrough peaks are observed depends on the size of the coupling constants and the inherent intensity of signals associated with a given amide, which can be subject to substantial variation, especially within membrane proteins. While this could potentially lead to some confusion during spectra evaluation, ^{15}N and $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids preceded by $^{13}\text{C}/^{15}\text{N}$ -labeled ones are unambiguously identified by their appearance in HN(COCA) and DQ-HN(CA) spectra, respectively. Spurious peaks due to $^1J(^{15}\text{N}, ^{13}\text{C}')$ mismatch were not observed in either HSQC or HN(CA) subspectra of any ts-HN(CO)/HN(CA) experiment in this study.

For comparison, separately recorded, regular 2D HN(CO), HN(CA) and (CO)HN(CA) spectra of TMD0 are shown in the supplementary material (Fig. S1). Since the delay durations τ and δ are not constrained by spectral editing they were optimized empirically for sensitivity, resulting in somewhat shorter values than in the time-shared experiment. Using

otherwise identical acquisition parameters, the (CO)HN(CA) was obtained in the same measurement time as the entire ts-HN(CO)/HN(CA) dataset and yields the same correlations as the corresponding subspectrum of the latter with overall very similar intensities. The same number of scans/FID (128) as in the (CO)HN(CA) were employed for recording the HN(CA) while 64 scans/FID were sufficient to obtain an HN(CO) of good quality. Taken together the measurement of the separately recorded experiments was 20 h compared to only 8 h for the time-shared experiment (the time for recording the HSQC (16 scans/FID, 1h) is not taken into account as, realistically, such spectra would anyway be recorded prior to setting up any type of triple-resonance experiment).

Since no discrimination against any ^{13}C moiety is applied, each of the regular HSQC, HN(CO), and HN(CA) spectra contains more cross peaks than their counterparts from the time-shared experiments. The crucial information as to whether a given amide nitrogen is coupled to ^{13}C or $^{13}\text{C}^\alpha$, to both, or to neither of the two must be retrieved by spectra superposition. In contrast, the ^{13}C labeling pattern is immediately apparent from individual subspectra of the ts-HN(CO)/HN(CA) in a convenient manner. More importantly, ambiguities caused by spectral overlap can be resolved (vide infra). Also shown in Fig. S1 (d, e) are “conventional” HN(CO) and HN(CA) spectra that have been reconstructed from the time-shared dataset. Resulting from linear combinations of only two of the four separately stored transients their signal-to-noise is, however, lower than that of the HN(CO) and HN(CA) subspectra with discrimination against the respective other ^{13}C type.

The theoretical dependence of signal intensities in the four subspectra on the setting of delay in the ts-HN(CO)/HN(CA) sequence is depicted in Fig. 4. Curves are drawn for three apparent ^{15}N relaxation times T_2^* , i.e. 100 ms, 50 ms, and 30 ms, approximately covering the range observed for the TROSY coherence in 2D [^{15}N , ^1H]-TRACT experiments of the proteins studied here. Uniform $^1J(^{15}\text{N}, ^{13}\text{C}^\beta)$ coupling constants of 15 Hz were assumed for calculating transfer efficiencies in HN(CO) (Fig. 4b) and (CO)HN(CA) (Fig. 4d) subspectra, whereas values of 10.7 and 9.5 Hz, corresponding to the overall average and the average of residues in α -helical conformations [96, 98], respectively, have been considered for $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ coupling constants, relevant for HN(CA) (Fig. 4c) and (CO)HN(CA) subspectra. Invariably large sensitivity penalties arise in HSQC-subspectra for typical durations employed in the ts-HN(CO)/HN(CA) as compared to a regular HSQC. Note, however, that this comparison does not take into account the loss of ^{15}N coherence during the variable t_1 evolution time in the HSQC, which does not occur in the time-shared experiment owing to the constant-time nature of t_1 . In HN(CO) subspectra, reductions of signal intensities range from approximately 20% (for $T_2^* = 100$ ms) to 50% (for $T_2^* = 30$ ms) compared to conventional HN(CO) spectra acquired with sensitivity-optimized δ delays, again ignoring relaxation effects during the semi-constant time t_1 period in the latter. As a consequence of smaller active ^{15}N - ^{13}C coupling constants, the effect of prolonged delay durations in the time-shared experiment is less pronounced in both HN(CA) and (CO)HN(CA) subspectra. Here, the signal attenuation amounts to about 40% in the worst case (large $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$, short T_2^*), while moderate enhancements (10–20%) are apparent for small $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ coupling constants in combination with long relaxation times. Overall, the calculated range of intensity ratios between signals in time-shared and in

separately recorded spectra roughly corresponds to what was experimentally observed in the current study.

Applications of time-shared HN(CO)/HN(COCA) and SQ-HN(CA)/DQ-HN(CA) pulse sequences to TMD0 are presented in Fig. 5. Except for a tryptophan side-chain NH correlation all signals detected in the ^{15}N -HSQC shown at the top are also observed in the time-shared triple-resonance experiments, distributed among three subspectra in each case. The ts-HN(CO)/HN(COCA) was acquired with delay settings close to their respective average $1/(2\ ^1J)$ values ($\delta = 33$ ms and $\varepsilon = 8$ ms), resulting in subspectra that contain all expected correlations and yield a clean separation between $^{12}\text{C}^\alpha\text{-}^{12}\text{C}'\text{-}^{15}\text{N}$, $^{12}\text{C}^\alpha\text{-}^{13}\text{C}'\text{-}^{15}\text{N}$, and $^{13}\text{C}^\alpha\text{-}^{13}\text{C}'\text{-}^{15}\text{N}$ moieties (Fig. 5, left panels). A conventional HN(COCA) spectrum of very similar quality as the one in Fig. 5d was obtained with an identical number of scans (128), but using sensitivity-optimized magnetization transfer times of $\delta = 24$ ms and $\varepsilon = 7$ ms (supplementary material, Fig. S2a). Cross peaks from amide ^{15}NH groups sequentially following either $^{12}\text{C}^\alpha\text{-}^{13}\text{C}'$ or $^{13}\text{C}^\alpha\text{-}^{13}\text{C}'$ labeled residues were obtained in a separately recorded HN(CO) spectrum (Fig. S2b). The relatively high sensitivity of the HN(CO) experiment allows for a reduction of the number of transients by a factor of two compared to the time-shared experiment while a further reduction to 32 resulted in insufficient signal-to-noise. Thus, time savings afforded by the time-shared HN(CO)/HN(COCA) experiment amounts to approximately one third (6h15' vs. 9h20'), not taking into account the measurement time of the regular HSQC.

The three subspectra of the ts-SQ-HN(CA)/DQ-HN(CA) experiment of TMD0 are shown at the right-hand side of Fig. 5. The single sequential pair of $^{13}\text{C}/^{15}\text{N}$ -labeled residues present in this sample (W134-W135) is readily identified in the DQ-HN(CA) subspectrum (panel g). The SQ-HN(CA) subspectrum (panel f) is virtually identical to a separately recorded conventional HN(CA) spectrum (supplementary material, Fig. S2c). The presence of a W135 cross peak implies that there is no discrimination against DQ-HN(CA) correlations in this type of subspectra as it is usually impossible to simultaneously tune the $^{15}\text{N}\text{-}^{13}\text{C}^\alpha$ transfer time (here 40 ms) to $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ and $^2J(^{15}\text{N}, ^{13}\text{C}^\alpha)$. For the same reason, the HSQC subspectrum (Fig. 5e) shows some weak breakthrough signals due to $^2J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ correlations that are, however, easily tagged by their presence in a HN(COCA) spectrum (see Fig. 5d). It should be noted that only half the total amount of transients acquired in a ts-SQ-HN(CA)/DQ-HN(CA) experiment contribute to its SQ-HN(CA) subspectrum. Nevertheless, since the DQ-HN(CA) has the lowest sensitivity of the pulse sequences associated with triple-selective combinatorial labeling data accumulation often takes longest, such that the SQ-HN(CA) subspectrum still results from a number of scans that typically corresponds to what is employed for a conventional HN(CA), but does not utilize extra measurement time.

3.3. Spectral simplification

In any dual- or triple-selective combinatorial labeling scheme, complications arise whenever ^1H and ^{15}N resonance frequencies of two or more backbone amides are degenerate. As a simple example, consider two overlapping amides one of which is preceded by a carbonyl labeled amino acid and the other by an unlabeled one. There will be only one

apparent signal in both the HSQC and HN(CO) spectra and, hence, the latter amide may be overlooked. Such problems can be overcome with the help of the spectral editing capabilities of time-shared 2D triple resonance techniques. This is demonstrated in the following with the voltage-sensor domain (VSD) of the voltage-dependent K⁺ channel from *Aeropyrum pernix* (KvAP), which forms a four-helix bundle as determined by solution NMR [99, 100] and X-ray crystallography [101]. Published resonance assignments are mostly based on 3D spectra of uniformly ²H/¹³C/¹⁵N labeled *E.coli*-expressed protein in diC7PC micelles [99] and in a DPC/LDAO detergent mixture [100]. Using the same detergent mixture we have previously obtained backbone assignments via combinatorial triple-selective labeling in combination with a single 3D HNCA [32] using exclusively non-deuterated protein samples. Resonance frequencies resulting from the latter approach are in overall agreement but are not identical to the published ones [99, 100] due to the use of a different protein construct and slightly different sample conditions.

The combinatorial triple-selective labeling based assignment strategy relies on a clear distinction as to the presence or absence of a ¹H-¹⁵N correlation in each of six experiment types. In a few cases, an unambiguous assignment was compromised by signal overlap in some of these 2D spectra. The region plotted in Fig. 6, which compares subspectra of the various time-shared experiments recorded on a [¹³C/¹⁵N-Ile; ¹⁵N-Ala, Phe, Thr, Tyr; 1-¹³C-Asp, Leu]-labeled sample of KvAP VSD with their conventional counterparts, comprises two such hot spots. Coincidentally, one phenylalanine and two isoleucine residues are involved in both cases: (1) At ca. 8.0/118.7 (¹H/¹⁵N) ppm the two consecutive residues I130 and I131 partially overlap and F137 virtually fully overlaps with I130. Since I130 is preceded by a 1-¹³C-labeled leucine residue, a broad cross peak due to both isoleucines appears in each of the individually recorded HSQC, HN(CO), HN(CA) and (CO)HN(CA) spectra (Fig. 6a), and there is no hint that it conceals a third amide signal. In contrast, the presence of a cross peak in the HSQC subspectrum of the ts-HN(CO)/HN(CA) experiment (Fig. 6d) indicates that there must be an amide resonance at the same position as I130 that is not coupled to either ¹³C' or ¹³C^α, thus allowing for the identification of F137. (2) In a region around 7.5/119.4 (¹H/¹⁵N) ppm, two fully degenerate isoleucines and a phenylalanine are only slightly displaced along the ¹⁵N dimension. Residue F116 is preceded by a 1-¹³C-labeled leucine, I40 by an unlabeled residue and I68 by another isoleucine. Again, there are unresolved cross peaks at this position in all four individually recorded 2D spectra and it remains unclear how many amides contribute to the signal in each case. In the ts-HN(CO)/HN(CA) experiment, however, F116, I40, and I68 exclusively appear in the HN(CO), HN(CA), and (CO)HN(CA) subspectra, respectively, showing that there are at least three residues belonging to different isotopomers.

Overlap in KvAP VSD is resolved by virtue of the isotope editing characteristics in time-shared HN(CO)/HN(COCA) and SQ-HN(CA)/DQ-HN(CA) experiments, too. Which of the three time-shared experiments is more adequate for this purpose depends on the particular combination of isotopomeric species involved in signal overlap. For instance, both the I40/I68/F116 and I130/I131/F137 triplets become fully separated into the three subspectra of the time-shared HN(CO)/HN(COCA) (Fig. 6e) and the phenylalanine and the pairs of isoleucine residues can be distinguished in the time-shared SQ-HN(CA)/DQ-HN(CA)

subspectra (Fig. 6f). It should be noted that although each of the three time-shared experiments provides HSQC-type subspectra and HN(CO)- and HN(CA)-type subspectra are produced twice, none of them is identical since the pulse sequences discriminate against different combinations of $^{13}\text{C}'$ and $^{13}\text{C}^{\alpha}$ labels.

3.4. Application to larger systems

One important caveat to be raised against the time-shared pulse sequence's editing features is that they require prolonged delay durations to obtain clean filtered subspectra. In conventional pulse sequences, periods δ , and ε for ^{15}N - $^{13}\text{C}^{\alpha}$, ^{15}N - $^{13}\text{C}'$ and $^{13}\text{C}'$ - $^{13}\text{C}^{\alpha}$ magnetization transfers, respectively, are usually optimized for sensitivity, often resulting in values considerably shorter than $1/(2\ ^1J)$. In contrast, delay settings should be reasonably close to $1/(2\ ^1J)$ in the time-shared experiments presented here, potentially leading to substantial signal losses for fast relaxing spin systems. By the standards of membrane proteins TMD0 and KvAP VSD, which have rotational correlation times of 25 ns and 18.5 ns, respectively, are only moderately slow tumbling and transverse relaxation did not pose serious restrictions to the performance of the time-shared pulse sequences. We have also applied the new methods to two more demanding systems to assess whether extended delay durations would become prohibitive.

As the first example, time-shared HN(CO)/HN(CA) and HN(CO)/HN(COCA) experiments were tested on a [^{13}C / ^{15}N -Tyr; ^{15}N -Arg, Met, Phe; 1- ^{13}C -Ile, Val]-labeled sample of the seven-transmembrane-helix protein proteorhodopsin in diC7PC micelles. The ts-SQ-HN(CA)/DQ-HN(CA) experiment was not attempted since the amino acid sequence of proteorhodopsin does not contain Tyr-Tyr pairs. Under the conditions employed here, the rotational correlation time of the protein is 32 ns as determined with a two-dimensional version of the [^{15}N , ^1H]-TRACT experiment [102]. In a previous study [103] a value of 24 ns was measured at 50°C. The slower tumbling in the current sample can be attributed to the lower temperature (45°C) and higher viscosity due to higher protein and detergent concentrations.

Fig. 7 compares the three subspectra of the ts-HN(CO)/HN(COCA) experiment with individually recorded HSQC, HN(CO), and HN(COCA) spectra. In the time-shared experiment, delays δ and ε were adjusted to 30 ms and 8 ms, respectively, slightly shorter than the average values of $1/(2\ ^1J(^{15}\text{N}, ^{13}\text{C}'))$ and $1/(2\ ^1J(^{13}\text{C}', ^{13}\text{C}^{\alpha}))$, whereas $\delta = 28$ ms and $\varepsilon = 7.6$ ms was employed in the conventional HN(CO) and HN(COCA) experiments. Both HN(COCA) type spectra (c) and (f), which were acquired with the same total number of scans, show the four signals predicted for the chosen labeling scheme of proteorhodopsin. Complete sets of expected correlations are also detected in the HN(CO) (b, e) and HSQC spectra (a, d). However, the HSQC and HN(CO) subspectra of the time-shared experiment, lack HN(CO) and HN(COCA) correlations, respectively, and are therefore less prone to overlap. In the current example, the cross peak for M190 which is hidden under the almost degenerate F137 correlation in the regular HSQC (a) was discovered in the HSQC subspectrum (d) of the time-shared experiment. Importantly, no breakthrough peaks are observed in the HSQC and HN(CO) subspectra despite small mismatches between $^1J(^{15}\text{N}, ^{13}\text{C}')$ or $^1J(^{13}\text{C}', ^{13}\text{C}^{\alpha})$ and the respective transfer delays. However, small

amounts of ^{15}N labeled degradation products present in the sample give rise to spurious signals in the HSQC subspectrum in the time-shared experiments, whereas they are hardly visible in the regular HSQC. The reason for this is that in contrast to the signals from intact protein their intensity is almost unaffected by transverse relaxation during the fixed delays δ and ε in the ts-HN(CO)/HN(COCA) sequence and therefore appear boosted.

A similar comparison between time-shared and conventional versions of HN(CO)/HN(CA) experiments on proteorhodopsin is presented in the supplementary material (Fig. S3). Remarkably, although the value of the delay required to avoid $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ breakthrough peaks in HSQC and HN(CO) subspectra of the time-shared experiment (40 ms) was significantly longer than the one empirically optimized for sensitivity (28 ms), the signal-to-noise ratio was again sufficient to detect all expected signals in this region.

As an example for an integral membrane protein in native-like environment, the performance of time-shared 2D triple resonance experiments was tested on the lipoprotein signal peptidase II (LspA) that has been expressed into nanodiscs. The protein consists of 194 amino acids including a 30-residue N-terminal purification tag. Backbone resonance assignments are currently in progress in our laboratory. Using MSP1D1 H5 and DMPC in a 1:50 ratio for the preparation of nanodiscs [65] a complex was obtained with an apparent correlation time of 52 ns at 318 K as determined by 2D $[^{15}\text{N}, ^1\text{H}]$ -TRACT. For such slowly tumbling species perdeuteration is mandatory even for obtaining analyzable HSQC spectra. Consequently a triple-selectively labeled sample of protein was prepared exclusively using deuterated amino acids, with the exception of the $1\text{-}^{13}\text{C}$ labeled ones as they are commercially unavailable in deuterated form. Both the time-shared HN(CO)/HN(COCA) and SQ-HN(CA)/DQ-HN(CA) pulse sequences were applied to a LspA sample containing $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labeled isoleucine, $^2\text{H}/^{15}\text{N}$ -labeled alanine, arginine, leucine, and threonine, $1\text{-}^{13}\text{C}$ -labeled phenylalanine and tyrosine and all other amino acid types ^2H -labeled.

Results from the ts-SQ-HN(CA)/DQ-HN(CA) experiment recorded with $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha)/^2J(^{15}\text{N}, ^{13}\text{C}^\alpha)$ de- and rephasing periods of 44 ms are shown in Fig. 8 (d–f). The conventional DQ-HN(CA) reference spectrum (panel c) employed an identical period and the same total number of scans/FID (432). Half this number of scans were accumulated for the regular SQ-HN(CA) spectrum (panel b) which corresponds to exactly the amount contributing to the SQ-HN(CA) subspectrum of the time-shared experiment (panel e). For a fair comparison, however, the duration of was reduced to a more customary value of 28 ms, slightly improving sensitivity for most residues observed in the separately recorded SQ-HN(CA). The amino acid sequence of LspA comprises two Ile-Ile pairs, that are readily observed in both versions of the DQ-HN(CA) (Fig. 8 c , f). Also, the HSQC and SQ-HN(CA) subspectra of the time-shared version contain all cross peaks observed in the regular HSQC and HN(CA) spectra, demonstrating that an extended period of 88 ms during which transverse ^{15}N relaxation takes place in the time-shared ts-SQ-HN(CA)/DQ-HN(CA) experiment does not preclude its application to large proteins, provided they are perdeuterated.

A closer inspection of the two HSQC-type spectra (Fig. 8 a , d) reveals that signal overlap is considerably reduced in the time-shared version as compared to the regular HSQC. There are two contributions: first, as already noted for KvAP and proteorhodopsin, signals observed in other subspectra of the time-shared experiment are absent in its HSQC portion and second, line widths in ^{15}N dimension are smaller. To illustrate the line-narrowing effect more clearly, cross sections through the partially overlapping resonances of L54, L78, and L139 in the regular HSQC and the HSQC subspectrum of the time-shared experiment are shown in Fig. 8g and 8h, respectively. Although both spectra were recorded with the same evolution time and processed identically, the resolution is appreciably higher in the time-shared experiment, which can be explained by the constant-time nature of the ^{15}N evolution period. The apparently higher signal-to-noise arises from the much larger number of scans/FID (432 vs. 16 in the regular HSQC) employed. It should be reiterated, however, that the HSQC subspectrum is obtained “for free” in terms of measurement time in the time-shared experiment.

For slowly tumbling proteins, transverse $^{13}\text{C}'$ relaxation can severely degrade sensitivity of some triple-resonance experiments. As opposed to protons and nitrogens, deuteration does not alleviate the effect in the case of carbonyls as their relaxation is dominated by the CSA contribution. Therefore LspA was also employed to assess the feasibility of the ts-HN(CO)/HN(COCA) experiment. Results are shown in the supplementary material (Fig. S4) and can be summarized as follows: (1) all six expected HN(COCA) correlations of LspA are detected with sufficient sensitivity in the corresponding subspectrum of the ts-HN(CO)/HN(COCA) experiment, (2) several cross peaks due to ^{15}N labeled residues preceded by unlabeled amino acids are identified in the HSQC subspectrum that were not apparent in the regular HSQC because of chemical shift degeneracy with HN(CO) type signals, (3) no breakthrough peaks due to J mismatch were observed in either HSQC or HN(CO) subspectra of the time-shared experiment. Remarkably, empirical optimization of the $^{13}\text{C}'$ - $^{13}\text{C}^{\alpha}$ transfer delay ε yielded a value of 6.8 ms, distinctively shorter than $1/(2\ ^1J(^{13}\text{C}', ^{13}\text{C}^{\alpha}))$, as a trade-off between sensitivity and suppression of J -mismatch. Note, however, that when using an HMQC-type transfer [75], $^{13}\text{C}'$ antiphase magnetization with respect to $^{13}\text{C}^{\alpha}$ builds up at a rate faster than the J coupling alone because of an additional contribution from cross-correlated relaxation between $^{13}\text{C}'$ CSA and $^{13}\text{C}'$ - $^{13}\text{C}^{\alpha}$ dipole-dipole coupling as noted previously [95].

From the above it follows that the ts-HN(CO)/HN(COCA) is a viable method even for larger proteins, when applied at moderate static fields such as 600 MHz. Carbonyl CSA auto-relaxation scales with the square of the B_0 -field, adversely affecting the performance of HN(CO)CA-type experiments with increasing field. Conversely, due to their TROSY nature, each of the pulse sequences presented here benefits from the mutual ^1H and ^{15}N CSA/dipole-dipole relaxation interference at higher field strengths. For protons, the narrowest TROSY linewidths are expected at approximately 1.2 GHz [66, 104], while for nitrogens the optimal field strength lies around 800 MHz [105], both exhibiting a relatively wide site-to-site distribution. Slower transverse ^{15}N relaxation mitigates the sensitivity losses due to the longer delays in the time-shared pulse sequences required for clean separation of isotopomers into different subspectra. If available, higher magnetic fields (e.g. 800 or 900

MHz) are therefore recommended for application of ts-HN(CO)/HN(CA) and ts-SQ-HN(CA)/DQ-HN(CA) experiments, whereas the ts-HN(CO)/HN(COCA) should be tested for the system under study to observe whether the increased carbonyl relaxation rates overcompensate instrumental and TROSY-related sensitivity gains.

4. Conclusions

The primary aim of the current study was the reduction of spectrometer time demands associated with the triple-selective combinatorial labeling strategy for backbone resonance assignment. The three time-shared 2D experiments proposed here provide sufficient information to unambiguously distinguish six isotopomeric dipeptide moieties that occur in triple-selectively labeled proteins. Time savings of over 50 % compared to separately recorded HN(CO), HN(CA), and (CO)HN(CA) data were achieved with the ts-HN(CO)/HN(CA) experiment. In applications to membrane proteins, smaller gains were realized with the ts-HN(CO)/HN(COCA) and ts-SQ-HN(CA)/DQ-HN(CA) methods because HN(COCA) and DQ-HN(CA) are significantly less sensitive than the incorporated experiments, i.e. HN(CO) and HN(CA), respectively. For less demanding systems where measurement time is governed by sampling rather than sensitivity issues, the savings can be larger.

The fact that the full set of time-shared experiments provides three HSQC-, two HN(CO)-, and two HN(CA)-type subspectra implies a certain degree of redundancy. A closer look, however, reveals that each of them contains a different subset of cross peaks: While isolated ^{15}N -labeled amino acids are observed in the HSQC subspectra of all three experiments, the HSQC subspectra of the ts-HN(CO)/HN(COCA) and ts-SQ-HN(CA)/DQ-HN(CA) experiments additionally contain signals from isolated $^{13}\text{C}/^{15}\text{N}$ -labeled amino acids and from ^{15}N -labeled amino acids preceded by $1\text{-}^{13}\text{C}$ -labeled ones, respectively, and none of them is identical to a standard HSQC spectrum. Similarly, a single isotopomeric species is detected in each of the HN(CO) and HN(CA) subspectra of the ts-HN(CO)/HN(CA) experiment, whereas extra peaks from different moieties are observed in the corresponding subspectra of the remaining time-shared experiments.

Finally, it has been demonstrated that the separation of the various isotopomers into different subspectra improves spectral resolution and allows identification of amides that are hidden under overlapping signals in conventional experiments. Taken together, compared to the original versions employed in the context of combinatorial triple-selective labeling, the time-shared 2D triple-resonance experiments are not only faster but leave fewer residues unassigned.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at ...

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Highlights

- New experiments improve residue-type distinction in selectively labeled proteins.
- Combined acquisition of 2D HN(CX) triple-resonance spectra saves measurement time.
- Amide signal overlap is reduced owing to $^{13}\text{C}/^{12}\text{C}$ editing into different subspectra.

Fig. 1. BEST- ^{15}N , ^1H -TROSY [72, 81] type pulse sequences for time-shared recording of two-dimensional HN(CO)/HN(CA) (a), HN(CO)/HN(COCA) (b), and SQ-HN(CA)/DQ-HN(CA) (c) spectra. RF pulses with flip angles of 90° and 180° are represented by filled and open symbols, respectively. Pulses on ^{15}N are applied at the highest available power level. The width of ^1H and ^{13}C pulses indicated in the following are valid for 600 MHz ^1H Larmor frequency and should be scaled by $600/X$ for applications at X MHz spectrometers. The first two ^1H 90° pulses have a PC9 [82] shape and a duration of 3 ms, the third and fourth

employ 1.92-ms time-reversed E-BURP-2 and regular E-BURP-2 [83] shapes, respectively. A RE-BURP [83] shape and a width of 2 ms is employed for ^1H 180° pulses. All proton pulses are centered in the amide region (ca. 8.5 ppm). The ^{13}C carrier frequency is placed in the C^α region, its exact position being determined by the $^{13}\text{C}/^{15}\text{N}$ -labeled amino acid type and its $^{13}\text{C}^\alpha$ chemical shift distribution in the respective protein to which the sequences are applied (here 64.5 ppm for isoleucine, 62.5 ppm for tyrosine and 61 ppm for methionine/tryptophan). Carbonyl-selective 90° and 180° pulses of 120 μs duration are applied at 176.5 ppm using phase modulation [84, 85] and have an amplitude envelope corresponding to the center lobe of a $\sin(x)/x$ function. For α -carbons, rectangular pulses are employed with RF fields of $15^{1/2}$ and $3^{1/2}$ for 90° and 180° flip angles, respectively, ν being the difference between $^{13}\text{C}^\alpha$ and $^{13}\text{C}'$ offsets in Hz. All pulsed field gradients are sine-bell shaped and are applied along the z-axis using the following durations and peak amplitudes: G_1 , 0.2 ms, −10 % (percentage of the maximum available gradient strength, i.e. ca. 70 G/cm); G_2 , 0.5 ms, −15 %; G_3 , 0.7 ms, 78.9 %; G_4 , 0.2 ms, 8 %; G_5 , 1.0 ms, 25 %; G_6 , 0.175 ms, 64 %; G_7 , 0.2 ms, −12 %, while RF pulse phases ϕ_5 and ϕ_6 are set to +y and +x, respectively. Coherence selection is achieved by the gradient pair G_3/G_6 . For each t_1 increment N- and P-type transients are collected alternately by inverting the polarity of G_3 along with phases ϕ_5 and ϕ_6 . The two FIDs are stored separately and then added and subtracted to form the real and imaginary parts of a complex data point with a 90° zero-order phase shift being added to one of the components [86]. Pulse phase ϕ_1 and the receiver reference phase are synchronously cycled x, −x. Both phases are inverted in every other increment to shift axial peaks to the edge of the spectrum in the ^{15}N dimension. In all three experiments $\tau = 5.4$ ms, $\tau_G = 0.38$ ms, and $\mu = 0.003$ ms. Pulse sequence specific delays are (a) $\approx 1/(2 \text{ } ^1J(^{15}\text{N}, ^{13}\text{C}^\alpha))$, $\delta \approx 1/(2 \text{ } ^1J(^{15}\text{N}, ^{13}\text{C}'))$ (see text), $\tau_P = 0.3$ ms (the first τ_P period refocuses carbonyl chemical shift evolution during the sequence $90^\circ\text{C}_\alpha - \mu - 90^\circ\text{C}_\alpha$ and also includes the duration $\tau_{90^\circ\text{C}'}$ and $\tau_{180^\circ\text{C}'}/2$), $T_N^a = (\tau + \tau_P)/2$, $T_N^b = (\tau - \tau_P)/2$, $T_N^c = (\tau - \tau_P - \tau)/2 - \tau_G$, $T_N^d = (\tau + \tau_P - \tau)/2 - \tau_G$, (b) $\delta \approx 1/(2 \text{ } ^1J(^{15}\text{N}, ^{13}\text{C}'))$, $\varepsilon \approx 1/(2 \text{ } ^1J(^{13}\text{C}', ^{13}\text{C}^\alpha))$, $T_N^a = (\delta + \varepsilon)/2$, $T_N^b = (\delta - \varepsilon)/2$, $T_N^c = (\delta - \varepsilon - \tau)/2 - \tau_G$, $T_N^d = (\delta + \varepsilon - \tau)/2 - \tau_G$, and (c) $\approx 1/(2 \text{ } ^1J(^{15}\text{N}, ^{13}\text{C}^\alpha))$, $\tau_P = 0.12$ ms (i.e. comprising the sequence $90^\circ\text{C}_\alpha - \mu - 90^\circ\text{C}_\alpha$), $T_N^a = \tau/2 + \tau_P/4$, $T_N^b = \tau/2 - \tau_P/4$, $T_N^c = (\tau - \tau)/2 - \tau_P/4 - \tau_G$, $T_N^d = (\tau - \tau)/2 + \tau_P/4 - \tau_G$. In experiments a and b pulse phases ϕ_2 and ϕ_3 are independently alternated between x and −x and the resulting FIDs stored separately. Pulse phase ϕ_4 must be −x in order to have identical signs of cross peaks in all meaningful combinations. An empirical correction of +30° was applied to ϕ_4 to compensate for Bloch-Siegert-like phase shifts on carbonyl carbons arising from the two preceding 90° pulses on α -carbons. In sequence c, pulse phase ϕ_2 is cycled x, y, −x, −y to obtain four separately stored spectra.

Fig. 2.

Spectral editing achieved with the ts-HN(CO)/HN(CA) (a) and ts-HN(CO)/HN(COCA) (b) experiments. The schematic spectra on the left-hand sides of (a) and (b) result from application of the pulse sequences of Figs. 1a and 1b, respectively, with $\varphi_2 = x$, $\varphi_3 = x$ (A), $\varphi_2 = -x$, $\varphi_3 = x$ (B), $\varphi_2 = x$, $\varphi_3 = -x$ (C), and $\varphi_2 = -x$, $\varphi_3 = -x$ (D) to samples containing either four (a) or three (b) different dipeptides. Positive and negative cross peaks are drawn in black and red, respectively. Panels E-H and I-K are linear combinations, where black arrows denote addition and red arrows indicate subtraction. The isotopomeric peptide fragments selectively observed in each these subspectra are indicated, where ^{13}C nuclei are highlighted in green and unlabeled carbons are drawn in black.

Fig. 3. Separation of different isotopomers in $[^{13}\text{C}/^{15}\text{N}\text{-Met, Trp}; ^{15}\text{N}\text{-Ala, Ile, Lys, Thr, 1-}^{13}\text{C}\text{-Phe, Val}]$ -labeled TMD0 in the four subspectra of the ts-HN(CO)/HN(CA) experiment. A standard BEST- $[^{15}\text{N}, ^1\text{H}]$ -TROSY-HSQC spectrum (a) is shown in the center. It is surrounded by the HSQC (b), HN(CO) (c), HN(CA) (d) and (CO)HN(CA) (e) subspectra of the time-shared experiment with sequence-specific assignments indicated. An unassigned tryptophan side-chain N^{H} signal resonating in the backbone amide region is marked with W^{sc} in (a). HN(CA)-type breakthrough peaks in the HN(CO) subspectrum are labeled with

residue identifiers in parentheses. The two strongest correlations from isolated ^{15}N -labeled amino acids in the HSQC (marked K70† and A73†) are observed in the HN(CA) at ^{13}C natural abundance. Residues 166, A69, and W83 give rise to a second, minor cross peak, possibly due to proline cis-trans isomerism.

Fig. 4. Calculated signal intensities in HSQC (a), HN(CO) (b), HN(CA) (c), and (CO)HN(CA) (d) subspectra of a ts-HN(CO)/HN(CA) experiment as a function of the delay duration Δ in the pulse sequence of Fig. 1. Transfer amplitudes apply to the isotopomers shown in the insets, where ^{13}C nuclei are highlighted in green. Solid, dashed, and dashed-dotted curves are plotted for ^{15}N T_2^* times of 100 ms, 50 ms, and 30 ms, respectively. In (b) and (d) sets of curves have been calculated for $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha) = 10.7$ Hz (overall average observed in proteins, blue lines) and for $^1J(^{15}\text{N}, ^{13}\text{C}^\alpha) = 9.5$ Hz (average of α -helical residues, red lines). Note that the transfer amplitudes in HN(CO) and (CO)HN(CA) subspectra depend on the duration of δ , which is assumed to be equal to $\angle$ for $\angle = 1/(2 \cdot ^1J(^{15}\text{N}, ^{13}\text{C}'))$ and kept constant at $\delta = 1/(2 \cdot ^1J(^{15}\text{N}, ^{13}\text{C}'))$ for larger values of $\angle$. Shaded areas indicate the range of values employed in the time-shared version of this study (light gray) and ^{15}N - ^{13}C transfer periods typically employed in separately recorded HN(CO) (b), HN(CA) (c), and (CO)HN(CA) (d) experiments (dark gray).

Fig. 5. Subspectra of time-shared HN(CO)/HN(COCA) (b–d) and SQ-HN(CA)/DQ-HN(CA) (e–g) experiments applied to [$^{13}\text{C}/^{15}\text{N}$ -Met, Trp; ^{15}N -Ala, Ile, Lys, Thr, 1- ^{13}C -Phe, Val]-labeled TMD0. Panel (a) shows a standard BEST- ^{15}N , ^1H -TROSY-HSQC spectrum which results from the same data set as the spectrum in Fig. 3a, but was processed with the number of t_1 data points as employed in the time-shared experiments. The HSQC subspectra from the two time-shared experiments are shown in (b) and (e) while HN(CO), HN(CO)CA, SQ-HN(CA), and DQ-HN(CA) subspectra are plotted in panels (c), (d), (f), and (g), respectively. Two

minor unassigned signals in (b), marked by asterisks, presumably arise from incorrectly folded protein. In panels (c) and (f) weak signals of residues K70 and A73 (marked with daggers) are detected at ^{13}C natural abundance. Correlations due to *J*-mismatch in the HSQC subspectrum of the ts-SQ-HN(CA)/DQ-HN(CA) (e) are indicated by residue identifiers in parentheses.

Fig. 6.

Removal of overlap in ^1H - ^{15}N correlation spectra achieved by editing of time-shared 2D triple-resonance experiments applied to the KvAP voltage-sensor domain. Left: overlay of separately recorded HSQC, HN(CO), HN(CA), and (CO)HN(CA) (a), HSQC, HN(CO), and HN(COCA) (b), as well as HSQC, SQ-HNCA, and DQ-HNCA (c) spectra. Right: overlay of subspectra from time-shared HN(CO)/HN(CA) (d), HN(CO)/HN(COCA) (e), and SQ-HN(CA)/DQ-HN(CA) (f) experiments. The color coding on individual spectra is indicated

in the upper right corner of each panel. The two regions discussed in the text are highlighted by rectangles. Peaks without annotations are due to a small fraction of unfolded protein.

Fig. 7.

Application of the ts-HN(CO)/HN(COCA) experiment to [$^{13}\text{C}/^{15}\text{N}$ -Tyr; ^{15}N -Arg, Met, Phe; 1- ^{13}C -Ile, Val]-labeled proteorhodopsin. Top: Separately recorded reference HSQC (a), HN(CO) (b), and HN(COCA) (c) spectra. Bottom: HSQC (d), HN(CO) (e), and HN(COCA) (f) subspectra acquired simultaneously in the time-shared manner. Cross peaks originating from ^{15}N -labeled degradation products of the protein in panels (a) and (d) are marked with asterisks. Additional correlations observed for residues F195, Y204, and Y208 are outside the plotted region.

Fig. 8. Comparison of conventional HSQC (a), HN(CA) (b), and DQ-HN(CA) (c) spectra of [u - ^2H ; $^{13}\text{C}/^{15}\text{N}$ -Ile; ^{15}N -Ala, Arg, Leu, Thr; 1- ^{13}C -Phe, Tyr]-labeled LspA with the corresponding HSQC (d), SQ-HN(CA) (e), and DQ-HN(CA) (f) subspectra of the ts-SQ-HN(CA)/DQ-HN(CA) experiment. Where available, tentative sequence-specific assignments are indicated. Residues for which only the amino acid type could be identified, are labeled with the corresponding one-letter code followed by 'xx'. Cross sections along the ^{15}N dimension of spectra (a) and (d), taken at the position marked with dashed vertical lines, are shown in (g) and (h), respectively.