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ARTICLE

Biosynthetically directed ^{2}H labelling for stereospecific resonance assignments of glycine methylene groups

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Abstract Stereospecific resonance assignments of the α-protons of glycine are often difficult to obtain by measurements of scalar coupling constants or nuclear Overhauser effects. Here we show that these stereospecific resonance assignments can readily be obtained by cell-free protein synthesis in D_2O, as the serine hydroxymethyltransferase, that is naturally present in *E. coli* cell extracts, selectively replaces the pro-2*S* proton of glycine by a deuterium. To encourage the conversion by serine hydroxymethyltransferase, we performed the cell-free reaction without the addition of any glycine, exploiting the capability of the enzyme to convert serine to glycine with the help of tetrahydrofolate. ^{13}C-HSQC spectra of ubiquitin produced with ^{13}C/^{15}N-serine showed that about a quarter of the glycine residues derived from serine were stereospecifically deuterated. Pulse sequences are presented that select the signals from the stereospecifically deuterated glycine residues.

Keywords Biosynthetically directed ^{2}H labelling · Cell-free protein synthesis · Glycine-2H · NMR spectroscopy · Stereospecific resonance assignments

Introduction

Stereospecific resonance assignments are an important ingredient of high-resolution structure determinations by NMR spectroscopy. The distinction between prochiral protons or methyl groups can be achieved by short-range structure restraints such as scalar coupling constants and NOEs (e.g. Güntert et al. 1989; Sattler et al. 1992; Grzesiek et al. 1993; Folmer et al. 1997; Melacini and Goodman 1998) or any other structural restraints derived from NMR data, such as residual dipolar couplings (Carlomagno et al. 2000; Tang et al. 2005) or pseudocontact shifts (John et al. 2007). Even differential ^{2}H isotope effects on the ^{13}C-chemical shifts and $^{1}J_{HC}$ coupling constants can be used for the stereospecific assignment of the α-protons of ^{13}C-labelled glycine residues, as elegantly shown by LeMaster et al. (1994). Alternatively, the stereospecific resonance assignments can be established, without having to rely on subtle differences in NMR parameters, by preparing protein samples with stereo-selective isotope labelling (Kainosho et al. 2006; Ohki and Kainosho 2008; Kainosho and Güntert 2009; Gans et al. 2010; Plevin et al. 2011). Stereo-selective isotope labelling involves the chemical or enzymatic synthesis of suitably isotope-labelled amino acids or precursors. A particularly prominent and sophisticated example of stereo-selective isotope labelling is presented by the SAIL scheme (Kainosho et al. 2006; Kainosho and Güntert 2009), which involves the replacement of every CH_2 group by a CHD group of defined chirality. SAIL thus delivers stereospecific assignments automatically. The cost of the requisite amino acids, however, makes this technology unattractive if its sole purpose is to achieve stereospecific resonance assignments.

An alternative method for obtaining stereospecific resonance assignments exploits the stereo-selectivity of the natural enzymes involved in amino-acid biosynthesis. For example, biosynthetic fractional ^{13}C-labelling is frequently used for stereospecific assignments of the methyl groups of valine and leucine, which display different ^{13}C-multiplet fine structures when the protein is prepared from a 1:9

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mixture of uniformly ^{13}C-labelled and unlabelled glucose (Senn et al. 1989; Neri et al. 1989).

In many cases, the complicated network of metabolic conversions in vivo leads to the randomisation of isotope labels in the amino-acid building blocks, obscuring the activities of individual enzymes (e.g. McIntosh and Dahlquist 1990; Shortle 1994). Most of the natural metabolic conversions can be suppressed by the use of cell-free systems for protein production, as many non-essential enzyme activities are impaired in the S30 cell-extract used for cell-free synthesis (Kigawa et al. 1995). Therefore, cell-free systems are used routinely for SAIL labelling (Kainosho et al. 2006). A number of metabolic enzymes, however, remain active in the S30 extract. This can be detrimental to the synthesis of selectively isotope-labelled proteins, calling for ways of inhibiting the undesired enzyme activities (Morita et al. 2004; Ozawa et al. 2004; Etezady-Esfarjani et al. 2007; Jia et al. 2009; Su et al. 2011; Tong et al. 2008; Tonelli et al. 2011; Yokoyama et al. 2011). Conversely, the residual activities of metabolic enzymes can also be regarded as a virtue, as they allow the production of isotope-labelled samples from an incomplete set of amino acids (Yokoyama et al. 2010).

Here we present a method for the stereospecific assignment of the α-protons of glycine (Fig. 1), which relies on the presence of serine hydroxymethyltransferase (SHMT) in *E. coli* cell-free extracts. SHMT is an enzyme that produces glycine from serine by transferring the CH_2O moiety of the side chain of serine to tetrahydrofolate (THF). The enzyme uses pyridoxalphosphate (PLP) as a cofactor. During the reaction, the aldehyde group of PLP forms a Schiff base with the amino group of the amino acid, enabling the breaking of the C^{α}–C^{β} bond of serine and recovery of the second H^{α} proton from, ultimately, water (Schirch and Szebenyi 2005). The reaction is stereo-selective, which has been demonstrated by performing the reaction in D_2O. In fact, SHMT substitutes $H^{\alpha 2}$ (the pro-2*S* proton) of glycine with very high stereo-selectivity by a solvent proton even in the absence of serine (Fitzpatrick and Malthouse, 1996; Malthouse et al. 1991). The selective deuteration of the pro-2*S* position opens a straightforward way of stereospecific resonance assignments of the glycine α-protons.

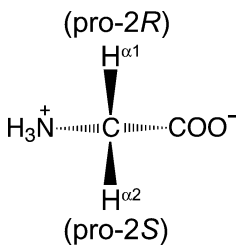

Fig. 1 The nomenclature used in this article follows conventions in the Protein Data Bank to distinguish the α-protons of glycine. Note that in IUPAC nomenclature α1 is renamed to α2 and α2 to α3 (Markley et al. 1998)

In the following we demonstrate that the SHMT activity present in *E. coli* cell-free extracts is sufficient to produce glycine from serine in quantities that are suitable for simultaneous protein synthesis in a cell-free coupled transcription/translation system without seriously compromising the protein yields. By performing the cell-free reaction in D_2O without any added glycine, all glycine produced from serine by the SHMT is stereo-selectively deuterated at the α_2-position. Using ^{13}C-serine in the reaction, the stereospecific assignment can be established by simple ^{13}C-HSQC or CH-group-selecting ^{13}C-HMQC spectra.

Materials and methods

Cell-free protein synthesis

Cell-free reactions were carried out at 30 °C for 14 h as described previously (Apponyi et al. 2008), using S30 extract from the *E. coli* strain BL21 Star (DE3) and a reaction volume of 4 mL inner buffer and 40 mL outer buffer, respectively. To encourage the production of ^{2}H-labelled glycine, the cell-free reaction was performed in D_2O with 0.68 mM folinic acid (corresponding to 10 times the usual amount) and 4 mM ^{15}N/^{13}C-labelled L-serine, while glycine was omitted. The remaining 18 amino acids were unlabelled and present in 1 mM concentration. No attempt was made to exchange the solvent of the S30 extract, the DNA template or other reagents for D_2O, somewhat reducing the final deuterium content of the solvent. The experiments were performed with human ubiquitin fused to a C-terminal Ser–His_6 tag. The protein was expressed from a linear PCR-amplified DNA template (Wu et al. 2007) and purified by using a HisTrap column (1 mL, GE Healthcare Life Sciences). After washing with 5 mL buffer A (50 mM sodium phosphate, pH 7.4, 300 mM sodium chloride) and 30 mM imidazole, the target protein was eluted with 5 mL buffer A containing 300 mM imidazole. The eluted protein was dialysed in spectra/por membrane tubing (MWCO 3500) against 2 × 2 L of 20 mM Tris buffer (pH 7.6) to remove imidazole. For NMR measurements in D_2O, the solvent was exchanged using an Amicon Ultracel concentrator (Millipore, MWCO 3000). The final protein yield was 2.4 mg. A control experiment carried out in H_2O under otherwise identical conditions yielded 4 mg of purified protein.

Preparation of NMR samples and NMR measurements

Samples of ubiquitin were prepared in either 95 % H_2O/5 % D_2O or 100 % D_2O without the addition of any buffer. The sample volumes were 0.53 mL each. NMR experiments were recorded at 25 °C on a Bruker 600 MHz NMR

spectrometer equipped with a cryoprobe. ^{13}C-HSQC spectra were recorded with decoupling during the evolution time, applying Waltz decoupling on ^{2}H and a 46 μs 180°(^{13}C) pulse on the carbonyl carbons, and refocusing the couplings with ^{1}H and ^{15}N by hard 180° pulses. A 2D H(N)CA spectrum was recorded using the pulse scheme of a standard 3D HNCA experiment, supplemented by Waltz decoupling on ^{2}H and a 46 μs 180°(^{13}C) pulse on the carbonyl carbons to achieve complete decoupling of the α-carbon resonances of the glycine residues during the evolution time. The CH-selected ^{13}C-HMQC spectrum was recorded as described in Fig. 3 and the design of the CH-selected HN(CA) experiment is detailed in Fig. 5.

Results

Stereospecific deuteration of glycine

To assess the production of stereo-selectively deuterated glycine during cell-free protein synthesis, we used human ubiquitin with a C-terminal Ser-His$_6$ tag for facilitated protein purification. ^{13}C/^{15}N-labelled serine was the only isotope-labelled amino acid supplied to the reaction mixture. Glycine was omitted and all other 18 amino-acids were provided in unlabelled form. The solvent of the cell-free reaction was D_2O.

To obtain singlets in the F_1 dimension, the ^{13}C-HSQC spectrum of the purified protein was recorded with decoupling of the couplings with ^{1}H, ^{15}N, ^{2}H and ^{13}C-carbonyl groups. Figure 2b shows the spectral region of the ^{13}C-^{1}H cross-peaks of the glycine residues. The cross-peaks of all non-serine and non-glycine amino acids were not perceptibly taller than expected for the background of natural isotopic abundance, indicating that the ^{13}C-enrichment provided with the ^{13}C/^{15}N-labelled serine had remained confined to serine and glycine. Figure 2b displays three cross-peaks for each glycine residue with non-degenerate H^{α} resonances, two weak cross-peaks arising from a $^{13}CH_2$ group and one intense cross-peak from the ^{13}CHD group with *S*-chirality ($H^{\alpha 1}$). Importantly, there was no evidence for the corresponding ^{13}CHD group with *R*-chirality ($H^{\alpha 2}$). At the signal-to-noise ratio of the experiment, the ^{13}CHD group with $H^{\alpha 2}$ gave signals at least 30-fold weaker than the ^{13}CHD group containing $H^{\alpha 1}$. This shows that the deuteration of glycine proceeds with very high stereo-selectivity, as expected for a reaction catalysed by SHMT (Malthouse et al. 1991). The occurrence of some $^{13}CH_2$ groups was expected because the cell-free reaction was carried out in a solvent that was only incompletely deuterated.

Analysis of the isotope distribution

To assess the existence of CD_2 groups that would not be observable in the ^{13}C-HSQC spectrum, we recorded a 2D H(N)CA experiment, in which the magnetization was transferred from the amide proton to the α-carbon via ^{15}N and back. The spectrum was recorded with decoupling of the ^{1}H, ^{15}N, ^{2}H and ^{13}C(carbonyl) spins in the indirect dimension, in complete analogy to the ^{13}C-HSQC spectrum described above. This allowed the separation of the cross-peaks from the CH_2, CHD and CD_2 groups, which are at different chemical shifts due to the ^{2}H isotope effect on the $^{13}C^{\alpha}$ chemical shift (Fig. 2a). This showed that about 65 % of the C^{α}-H^{N} correlations of glycine were from CHD groups, 25 % from CH_2 groups and 10 % from CD_2 groups. The H(N)CA spectrum also revealed that about 15 % of the serine residues had gathered a deuterium in the α-position (Figure S1). This is the same ratio as the abundances of CD_2 versus CHD groups in glycine, raising the possibility that many if not most of the deuteriums in the pro-2*R* position originated from the parent serine. PLP enzymes other than SHMT may be responsible for the H–D exchange in the α-position of serine.

The high abundance of $^{13}CH_2$ groups was unexpected, considering that the deuterium content of the solvent of the cell-free reaction was much greater than 75 %. The ^{13}C-label of the CH_2 groups unambiguously ties their origin to serine as the parent molecule. It is possible that an H/D isotope effect in the SHMT enzyme strongly favours the insertion of a proton over a deuterium in the pro-2*S* position. It is also possible that H/D isotope effects act in other enzymes that can exchange a pro-2*S* deuterium in glycine. The importance of isotope effects is highlighted by the fact that performing the cell-free reaction in H_2O rather than D_2O solution enhanced the protein yield by about 50 %.

To test whether transaminases in the S30 extract act on glycine, we recorded a ^{13}C-HSQC spectrum without decoupling of ^{15}N in the indirect dimension (but with decoupling of ^{1}H, ^{2}H and the carbonyl carbons). The cross-peaks of the $^{13}CH_2$ groups displayed the expected splitting by the ^{1}J ($^{13}C^{\alpha}$,^{15}N) coupling (Figure S2), indicating that the glycine produced was not appreciably affected by transaminases in the S30 extract.

^{13}C-HMQC experiment selecting for CH groups

As the emergence of $^{13}CH_2$ groups proved difficult to avoid during protein production, we designed an NMR experiment to suppress their cross-peaks by a simple filter. The pulse sequence is shown in Fig. 3. It is derived from a ^{13}C-HMQC experiment with pulsed field gradients (PFG) for coherence selection. The filter consists of a delay applied to the heteronuclear multiple-quantum coherence generated by the first 90°(^{13}C) pulse, during which $^{1}J_{HC}$ couplings are allowed to evolve. The filter delay $[1/(2^{1}J_{HC})]$ is designed to prevent the evolution of the

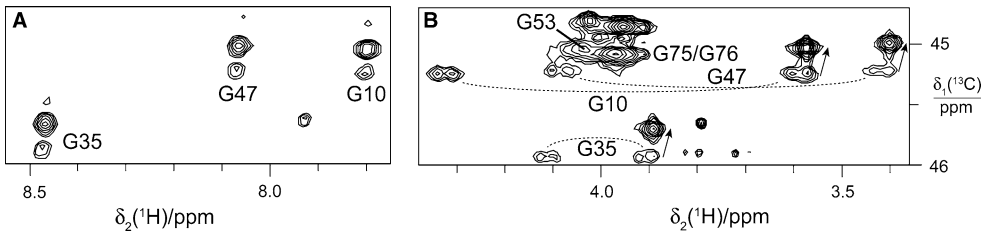

Fig. 2 ^{13}C-correlation spectra of a 0.25 mM solution of biosynthetically directed ^{2}H-glycine labelled ubiquitin. Both spectra were recorded with decoupling of ^{1}H, ^{15}N, ^{13}C-carbonyl and ^{2}H in the indirect dimension. **a** Selected spectral region from the 2D H(N)CA spectrum recorded in 95 % H_2O/5 % D_2O, pH 6.5. Isotope effects split the correlations of each glycine residue into three resolved cross-peaks that correspond to glycine with a CH_2, CHD and CD_2 group, respectively. The cross-peaks arising from the CD_2 group are very weak. **b** Selected spectral region from the ^{13}C-HSQC spectrum recorded in D_2O, pH 6.9. Pairs of cross-peaks from glycine CH_2 groups are connected by dotted lines. Arrows identify the related cross-peaks from $H^{\alpha 1}$ protons in glycine CHD groups. The absence of corresponding cross-peaks from $H^{\alpha 2}$ protons in glycine CHD groups demonstrates the high selectivity of deuteration. The cross-peaks from $^{13}CH_2$ groups are assigned. Due to the *C*-terminal Ser-His_6-tag in our ubiquitin construct, the cross-peaks of Gly75 and Gly76 overlap and are not assigned individually

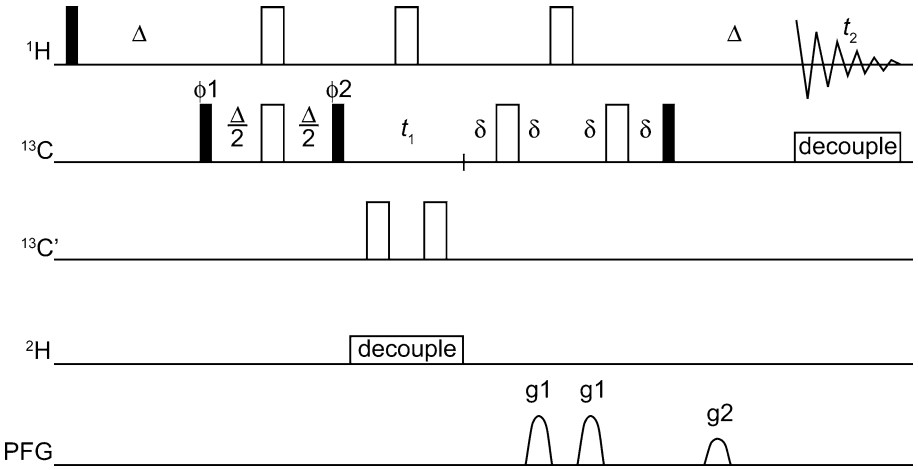

Fig. 3 Pulse sequence of a ^{13}C-HMQC experiment with selection of CH groups. *Narrow filled* and *wide open bars* indicate 90° and 180° pulses, respectively. The pulses are applied along the *x*-axis unless indicated otherwise. Parameters: $\Delta = 1/(2^{1}J_{HC})$ (3.4 ms), $\delta = 0.7$ ms, duration of the 180°(^{13}C) pulses = 46 µs, duration and amplitude of the PFGs: g1 = 0.5 ms at −20 G/cm, g2 = 0.5 ms at 10.05 G/cm. For quadrature detection in the F_1 dimension, the sign of the gradients g1 and the receiver phase are inverted in every second FID in an echo-antiecho scheme (Kontaxis et al. 1994). Phase cycle: $\phi_1 = \phi$(receiver) = 2[x,-x]; ϕ_2 = y,y,-y,-y. The ^{13}C-carrier frequency was set at 50 ppm. Broadband decoupling during the acquisition time t_2 was achieved by a train of adiabatic inversion pulses (Freeman and Hurd, 1997). ^{2}H decoupling during the evolution time t_1 was achieved by the Waltz-16 sequence. The 180°(^{13}C') pulses are applied at $t_1/4$ and at $3t_1/4$. In addition, 180°(^{15}N) pulses applied together with the 180°(^{13}C') pulses refocus couplings with ^{15}N (pulses not shown)

coherence of CHD groups while the coherence from $^{13}CH_2$ groups changes in phase by 90°, so that the subsequent 90°$_y$(^{13}C) pulse eliminates the transverse ^{13}C part of the coherence and the resulting magnetisation is no longer selected by the PFGs.

We recorded the experiment for the ubiquitin sample of Fig. 2. As expected, the only observable cross-peaks were those of the $C^{\alpha}H^{\alpha}$ groups of serine and of the ^{13}CHD groups of glycine (Fig. 4). Comparison with the ^{13}C-HSQC spectrum of Fig. 2b demonstrates the complete absence of the intense cross-peaks from the CH_2 groups of Gly75 and Gly76. Also the cross-peaks from the CH_2 groups of serine were absent (Figure S3).

HN(CA) experiment selecting for CH groups

Owing to the absence of a side chain, glycine residues readily assume positive values for the backbone dihedral angle ϕ. It has been shown that positive ϕ angles are associated with negative dipolar cross-correlation effects that, in the absence of ^{15}N-decoupling, lead to narrower (i.e. taller) outer lines for the H^N multiplet of four lines arising from the scalar $^{1}J_{HN,N}$ and $^{3}J_{HN,H\alpha}$ couplings (Crowley et al. 2000). This suggests a straightforward way for identifying glycine residues with positive ϕ angles as, following stereospecific deuteration, the amide proton of glycine couples to only a single α-proton, resulting in a

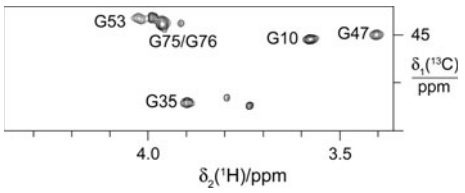

Fig. 4 ^{13}C-HMQC spectrum of biosynthetically directed ^{2}H-glycine labelled ubiquitin with selection of CH groups. The spectrum was recorded using the pulse sequence of Fig. 3. The spectral region shown displays the cross-peaks of glycine residues. The cross-peaks of CHD groups are labelled with their assignments. Some of the new peaks not visible in the spectrum of Fig. 2B indicate sample degradation, arising from the delay between both experiments, which involved long measurements and multiple changes of solvent and pH of the sample. The spectrum shown was recorded at pH 6.9. The strong cross-peaks of the CH_2-groups of Gly75 and Gly76 present in the ^{13}C-HSQC spectrum of Fig. 2B are clearly absent from the CH-selected ^{13}C-HQMC spectrum

correspondingly simplified multiplet fine-structure. The crystal structure of ubiquitin reports positive ϕ angles for Gly10, Gly35 and Gly47 (PDB ID 1UBQ; Vijay-Kumar et al. 1987). To observe the multiplet fine-structure of the glycine residues with CHD groups, we performed a 2D HN(CA) experiment with a simple filter to suppress the corresponding signals from CH_2 groups.

The pulse sequence is shown in Fig. 5. The filter relies on the evolution of antiphase magnetisation $2C_yN_z$ during the delay δ. In the case of a CHD group, this leads to the term $4C_xN_zH_z$, which is insensitive to the following $90°_x(^{13}C)$ pulse and will refocus during the following delay δ. In contrast, a CH_2 group will generate the term $8C_yN_zH_{1z}H_{2z}$, which is affected by the $90°_x(^{13}C)$ pulse and thus readily eliminated by phase cycling.

Figure 6 shows cross-sections taken along the ^{1}H dimension through two of the glycine cross-peaks of a 2D HN(CA) spectrum with CH-group selection. The $^{3}J_{HN,H\alpha}$ couplings are barely resolved, but the outer components appear to be taller as expected for positive ϕ angles. The $^{3}J_{HN,H\alpha}$ coupling of Gly47 was too small to give rise to any significant multiplet splitting.

In vivo experiments

Depending on the protein, the expression yields can be higher in vivo than in cell-free reactions. We therefore attempted to produce ubiquitin by in vivo expression in the presence of ^{15}N/^{13}C-serine in media prepared with D_2O. The ^{13}C-HSQC cross-peaks of the serine residues observed for the resulting protein samples turned out to be only little more intense than the cross-peaks of all the other amino acid residues, showing that most of the ^{13}C-label had been metabolized (data not shown). The expected cross-peaks of the ^{13}CHD groups of glycine residues could be detected but they were of similar intensities as the cross-peaks of all the other amino acids. This result was independent of whether the protein was produced using standard M9 medium supplemented by isotope-labelled serine or whether a minimal medium was used that contained isotope-labelled serine together with all other 18 non-glycine amino acids at natural isotopic abundance (and no ammonium chloride). In contrast, the samples prepared by cell-free synthesis showed much greater peak intensities for the isotope-labelled serine residues. Therefore, cell-free synthesis is much better at achieving the stereospecific resonance

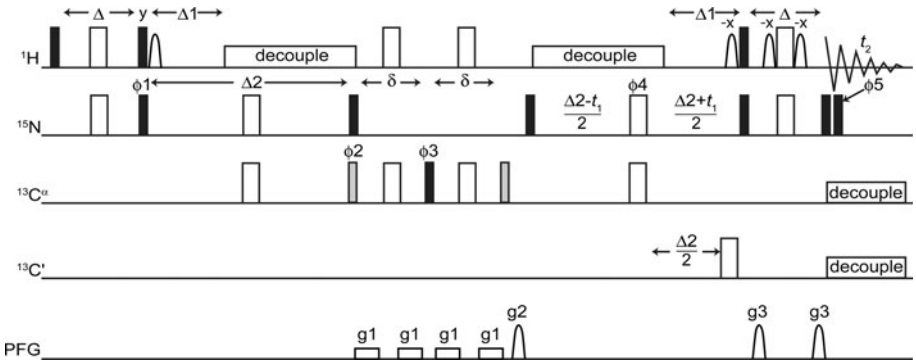

Fig. 5 Pulse sequence of a 2D HN(CA) experiment with selection of CH groups. *Narrow filled* and *wide open bars* indicate 90° and 180° pulses, respectively. $^{13}C^{\alpha}$ pulses were applied at 50 ppm and ^{13}C' pulses at 176 ppm. The *narrow black bar* at the $^{13}C^{\alpha}$ frequency is a rectangular 52 μs 90° pulse, while the *narrow shaded bars* represent 320 μs Q5 pulses (Emsley and Bodenhausen 1992). The $180°(^{13}C)$ degree pulses were 256 μs Q3 pulses, except during the CH-filter delay, when they were applied as 46 μs rectangular pulses. Water-selective shaped 90° pulses (centre lobe of the sinc function) were of 1.5 ms duration. All pulses are applied along the *x*-axis unless indicated otherwise. Parameters: $\Delta = 4.6$, $\Delta1 = 5.5$, $\Delta2 = 24$, $\delta = 1.72$ ms, duration and amplitude of the PFGs: g1 = 0.85 ms at 1 G/cm, g2 = 0.5 ms at 25 G/cm, g3 = 0.5 ms at 30 G/cm. Phase cycle: $\phi_1 = 4[x]4[-x]$; $\phi_2 = x,-x$; $\phi_3 = x, x,-x,-x$; $\phi_4 = 16[x]16[-x]$; $\phi_5 = 8[x]8[-x]$; ϕ(receiver) = x,−x,x,−x,−x,x,−x,x. Broadband ^{13}C-decoupling during the acquisition time t_2 was achieved by a train of adiabatic inversion pulses (Freeman and Hurd 1997). The broadband ^{1}H decoupling blocks (Waltz decoupling) were preceded by a $90°_y(^{1}H)$ and followed by $90°_{-y}(^{1}H)$ pulses to flip the water magnetisation back to the *z*-axis (pulses not shown)

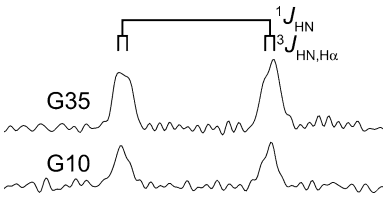

Fig. 6 Cross-sections through the cross-peaks of Gly10 and Gly35 in the 2D HN(CA) spectrum with selection of CH groups. The splittings due to the $^1J_{HN}$ and $^3J_{HN,H\alpha}$ coupling constants are identified. The spectrum was recorded at 25 °C, pH 6.5, on a 600 MHz NMR spectrometer

assignments of the α-protons of glycine residues by biosynthetically directed deuteration.

Optimized cell-free reaction conditions

The combined information from the ^{13}C-HSQC and H(N)CA cross-peaks of the serine and glycine residues indicated that only about a third of the glycine residues was labelled with ^{13}C, implying that most of the glycine residues were at natural isotopic abundance. A possible source of unlabelled glycine could be the amino acids used in the cell-free reaction, as even a small impurity of glycine in one of the amino acids used in the cell-free reaction would in principle be sufficient to produce all protein without any ^{13}C-labelled glycine.

In principle, conversion of threonine to glycine by threonine aldolase is another possible source of unlabelled glycine. To test for the activity of this pathway, we performed a cell-free synthesis of ubiquitin with 4 mM ^{15}N/^{13}C-serine and 4 mM ^{15}N/^{13}C-threonine as the isotope-labelled amino acids. No significant increase in the ^{13}C-glycine peaks could be detected.

The attempt to increase the conversion rate from serine to glycine by using 10-fold more folinic acid than usual (0.68 mM instead of 0.068 mM) did not lead to a significant change in the yield of ^{13}C-glycine. Nonetheless, the high folinic acid concentration reproducibly reduced the amount of precipitate accumulating during the cell-free reaction. Combining tenfold more folinic acid with 4 times more ^{15}N/^{13}C-labelled serine (4 mM instead of 1 mM), however, lead to a 50 % increase of the ^{13}C-glycine peaks relative to the ^{13}C-serine peaks. This suggests that the SHMT enzyme in the cell-free reaction is not saturated when serine is present in 1 mM concentration. We therefore recommend using the higher concentrations of folinic acid and serine.

Discussion

Glycine is the only amino acid without a side chain, leaving the backbone dihedral angles ϕ and ψ as the main conformational variables. In a folded protein, the backbone chain is usually quite rigid, placing the α-protons of glycine residues almost always in different chemical environments. As a result, their resonances usually appear at different chemical shifts. The absence of a side chain, however, makes it harder to determine the backbone dihedral angles, as the amide proton displays a more complicated multiplet fine structure and the H^{α}-resonances need to be assigned stereospecifically to extract their full structural information. The situation is compounded by the fact that glycine residues are just as likely to populate conformations with positive ϕ angles as conformations with negative ϕ angles. Without stereospecific resonance assignments, it becomes difficult to obtain precise NOE restraints for the glycine residues, resulting in correspondingly large uncertainties for these residues in protein structure determinations by NMR spectroscopy.

The strategy of biosynthetically directed ^{2}H labelling introduced here offers a straightforward way of stereospecific resonance assignments. It is inexpensive: the production of 1 mg ubiquitin in D_2O required less than $20 worth of ^{15}N/^{13}C-serine, comparable to the cost of the D_2O solvent. Although some of the isotope-labelled serine provided in the reaction ended up in Gly $^{13}CH_2$ groups rather than in Gly ^{13}CHD groups, and a substantial fraction of the glycine residues showed no ^{13}C-label at all, the samples obtained are still highly suitable for stereospecific resonance assignments. Notably, the resonance assignment can be established with ^{13}C-HSQC or ^{13}C-HMQC experiments that are less prone to magnetisation losses by relaxation than the experiments employed for stereospecific distinction of the methyl groups of valine and leucine in the biosynthetically directed fractional ^{13}C-labelling scheme (Neri et al. 1989; Hilty et al. 2003; Plevin et al. 2011). Furthermore, even if only a quarter of the glycine contains the desired ^{13}CHD group, this still presents a higher yield than what is usually obtained in the experiments performed for stereospecific assignments of valine and leucine methyl groups, which aim for no more than 10 % ^{13}C-enrichment unless specifically ^{13}C-labelled precursors are added to the medium (Gans et al. 2010; Plevin et al. 2011). Finally, the simplified multiplet fine structure and reduced ^{13}C relaxation rate in a CHD versus a CH_2 group further enhance the peak heights, as demonstrated by the spectrum of Fig. 2b. The CH-group-selecting ^{13}C-HMQC experiment of Fig. 4 makes full use of these features. Importantly, our present work established that ^{13}CHD groups of the opposite chirality, that could confuse the stereospecific assignments, are practically absent.

With the commercial availability of SAIL amino acids (SAIL Technologies, Yokohama, Japan), an obviously simpler way of stereospecific assignments of glycine would be the use of stereospecifically deuterated glycine. Using

ubiquitin Kainosho and co-workers demonstrated, however, that a cell extract prepared from BL21 (DE3) can lead to partial loss of the pro-2*S* deuterium in glycine, while a wheat germ cell-free extract is even more prone to scrambling of the ^{2}H label, even in the presence of inhibitors of PLP enzymes (Tonelli et al. 2011). Use of stereospecifically deuterated glycine in the cell-free reaction thus does not necessarily simplify the NMR spectrum over that of Fig. 2b, although the protein yield may be higher. When we produced ubiquitin with selectively (pro-2*S*) deuterated, ^{13}C/^{15}N-labelled glycine using our cell-free extract, we found no evidence for isotope scrambling of the stereospecifically deuterated glycine, as measured by the absence of any cross-peaks from $^{13}CH_2$ groups at 5 % of the peak height of the desired ^{13}CHD cross-peaks (data not shown). The origin of the isotope scrambling observed by Tonelli et al. (2011) is thus unclear. Notably, no evidence of isotope scrambling was observed in earlier work using synthetic, stereoselectively ^{2}H-labelled glycine in different in vivo *E. coli* protein expression systems (Kushlan and LeMaster 1993; Curley et al. 1994).

In conclusion, the biosynthetic fractional ^{2}H labelling scheme presented here offers a practical and economical way of establishing the stereospecific resonance assignments of glycine $C^{\alpha}H_2$ groups for improved protein structure analysis. Combined with suitable CH-group-selecting NMR experiments, the biosynthetic fractional ^{2}H-labelling scheme can yield most valuable structure restraints.

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