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Stereospecific assignments of glycine in proteins by stereospecific deuteration and ^{15}N labeling

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SUMMARY

A method is described for stereospecifically assigning the α-protons of glycine residues in proteins. The approach involves the stereospecific deuteration and ^{15}N labeling of glycine and subsequent selective incorporation of this residue into the protein. The stereospecific assignments of the glycine α-protons are obtained from a comparison of a 3D ^{15}N-resolved TOCSY spectrum of the uniformly ^{15}N-labeled protein with a 2D/3D ^{15}N-edited TOCSY spectrum of the protein, containing the stereospecifically deuterated and ^{15}N-labeled glycine. The approach is demonstrated by stereospecifically assigning the glycine α-protons of the FK506 binding protein when bound to the immunosuppressant ascomycin.

INTRODUCTION

Stereospecific assignments are important for accurately determining the 3D structures of proteins (Driscoll et al., 1989; Güntert et al., 1989). In some cases, these assignments can be determined from an analysis of homonuclear and heteronuclear vicinal coupling constants or from NOE data (Hyberts et al., 1987; Wagner et al., 1987; Arseniev et al., 1988; Nilges et al., 1990; Xu et al., 1992). Alternatively, isotope labeling has been employed to unambiguously obtain stereospecific assignments (Fischman et al., 1980; LeMaster, 1987; LeMaster and Richards, 1988; Neri et al., 1989). For example, the β-protons of the aspartic acid and asparagine residues in thioredoxin have been stereospecifically assigned from an analysis of a homonuclear COSY spectrum of the protein in which stereospecifically deuterated aspartic acid was incorporated (LeMaster, 1987).

It is especially important to stereospecifically assign the glycine α-protons in proteins, because of the potential for different backbone conformations of this residue. These stereospecific assign-

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ments are critical for interpreting the $^{3}J_{H^{N},H^{\alpha}}$ coupling constants and NOEs involving the glycine α-protons which define the conformation of this part of the protein. However, it is often difficult to unambiguously obtain the stereospecific assignments for the glycine residues.

In this report we describe the synthesis of stereospecifically deuterated and ^{15}N-labeled glycine, using a slightly modified procedure, originally described by Woodard and co-workers for the stereoselective deuteration of glycine (Ramalingam et al., 1988). The isotopically labeled glycine was incorporated into the FK506 binding protein (FKBP) (Harding et al., 1989; Siekierka et al., 1989) and used to unambiguously determine the stereospecific assignments of the glycine residues of FKBP when complexed to the immunosuppressant ascomycin (Arai et al., 1962; Hatanaka et al., 1988).

MATERIALS AND METHODS

Preparation of (R)-[2-^{2}H,^{15}N]-Gly

The synthesis of (R)-[2-^{2}H,^{15}N]-Gly followed closely the procedure for the preparation of (*R*)-[2-^{2}H]-Gly of Woodard and co-workers (Ramalingam et al., 1988), with necessary modifications. As illustrated in Fig. 1, the deuterated morpholinonitrile **2** was prepared in good yield (~70%) with 98% deuterium incorporation from 4-methoxybenzaldehyde (**1**), by using more forcing conditions (2 equiv. NaH, refluxed in THF for 4 h before $^{2}H_2O$ quench) than those previously described. Acid-catalyzed hydrolysis of **2** regenerated ^{2}H-*p*-anisaldehyde **3** in 88% yield after chromatography (10% ethylacetate/hexane on silica gel). Reduction of **3** with (*R*)-Alpine borane produced (*S*)-[2-^{2}H]-*p*-anisyl alcohol (**4**) in high yield (95%) after step-gradient silica gel chromatography (10%, then 50% ethylacetate/hexane). A diethylazodicarboxylate-mediated Mitsunobu reaction (Mitsunobu et al., 1972) of [^{15}N]-phthalimide (Cambridge Isotopes) with **4** gives doubly labeled (*R*)-phthalimide **5** in modest (49%) yield. Exchange of the phthalimide

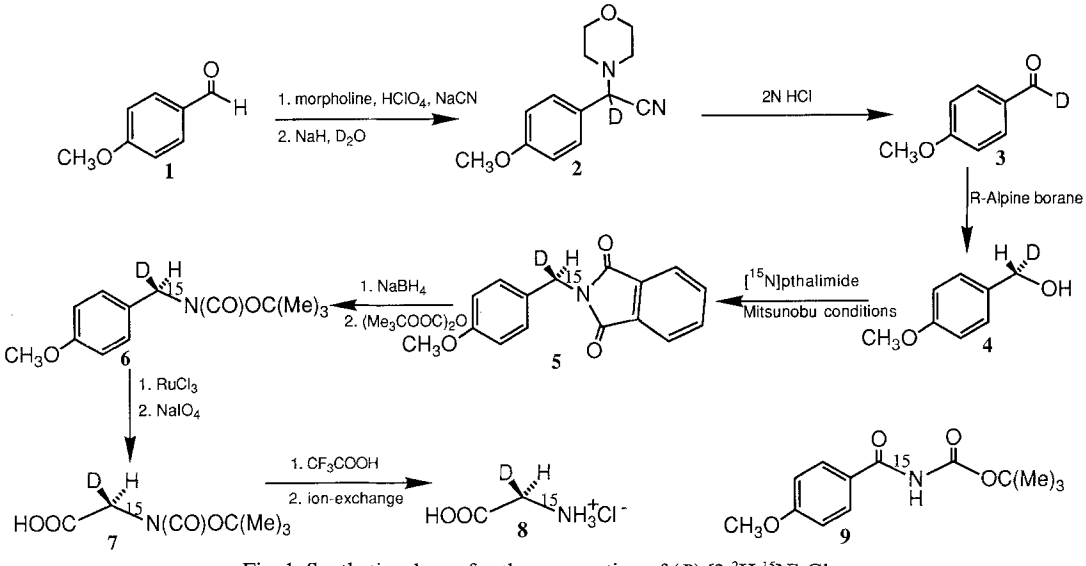

Fig. 1. Synthetic scheme for the preparation of (*R*)-[2-^{2}H,^{15}N]-Gly.

protecting group to a *t*-butyloxycarbonyl group went smoothly (86% yield) to give **6** according to the published procedure. However, oxidative degradation of the aromatic ring to the carboxylic acid **7** according to the published method (Ramalingam et al., 1988) gave a disappointing yield in our hands, providing predominantly the undesired *p*-anisamide **9**. Improved yields (45%) of **7** were obtained by strictly adhering to the reagent stoichiometry (20 equiv. $NaIO_4$, 2.2 mol % $RuCl_3$) and solvent ratios ($CCl_4/CH_3CN/H_2O$ 4:4:6 ml/mmol) as described by Sharpless (Carlsen et al., 1981). Trifluoracetic acid hydrolysis of the *t*-Boc protecting group and isolation by ion exchange (Dowex 50W-X8, H^+-form) gave the desired (*R*)-[2-^{2}H,^{15}N]-Gly (**8**) in 92% yield. The final product (**8**) was estimated to be ca. 80% optically pure by ^{1}H NMR analysis of the amide made between (*R*)-**8** and 1-(*S*)-(−)-camphanic acid chloride. This purity is virtually identical to that estimated by this method by Woodard and co-workers for (*R*)-[2-^{2}H]-Gly (Ramalingam et al., 1988).

Based on the suggestion that the ruthenium tetraoxide oxidation proceeds more efficiently on 3,5-dimethoxyarenes (Kobayashi et al., 1983), the procedure was repeated using 3,5-dimethoxybenzaldehyde as starting material. Unfortunately, while the use of this costly starting material resulted in considerably better yields in the Mitsunobu reaction (~70% in our hands), the overall yield of the final product was about the same, due to the lower yield obtained in the oxidation step.

Isotopically labeled FKBP

Recombinant human FKBP was cloned from a Jurkat T cell cDNA library and expressed at high levels in *E. coli*, using translational coupling to the 5′ end of the *E. coli* kds B gene (Pilot-Matias et al., 1993). [U-^{15}N]FKBP was prepared from these cells grown on a minimal medium, containing [^{15}N]NH_4Cl as the nitrogen source. FKBP, selectively labeled with (*R*)-[2-^{2}H,^{15}N]-Gly, was obtained by growing cells that overexpress FKBP on a medium containing unlabeled amino acids (Muchmore et al., 1989) and 250 mg/l of (*R*)-[2-^{2}H,^{15}N]-Gly. Isotopically labeled FKBP was purified using ion exchange and size-exclusion chromatography as previously described (Edalji et al., 1992).

NMR samples were prepared by exchanging the protein into an H_2O solution (pH = 6.5), containing potassium phosphate (50 mM), sodium chloride (100 mM), and dithiothreitol-d_{10} (5 mM), and concentrating the solution to ~500 µl using a centricon-10 microconcentrator. The final FKBP concentration in both samples was ~3 mM. The FKBP–ascomycin complex was prepared by incubating excess unlabeled ascomycin with the concentrated protein solution for 24–48 h at room temperature. Excess ascomycin was removed by centrifugation.

NMR spectroscopy

All NMR spectra were collected at 30 °C on Bruker AMX 500 or AMX 600 NMR spectrometers. NMR spectra were processed and analyzed using in-house written software on Silicon Graphics computers.

^{15}N-resolved 3D TOCSY-HSQC spectra (Marion et al., 1989) of [U-^{15}N]FKBP were acquired at 600 MHz as previously described (Xu et al., 1993) using a mixing time consisting of a 25-ms MLEV-17 spin-lock period, modified for the suppression of rotating-frame NOE contributions (Griesinger et al., 1988). The data set contained 96 (t_1) × 42 (t_2) × 1024 (t_3) complex points with spectral widths of 10 000 (ω_1,^{1}H), 2128 (ω_2,^{15}N), and 10 000 Hz (ω_3,^{1}H). Water suppression was

accomplished with a 2.5-ms spin-lock pulse using the method of Wüthrich and co-workers (Messerle et al., 1989).

The ^{15}N-edited 2D TOCSY spectrum of (*R*)-[2-^{2}H,^{15}N-Gly]FKBP was acquired at 30 °C, using a mixing time of 25 ms. A total of 32 scans were acquired for each of the 256 complex t_1 increments.

RESULTS AND DISCUSSION

Several enzymatic and chemical methods have been reported for preparing stereospecifically deuterated glycine (Armarego et al., 1976; Belokon et al., 1980; Kakinuma et al., 1982; Ohrui et al., 1985; Santaniello et al., 1985; Sinclair et al., 1986; Ramalingam et al., 1988). We chose to employ the method of Woodard and co-workers (Ramalingam et al., 1988) because of the relatively low cost of the starting materials and the applicability of the synthetic procedure for incorporating the ^{15}N label. However, it was important to slightly modify the published procedure in order to obtain a high level of deuterium incorporation, which was critical for the success of this approach. Using the procedure described in the Materials and Methods section, more than

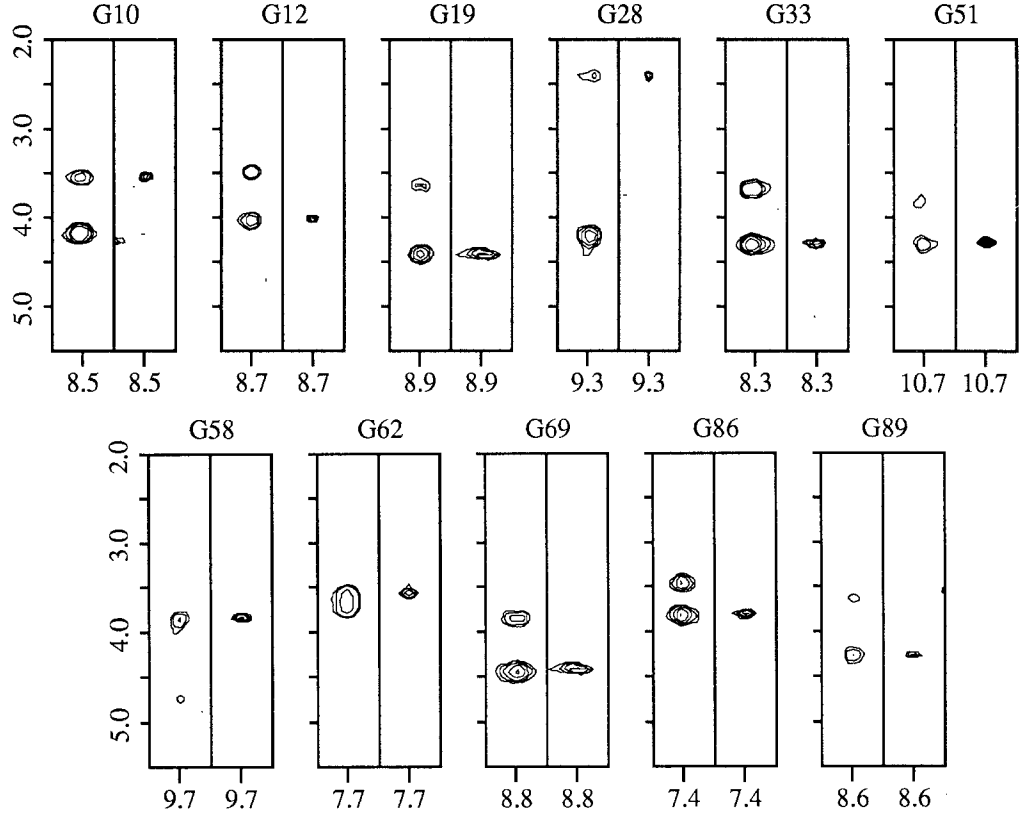

Fig. 2. Left panels: ^{1}H,^{1}H planes from a 3D ^{15}N-resolved TOCSY spectrum of [U-^{15}N]FKBP–ascomycin extracted at the ^{15}N chemical shifts of the glycine residues given at the top of the spectra. Right panels: selected regions from a 2D ^{15}N-edited TOCSY spectrum of [(*R*)-2-^{2}H,^{15}N-Gly]FKBP–ascomycin.

TABLE 1
STEREOSPECIFIC ASSIGNMENTS OF THE α-PROTONS OF THE GLYCINE RESIDUES[a] OF FKBP IN THE FKBP–ASCOMYCIN COMPLEX

Residue	δ pro-*R* (ppm)	δ pro-*S* (ppm)	Residue	δ pro-*R* (ppm)	δ pro-*S* (ppm)
Gly^{10}	4.17	3.55	Gly^{58}	4.02	3.87
Gly^{12}	3.50	4.05	Gly^{62}	3.75	3.63
Gly^{19}	3.67	4.42	Gly^{69}	3.87	4.43
Gly^{28}	4.20	2.42	Gly^{86}	3.47	3.84
Gly^{33}	3.67	4.30	Gly^{89}	3.64	4.28
Gly^{51}	3.85	4.31			

[a] The α-protons of Gly^{1} and Gly^{83} were not stereospecifically assigned.

1.5 g of (*R*)-[2-^{2}H,^{15}N]-Gly was prepared with >98% deuterium incorporation at a total cost of ~US$ 600/g. Using the same synthetic scheme, (*S*)-[2-^{2}H]-Gly was also prepared by substituting (*S*)-Alpine borane in the aldehyde reduction step. However, as noted previously (Ramalingam et al., 1988) the (*S*)-enantiomer was found to be less optically pure than the (*R*)-enantiomer, due to the lower optical purity of the (*S*)-Alpine borane. Thus, the (*R*)-enantiomer was selected for incoporation into FKBP. As judged from an ^{15}N HSQC spectrum, the only other amino acid that was labeled to any extent (60%) were the serine residues.

In order to stereospecifically assign the α-protons of glycine, individual 2D planes from a 3D ^{15}N-resolved TOCSY spectrum of the [U-^{15}N]FKBP–ascomycin complex (Fig. 2, left panel of each pair) were compared to a 2D ^{15}N-edited TOCSY spectrum of [(*R*)-2-^{2}H,^{15}N-Gly]FKBP–ascomycin (Fig. 2, right panel of each pair). In the ^{1}H,^{1}H planes extracted from the 3D ^{15}N-resolved TOCSY spectrum, both glycine α-protons were observed, whereas only one of the α-proton signals was observed in the ^{15}N-edited TOCSY spectrum of [(*R*)-2-^{2}H,^{15}N-Gly]FKBP–ascomycin. By comparing the two sets of spectra shown in Fig. 2, all of the α-protons of the glycine residues of FKBP in the FKBP–ascomycin complex were stereospecifically assigned (Table 1), with the exception of Gly^{83} (whose signals were too broad to be observed) and the N-terminal glycine. These stereospecific assignments were easily obtained from a simple inspection of the TOCSY data and are very reliable, since they are based on deuterium labeling. The ^{15}N labeling of the glycine residues was important to simplify the analysis of the spectra. For the FKBP–ascomycin complex, a 2D ^{15}N-edited TOCSY spectrum was sufficient to resolve the signals, since the glycine amide proton signals did not overlap. However, for more complicated spectra, a 3D ^{15}N-resolved TOCSY experiment may be required.

CONCLUSIONS

A method is described for stereospecifically deuterating and ^{15}N labeling glycine residues of proteins. From the H^N/H^α cross peaks observed in ^{15}N-resolved TOCSY spectra, the stereospecific assignments of the glycine residues can be unambiguously obtained, as demonstrated here for FKBP when bound to the immunosuppressant ascomycin. These assignments are important for interpreting vicinal $^3J_{H^N,H^\alpha}$ coupling constants of the glycine residues, as well as for more

accurately interpreting the NOE restraints involving the glycine α-protons, resulting in more accurate protein structures.

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