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Toward the Complete Assignment of the Carbon Nuclear Magnetic Resonance Spectrum of the Basic Pancreatic Trypsin Inhibitor[†]

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ABSTRACT: A total of 54 of the 58 α-carbon resonances and numerous side-chain carbon signals were individually assigned in the basic pancreatic trypsin inhibitor by using two-dimensional heteronuclear correlated and relayed coherence transfer spectroscopy with proton detection. No isotope enrichment was used, and the spectra were recorded in 5-mm sample tubes. The pulse sequences were optimized to eliminate, prior to phase cycling, the signals of protons attached to ^{12}C. We have concentrated on assignments of carbons bearing a single hydrogen in view of a relatively easy interpretation of carbon relaxation times, and most of these carbon resonances could be assigned. Furthermore, we demonstrate that two-dimensional heteronuclear correlated and relayed coherence transfer spectra can be used to elucidate connectivities between degenerate resonances within proton spin systems that often occur in threonines and aromatic side chains.

Identification of carbon resonances of macromolecules is of interest for the analysis of internal motions in proteins. Since the relaxation of the carbon resonances is dominated by the dipole–dipole interaction with the directly coupled protons at a fixed intramolecular distance (Allerhand, 1971), the carbon relaxation rates can be used to map the spectral density of the motions of the carbon–proton bond at frequencies of the nuclear transitions. This information is also relevant for the accurate interpretation of proton–proton nuclear Overhauser enhancements (NOEs)[1] for distance measurements in proteins and nucleotides, which yield the most valuable source of parameters for the structure determination of such molecules in solution.

Individual assignments of carbon resonances are also of interest for studies of protein reactions. Since the carbon chemical shifts are sensitive to the hybridization of the electron orbitals, distortions of standard bond geometries may be identified from the carbon spectrum, for example, in an enzyme–substrate or an enzyme–inhibitor complex (Richarz et al., 1980; Baillargeon et al., 1980).

Heteronuclear COSY experiments as described in this paper may provide access to larger protein systems than those accessible to homonuclear ^1H COSY: because the direct carbon–proton coupling constants are large compared to the line widths, there is less cancellation of antiphase components than in homonuclear ^1H COSY.

Hitherto, few individual assignments of carbon resonances in proteins and nucleotides have been available, and these were mainly restricted to methyl groups, which often can be assigned even with one-dimensional NMR techniques. The interpretation of the relaxation rates of methyl groups is complicated, however, by the methyl rotation. Here we desribe an effort to assign the resonances of carbons bearing a single proton with a further goal to measure their relaxation rates. These carbon resonances constitute a larger group of probes than the methyl resonances for investigations of internal mobility, and the interpretation of relaxation rates is more straightforward.

The complete individual assignments of carbon or nitrogen spectra of proteins have become possible after the complete individual assignments of proton protein spectra had been

achieved (Wagner & Wüthrich, 1982). Thus, the assignments of the protonated carbons could, in principle, be obtained from heteronuclear COSY experiments. For such experiments, schemes with proton detection provide the highest sensitivity (Maudsley & Ernst, 1977; Maudsley et al., 1977; Bodenhausen & Ruben, 1980). Among these, experiments with a heteronuclear two-spin coherence evolution period have proven to be particularly valuable (Müller, 1979; Bendall et al., 1983; Bax et al., 1983 a, b; Frey et al., 1985; Otvos et al., 1985; Live et al., 1985).

In these experiments, the proton polarization is converted to heteronuclear two-spin coherence, labeled subsequently with the carbon frequencies and finally converted back to proton coherence for detection. Thus, these experiments have a sensitivity similar to proton–proton COSY, reduced only by the low natural abundance of ^{13}C. The cross peaks in such heteronuclear COSY experiments correlate carbon resonances (along ω_1) with the resonances of the directly attached protons (along ω_2), and the carbon resonances can be identified if the proton assignments are known. The heteronuclear RELAY experiments described in this paper yield the same direct connectivities and, in addition, cross peaks between the carbons and remote protons (e.g., between C^α and H^β). This facilitates carbon assignments if the resonances of the directly bound protons are overlapped. With these experiments we have achieved almost complete assignments of the α-carbons and a part of the side-chain carbons of the basic pancreatic trypsin inhibitor. To our knowledge, this is the first case of such a complete assignment for a protein.

MATERIALS AND METHODS

The basic pancreatic trypsin inhibitor (BPTI, Trasylol) was obtained as a gift from Farbenfabriken Bayer, Leverkusen. All measurements were carried out with a 20 mM solution of the protein in D_2O, pD 4.6, at 36 °C. No isotope enrichment was employed. All experiments were carried out in 5-mm sample tubes.

The NMR experiments were recorded on a Bruker AM 360 spectrometer. Two experimental schemes were used for the assignments. A heteronuclear COSY was recorded with the pulse sequence (Müller, 1979; Brühwiler & Wagner, 1986)

$$90_x(^1H)-\tau_0-180(^1H,^{13}C)-\tau_0-90_x(^1H),90_{\phi_1}(^{13}C)-t_1/2-$$
$$180(^1H)-t_1/2-90_{\phi_2}(^{13}C)-\text{acquire}_{\phi_3}(^1H) \quad (1)$$

The phases were cycled as follows: ϕ_1, $+x$, $-x$, $+x$, $-x$; ϕ_2, $+x$, $+x$, $-x$, $-x$; ϕ_3, $+$, $-$, $-$, $+$. In addition, the phases of the 180° pulses were inverted every other four scans. This sequence selectively excites protons coupled to ^{13}C provided the delay τ_0 is tuned for the large (^1H, ^{13}C) coupling constant of 140 Hz ($\tau_0 = 1/4J_{HC}$). Protons not coupled to ^{13}C are strongly suppressed by this pulse sequence (Müller, 1979; Brühwiler & Wagner, 1986) since the second $90_x(^1H)$ pulse converts most of this magnetization back to the z axis. The cross peaks show the heteronuclear coupling along ω_2 in antiphase but no heteronuclear coupling along ω_1. In our experience, this pulse sequence was superior to experiments that included an additional refocusing period $-\tau_0-180(^1H,^{13}C)-\tau_0-$ prior to detection, which probably detected in-phase multiplet components. The antiphase multiplet structure facilitates the recognition of the multiplet patterns in crowded spectral regions. Furthermore, it permits application of filter functions, which are matched for these antiphase signals (e.g., a trapezoidal window that is 0 at $t_2 = 0$ and reaches 1 at $t_2 = 1/2J_{CH}$) and which thus suppress all in-phase signals present due to nonoptimum instrument performance. Concomitantly, filters matched for antiphase signals yield flatter base lines than those

optimized for in-phase multiplets. For the same reason we did not attempt to acquire refocused spectra with ^{13}C decoupling during t_2 although an apparent simplification of the spectra could be expected.

Heteronuclear RELAY experiments with ^1H detection were recorded with the pulse sequence (Brühwiler & Wagner, 1986)

$$90_x(^1H)-\tau_0-180(^1H,^{13}C)-\tau_0-90_y(^1H)-\text{homospoil}-\tau_1-$$
$$90_{-y}(^1H),90_{\phi_1}(^{13}C)-t_1/2-180(^1H)-t_1/2-90_{\phi_2}(^{13}C)-\tau_0-$$
$$180(^{13}C)-\tau_2-180(^1H)-\tau_2-\tau_0-90_y(^1H)-\text{acquire}_{\phi_3}(^1H) \quad (2)$$

The same phase cycling was used as for the hetero-COSY. The phase of the last $90_y(^1H)$ pulse was inverted every other four scans. the extra delay τ_2 was inserted to allow for a sufficient evolution of proton antiphase magnetization necessary for relayed coherence transfer. The sequence produces 2D absorptive cross peaks for direct connectivities, which show the heteronuclear coupling along ω_2 as an in-phase absorptive splitting. The homonuclear splittings are in-phase absorptive in both frequency dimensions. The intensities of the direct cross peaks are often rather low due to the efficient coherence transfer to the remote nuclei. The remote connectivities show cross peaks that are antiphase dispersive, with respect to the active homonuclear coupling, along both frequency dimensions. The signals of the protons not coupled to ^{13}C are destroyed by the homospoil pulse after the second $90_y(^1H)$ pulse while the magnetization of interest is saved as heteronuclear longitudinal two spin order, I_zS_z (Brühwiler & Wagner, 1986).

RESULTS AND DISCUSSION

The assignments of carbon resonances in BPTI as described in this paper were obtained by heteronuclear COSY and RELAY experiments using the known ^1H resonance assignments (Wagner & Wüthrich, 1982). For an accurate comparison of ^1H chemical shifts, a homonuclear ^1H NOESY spectrum was used, which was recorded under identical conditions of temperature and pD.

Figure 1 shows the region of the (C^α,H^α) cross peaks of BPTI. The ^1H spectrum is along ω_2, and the ^{13}C spectrum is along ω_1. The 140-Hz splitting along ω_2 due to the scalar coupling $^1J_{HC}$ is indicated with curved lines. For each of these cross peaks the two doublet components have opposite signs (not indicated in Figure 1). Occasionally, the negative right-hand component of one doublet comes close to, or coincides with, the positive left-hand component of another doublet. This leads to partial or complete cancellation of the coinciding doublet components (see, for example, K26 and R42 in Figure 1). With the H^α proton assignments of Wagner and Wüthrich (1982), 42 α-carbon resonances with unique ^1H resonance positions could be assigned immediately. These 42 resonance positions are labeled with a C in Table I. For the remaining 16 residues cross peaks were observed in Figure 1, but the ^1H resonances are not unique. They can be divided into five groups with overlapping H^α resonance positions at five chemical shift positions. These five H^α positions are at 4.59 (F4 and E7), 4.50 (L6, T11, P13, and S47), 4.30 (A16, I19, Y23, and A27), 4.07 (K26, A40, and T54) and 4.87 ppm (F33 and Y35). In addition, H^α of A58 at 4.00 ppm partially overlaps with one H^β of C38 (see Figure 1).

The methyl groups show the most intense cross peaks in the hetero-COSY spectrum (Figure 2). The assignments of these groups were straightforward, and the chemical shifts are listed in Table I. A large part of these assignments has been obtained previously by Richarz and Wüthrich (1978) with 1D heteronuclear decoupling experiments (see Table I). They are consistent with our data.
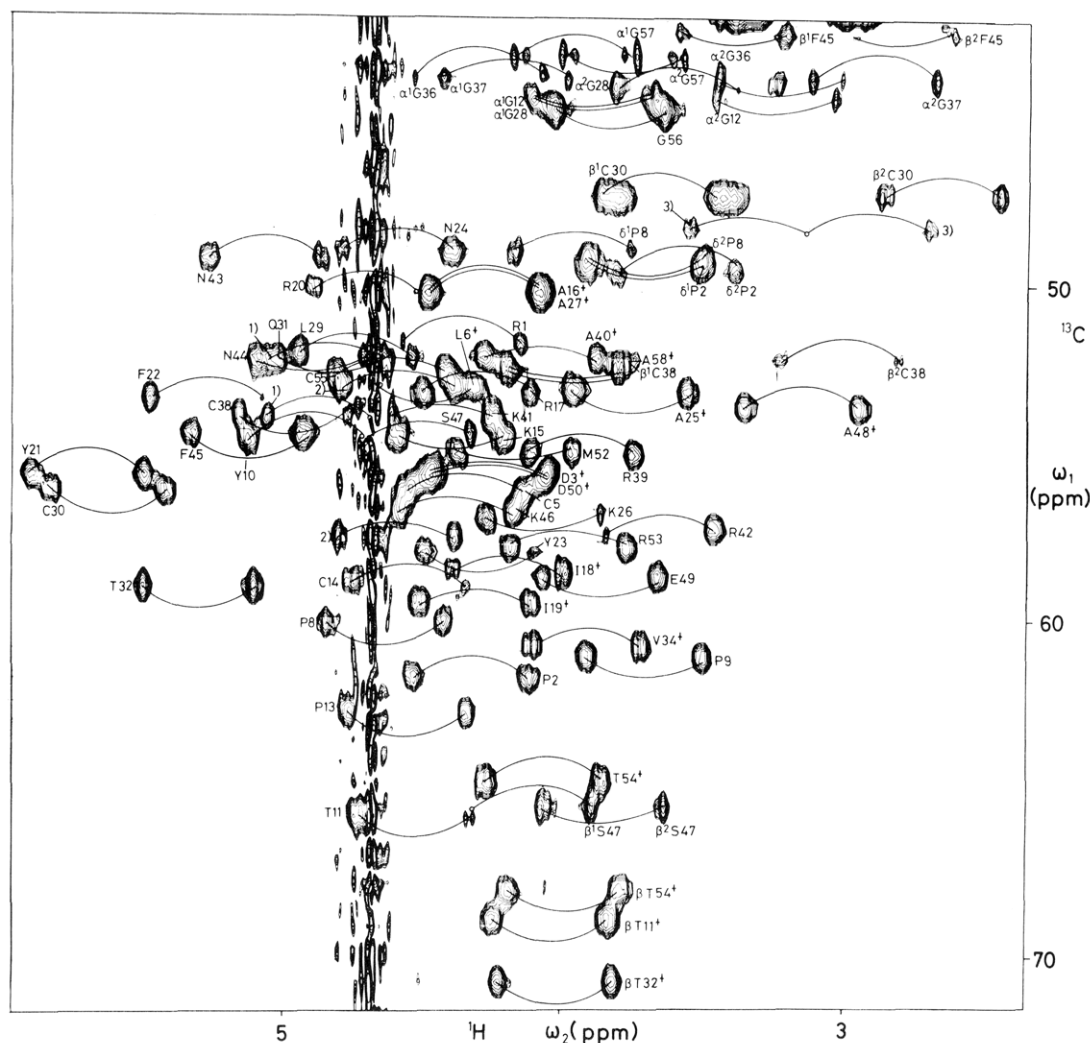
FIGURE 1: Region of the (C^α,H^α) cross peaks of a hetero-COSY experiment of a 20 mM solution of BPTI in D_2O, recorded in a 5-mm sample tube with the pulse sequence of eq 1. No isotope enrichment was used. The total measuring time was 90 h. The heteronuclear splitting along ω_2 of the cross peaks is indicated with curved lines, and the cross peaks are identified with the one-letter symbols for amino acids and the sequence positions. A cross indicates that remote connectivities between the α-carbons and β-protons were observed in a RELAY experiment (eq 2). The labels 1, 2, and 3 indicate that no individual assignments were obtained within the group of (1) F33 and Y35, (2) F4 and E7, and (3) C^β F4 and C^δ P9.

A part of the methylene groups could also be assigned directly (Table I). In Figure 1 some of these assignments are indicated for CH_2 groups that fall in this spectral region. The assignment of the methylene carbons was, in one respect, facilitated by the fact that the cross peaks to both methylene protons must appear at the same carbon chemical shift. This was particularly valuable for the assignment of the α-carbon of G36 where one H^α overlaps with those of the four residues A16, I19, Y23, and A27 while the other H^α resonance has a unique chemical shift. On the other hand, these assignments were more difficult since the region of the methylene groups is very crowded and most methylene cross peaks apparently have lower intensities (Figure 1). The β-carbons of the three threonines could also be assigned directly. They appear at the low-field end of the aliphatic carbon spectrum (Figure 1).

Most of the protonated aromatic carbons could also be assigned directly. Figure 3 shows the cross peaks of the aromatic side chains. All carbon resonances could be identified with the exception of the 2,6 and 3,5 carbons of F45, which have, at 36 °C, broadened proton resonances due to a slow side-chain rotation (Wagner et al., 1976). Note that the assignments of the aromatic proton spin systems of F22 and F33 are exchanged relative to previous publications (Wagner & Wüthrich, 1982). Furthermore, no evidence was found in

Figure 3 that either F22 or F33 is slowly rotating. This is in contrast to an earlier statement, derived from 1D NMR studies, that one of the phenylalanine side chains is slowly rotating even at high temperatures of 72 °C (Wagner et al., 1976). Details of this reinvestigation of the aromatic spin systems will be described elsewhere.

For further assignments two heteronuclear RELAY experiments (eq 2) with two different tuned delays, τ_2, were recorded. For the direct connectivities, these experiments show the same two doublet components as the normal hetero-COSY but an extra cross peak appears at the ω_1-position of the same carbon resonance at the ω_2-position of the remote proton. As an example, Figure 4 shows a cross section along ω_2 at the ω_1-position of the C^β of Thr-54. The two doublet components of the direct connectivity to H^β are in-phase absorptive. In addition, this cross section shows remote connectivities to the α-proton and to the γ-methyl protons of this residue. The remote connectivities are antiphase dispersive with respect to the active (^1H,^1H) coupling.

In Figure 4 we show the cross section for a threonine spin system to demonstrate that the heteronuclear RELAY experiment (Eq 2) can solve the long-lasting problem of distinguishing Ala proton spin systems from Thr spin systems where the α- and β-protons are degenerate. In proton COSY spectra
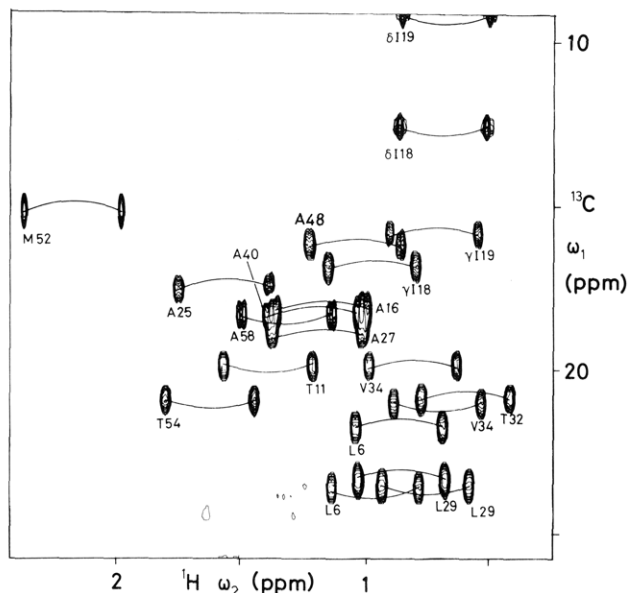
FIGURE 2: Region of the methyl cross peaks of the same spectrum shown in Figure 1. The assignments given are consistent with the earlier one-dimensional studies by Richarz and Wüthrich (1978). In this earlier study all methyl carbon resonances had been observed, but they could only in part be individually assigned due to spectral overlap.
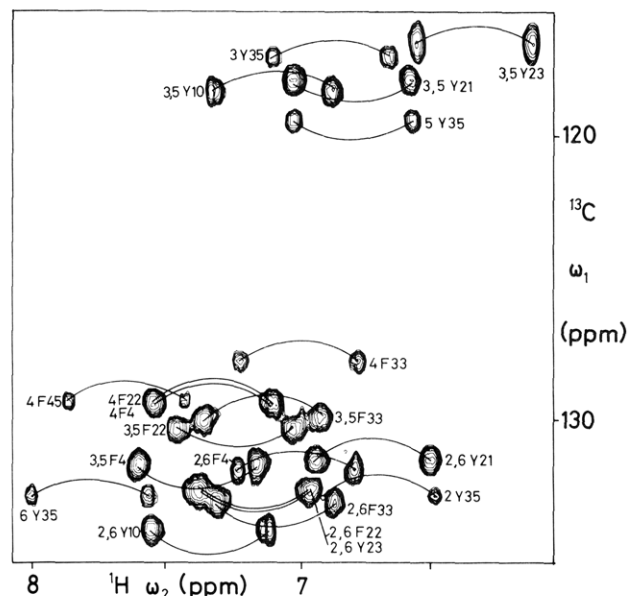

FIGURE 3: Region of the aromatic cross peaks of the same spectrum shown in Figure 1.

the α–β cross peak for such threonines coincides with the diagonal, and it cannot be distinguished from alanines. To solve this problem, use of multiple quantum spectroscopy and sophisticated editing techniques (Levitt & Ernst, 1985) has been proposed. Compared to those techniques, the hetero-RELAY experiment provides a more direct solution for this problem. Since the resonance of the β-proton observed is split by the coupling with ^{13}C (Figure 4), the cross peak of the connectivity to the α-proton can readily be observed, even if the α-proton is exactly degenerate with β-proton. This feature also promises to be rather valuable for the elucidation of aromatic proton spin systems, which often suffer from resonance degeneracies. In BPTI we observed all possible heteronuclear RELAY cross peaks for all aromatic spin systems with the exception of the cross peak between the 3,5 carbons and the 2,6 protons of Y23 and all cross peaks of F45 where

Table I: ^{13}C Chemical Shifts of BPTI Relative to TSP[a]

residue	C^α	C^β	others
Arg-1	52.0 C		
Pro-2	62.0 C		C^δ 50.0
Asp-3	56.0 C		
Phe-4 (Glu-7)	53.0 (58.0)		C^δ 131.5, C^ϵ 131.0, C^ζ 129.0
Cys-5	56.5 C		
Leu-6	53.0 R		C^γ 26.5, C^δ 22.0,[b] 23.5
Glu-7 (Phe-4)	58.0 (53.0)		
Pro-8	60.5 C		C^δ 49.0
Pro-9	61.5 C	28.5	C^γ 24.5
Tyr-10	55.0 C		C^δ 133.5, C^ϵ 118.5
Thr-11	65.5 F	69.0	C^γ 20.0[b]
Gly-12	45.0 C		
Pro-13	63.0 F		
Cys-14	58.5 C		
Lys-15	54.5 C		
Ala-16	50.5 R	18.5[b]	
Arg-17	53.5 C		
Ile-18	58.5 C		$C^\gamma(H_3)$ 17.0, C^δ 12.5
Ile-19	59.5 R		$C^\gamma(H_3)$ 16.0, C^δ 9.0
Arg-20	50.5 C		
Tyr-21	56.0 C		C^δ 131.5, C^ϵ 118.0
Phe-22	53.5 C		C^δ 132.0, C^ϵ 130.0, C^ζ 129.0
Tyr-23	58.0 E		C^δ 132.0, C^ϵ 116.5
Asn-24	49.0 C		
Ala-25	53.0 C	17.5[b]	
Lys-26	57.0 E		
Ala-27	50.5 R	19.0[b]	
Gly-28	44.5 C		
Leu-29	52.0 C		C^γ 24.5, C^δ 23.5, 23.5
Cys-30	56.5 C	48.0	
Gln-31	52.5 C		
Thr-32	59.5 C	71.0	C^γ 21.0[b]
Phe-33 (Tyr-35)	52.5 (54.0)		C^δ 132.5, C^ϵ 130.0, C^ζ 128.0
Val-34	61.0 C	30.5	C^γ 20.0, 21.0
Tyr-35 (Phe-33)	54.0 (52.5)		C^δ 132.5, 132.5, C^ϵ 117.0, 119.5
Gly-36	44.0 C		
Gly-37	44.0 C		
Cys-38	54.0 C		
Arg-39	55.0 C		
Ala-40	52.5 R	18.5[b]	
Lys-41	54.0 C		
Arg-42	57.5 C	28.0	
Asn-43	49.5 C	33.5	
Asn-44	52.5 C		
Phe-45	54.5 C		C^ζ 129.0
Lys-46	56.5 C		
Ser-47	54.5 F	65.5	
Ala-48	53.5 C	16.0[b]	
Glu-49	59.0 C		
Asp-50	56.0 C		
Cys-51	57.5 C		
Met-52	55.5 C		C^ϵ 15.0[b]
Arg-53	58.0 C		
Thr-54	65.0 R	68.0	C^γ 21.0[b]
Cys-55	53.0 C		
Gly-56	45.0 C		
Gly-57	43.5 C		
Ala-58	53.0 R	18.5[b]	

[a] No individual assignments were obtained within the groups for C^α resonances of Phe-4 and Glu-7 and for C^α resonances of Phe-33 and Tyr-35 since the two residues in either group have degenerate α-proton resonances. The letters C, R, F, and E indicate that the assignments were obtained by hetero-COSY, RELAY, fine structure analysis, and exclusion, respectively (see text). [b] The individual assignments of these methyl carbons have been obtained previously by Richarz and Wüthrich (1978) using one-dimensional decoupling experiments.

the resonances are broadened by the rotational motions of the side chain.

The RELAY experiments were used to complete the assignments of the α-carbon resonances. For all cross peaks of Figure 1, which are identified with a cross, a RELAY cross peak was observed between the C^α and the H^β resonances. For
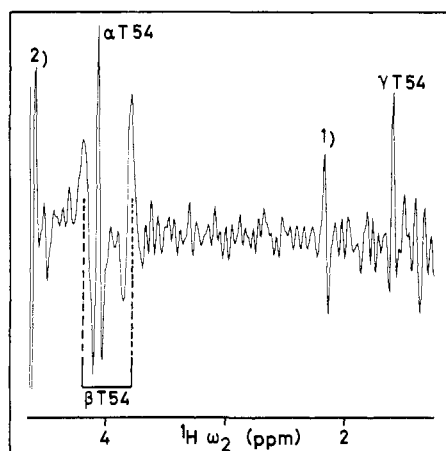
FIGURE 4: Cross section along ω_2 in a RELAY experiment at the C^β-position of T54 (68.0 ppm). The RELAY experiment was recorded with the pulse sequence of eq 2. The total measuring time was 60 h. The signals identified by "1" and "2" originate from t_1 noise of the ϵ-methyl group of M52 and the residual water signal, respectively.

the seven residues A16, A27, A40, A58, T54, L6, and I19 these RELAY connectivities were essential for unambiguous assignment since the H^α resonances are not unique. This is indicated with a letter R in Table I. Thus, the hetero-COSY and the hetero-RELAY yield in total 49 assignments of α-carbon resonances. After the assignment of A40 and T54 by the RELAY experiment, K26, which has a degenerate H^α resonance with these two residues, can be assigned by exclusion. The same holds for Y23 after the assignment of A16, I19, and A27 by the RELAY experiment. To indicate this, we have labeled the corresponding resonances in Table I with the letter E. In the group of L6, T11, P13, and S47, which have the same H^α chemical shift, L6 was assigned by the RELAY experiment. For the other three the fine structure of the ^1H resonance could be used for unambiguous assignment. Information about this fine structure was obtained from a homonuclear ^1H NOESY experiment where the cross peak assignments are known. In the NOESY we recognize that T11 has a clear doublet fine structure with a splitting of ca. 12 Hz. S47 shows a narrow triplet fine structure as typical for a gauche–gauche side-chain conformation whereas P13 has a broad "doublet of doublets" multiplet structure as typical for a gauche–trans side-chain conformation. These features can clearly be recognized in Figure 1 and lead to unambiguous assignment of the corresponding α-carbon resonances. In Table I these resonances are labeled with the letter F. Finally, only the α-carbon resonances of F4 and E7 could not be distinguished since the attached protons have degenerate chemical shifts, and also within the group of F33 and Y35 no individual assignments were obtained for the same reason. For these residues also, the RELAY experiments failed to give cross peaks to the β-protons.

The heteronuclear COSY experiments have a further attractive feature for elucidation of the ^1H spin systems. Due to the large direct coupling constants $^1J_{CH}$, no cancellation of antiphase components within one multiplet component occurs so that $C^\alpha H$ cross peaks for all residues can readily be observed. This is in contrast to homonuclear ^1H COSY experiments where the H^α resonance positions have to be identified from the (H^α,H^β) cross peaks. Since these cross peaks are often weak due to mutual cancellation of antiphase com-

ponents split by small coupling constants, usually the H^α resonances of some residues are difficult to detect. This makes $^1H-^{13}C$ COSY experiments attractive for proteins with higher molecular weights where $^1H-^1H$ COSY spectra suffer heavily from such cancellation effects because of the larger line widths. Even for BPTI the H^α resonances of P13 and R1 had previously not been identified in ^1H COSY spectra. In the hetero-COSY experiments there were two $C^\alpha H$ cross peaks left after the identification of all cross peaks with assigned H^α resonances. In a search for NOESY cross peaks of these two H^α resonances, sequential connectivities could be detected, so that they could be assigned to R1 and P13.

The 2D NMR methods described in this paper make the assignments of ^{13}C resonances of proteins straightforward, provided the ^1H spectrum has been assigned already. The complete assignment of the ^{15}N spectrum appears also to be possible without isotope enrichment (work in progress). The most serious problem for these assignments is the occasional degeneracy of the NH resonances. The assignments described in this paper will be the basis for a screening of ^{13}C relaxation rates in this protein. In order to make use of the assignments, two-dimensional NMR experiments will be necessary for the measurements of the relaxation rates.

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