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Extensive ^{1}H NMR resonance assignment of proteins using natural abundance gradient-enhanced ^{13}C-^{1}H correlation spectroscopy

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The reliability and completeness of ^{1}H NMR resonance assignment can be improved by the use of ^{13}C-^{1}H HSQC correlation spectra on unlabelled protein samples using pulsed field gradients. This technique is illustrated on a 5.2 mM sample of the 79 residue *Desulfovibrio vulgaris* ferrocytochrome c_{553}. Protons attached to the same carbon can be unambiguously paired in a HSQC spectrum. Contrary to ^{1}H, most amino acids exhibit characteristic ^{13}C chemical shift ranges, which can be used for ^{13}C assignment. This technique is especially useful for long side chain residues, such as Gln, Glu, Lys, Arg.

Nuclear magnetic resonance; Cytochrome; Chemical shift; Secondary structure; Carbon-13; Resonance assignment

1. INTRODUCTION

NMR resonance assignment is a prerequisite for conformational study of proteins in solution. Generally, this tedious process is perfomed according to the method proposed by Wüthrich and co-workers [1] in the early 80's. Intra-residue connectivities are first identified using COSY and HOHAHA spectra (either in H_2O or D_2O) and then each residue is correlated to its nearest neighbours using NOESY or ROESY spectra. The increase in the size of the proteins under investigation leads not only to more crowded spectra, but also to a less efficient HOHAHA transfer. On the other hand, the COSY experiment has several drawbacks, especially for larger proteins: it exhibits poor sensitivity and does not always distinguish the C^β protons from the rest of the side chain. Finally, the antiphase nature of the cross-peaks is often inconvenient. The outcome of these combined effects is usually an incomplete assignment of the ^{1}H NMR spectrum, which, in return, limits the reliability and accuracy of the structure derived on this basis.

Over recent years, a shortcut to this problem has been supplied by heteronuclear 3D techniques [2], only feasible on ^{15}N *and* ^{13}C-labelled samples: the second isotope, unfortunately the more expensive, is in fact required to completely identify the side chains. As a large fraction of proteins studied by NMR cannot be over-expressed and thus labelled at a reasonable cost, these powerful techniques are not universally applicable. However, as pulsed field gradients (PFGs) become available for high-resolution spectrometers [3], natural abundance ^{13}C-^{1}H correlations [4] can now be implemented without too much demand on the dynamical range, and thus giving a reasonable sensitivity. In this communication, we demonstrate the great wealth of information available in these experiments in order to back up the ^{1}H assignment work based on classical methods. An example is provided on a monohemic cytochrome c_{553} from the sulfate-reducing bacterium, *Desulfovibrio vulgaris* Hildenborough [5].

2. MATERIALS AND METHODS

D. vulgaris Hildenborough cytochrome c_{553} was purified as previously reported by Le Gall and Bruschi-Heriaud [6]. Reported results were collected at 37°C on a 5.2 mM (phosphate buffer 0.1 M; pH ≈ 5.9; D_2O) reduced sample (ferrocytochrome), obtained by the addition of a 2-fold excess of disodium dithionite in phosphate buffer (0.1 M; pH 8.0) after deoxygenating the protein solution with argon gas. The data were recorded on a Bruker AMX-600 (^{1}H = 600 MHz) equipped with a standard 5 mm inverse detection Bruker probe with internal B_0 gradient coil. This study was based on the resonance assignments recently published [7], obtained solely on the basis of ^{1}H homonuclear experiments (2D and 3D).

The assignment of the ^{13}C resonances and the consequent check of the ^{1}H resonances is based on two experiments: a heteronuclear single quantum coherence (HSQC) [8] and a relayed HSQC-HOHAHA experiment. Schematically, the HSQC sequence can be visualized as:

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Abbreviations: NMR, nuclear magnetic resonance; NOESY, homonuclear nuclear Overhauser enhancement specroscopy; HOHAHA, homonuclear Hartman-Hahn spectroscopy, HMQC, ^{1}H-detected heteronuclear multiple-quantum coherence spectroscopy; HSQC, ^{1}H-detected heteronuclear single-quantum coherence spectroscopy; PFG, pulsed B_0 field gradient; INEPT, insensitive nuclei enhanced by polarization transfer.

[90°] [INEPT $^1H \rightarrow {}^{13}C$] [Evolution (t_1)] [retro-INEPT $^{13}C \rightarrow {}^1H$] [Detection (t_2)]

The coherences are carried by the ^{13}C spin during τ_1 and by the ^1H spin during τ_2 and decoupled from the other spin. The coherences of the ^1H bound to ^{12}C (about 99%) should be cancelled, either by phase cycling or by PFG. As far as the dynamical range of the receiver is concerned, gradients are preferable to phase cycling, as the cancellation of undesired signals occurs before the analog-to-digital convertor. Let us recall the INEPT sequence:

^1H: τ–180°–τ–90°
^{13}C: τ–180°–τ–90°

(with $\tau = 0.25 \times J_{HC}$) which transfers the proton magnetization from ^1H to ^{13}C. As pointed out by Bax and Pochapsky [9], the two pulses need not be applied simultaneously and the selection of the coherence pathway via the ^{13}C spin can be done by a PFG in between.

^1H:	τ	180°	τ	90°	
^{13}C:	τ	180°	τ		90°
field gradient:	τ		τ	pulse	

Because of the symmetry of the HSQC sequence, filtering can alternatively be done between the two (^{13}C,^1H) 90° pulses at the beginning of the retro-INEPT sequence. A 200 μs PFG pulse (15–20 gauss/cm) followed by a recovery period of 400 μs was used. Other significant parameters are: 90° (^1H) = 10 μs, 90° (^{13}C) = 13 μs, ^1H spectral width = 5.5 ppm, ^{13}C spectral width = 73.36 ppm, 128 scans/τ_1 increment (overall experimental time = 25 h).

The HSQC-HOHAHA experiment is derived from the HSQC by inserting an isotropic mixing before the acquisition period. The Waltz-17 sequence was modified in order to balance the longitudinal and rotating frame nuclear Overhauser effect ('Clean-TOCSY' method [10]). The isotropic mixing time was 70.4 ms (including delays for a overall duration of 28.2 ms) and the experimental time was 50 h (256 scans/increment).

While selecting the coherence pathway through the ^{13}C, the PFG is also able to eliminate, to a large extent, the (residual) water resonance. Similar experiments have been successfully carried out on proteins disolved in H_2O, for lack of a D_2O sample. In any case, the receiver can be set to a higher gain, if the relaxed water resonance is further eliminated by a z-filter prior to acquisition (in HSQC and HSQC-HOHAHA experiments). By adding the following building block:

[90°x] [PFG] [90°$-x$]

The water signal (brought to the transverse plane by the first ^1H 90° pulse of the retro-INEPT) can be selectively eliminated because it is 90° out-of-phase with respect to the protein signal before this purging block.

3. RESULTS AND DISCUSSION

^1H-detected heteronuclear pulse schemes can basically be devided into two types: experiments that utilize heteronuclear multiple-quantum coherence (HMQC) [11] and experiments that rely on low-γ transverse coherence (HSQC) [8]. Bax et al. [12] have shown that the latter offer improved resolution, because the dipolar transverse relaxation of single-quantum coherences is slower than that of multiple-quantum coherences. Gradients have been implemented in both HSQC and HMQC experiments [13] with, in some cases, extensive modifications of the original pulse sequence. A major difference between phase cycling and PFG deserves special emphasis: phase cycling is able to select positive and negative coherence order (± j) simultaneously, but a set of PFGs will keep *exclusively* either of the two orders (+ j *or* −j), except when j is equal to zero. Such a dramatic decrease in sensitivity is a severe penalty when working on unlabelled samples. With minor modifications and without altering the sensitivity, the HSQC scheme can be adapted to include PFG, as reported above. The heteronuclear coherence transfer between proton and carbon is achieved by the final 90° pulses of the INEPT building block and can be described in terms of product operators as [14]:

$$\pm \text{HyCz} \rightarrow [90°_x(^1H)] \rightarrow \pm \text{HzCz} \rightarrow [90°_x(^{13}C)] \rightarrow \pm \text{HzCy}$$

A PFG applied between the two pulses scrambles the transverse coherences (for ^1H attached to ^{12}C) and retains longitudinal magnetizations as the longitudinal zz-order H_zC_z, to be later converted into antiphase ^{13}C coherence (H_zC_y) by the ^{13}C 90° pulse. This location of the PFG exhibits two further advantages: the resulting spectrum is almost free of linear phase gradient (as no

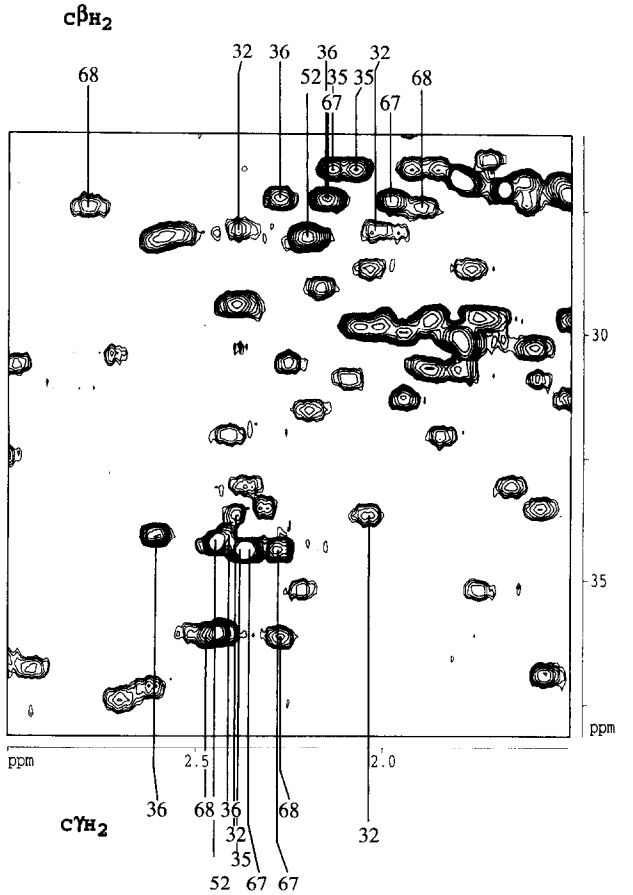

Fig. 1. HSQC spectrum of ferrocytochrome c_{553} from *D. vulgaris* Hildenborough. The side chains of all Glu and Gln residues in the proteins are labelled: Gln-32, Glu-35, Glu-36, Glu-52, Glu-67 and Glu-68. Note the large chemical shift difference between the C^β (near 25 ppm) and the C^γ (near 34 ppm).

delay is added when the spins are precessing) and the slower relaxation of the longitudinal magnetization (than for transverse coherence) prevents a large decay of the signal.

Figs. 1 and 2A show a selected area of the HSQC spectrum recorded on ferrocytochrome c_{553} from *D. vulgaris* Hildenborough. As compared to homonuclear spectra (either COSY or HOHAHA), two types of information can be extracted from ^{1}H-^{13}C correlations: protons attached to the same carbon can be unambiguously identified and additional evidence for their specific assignment is given by the ^{13}C chemical shift. In

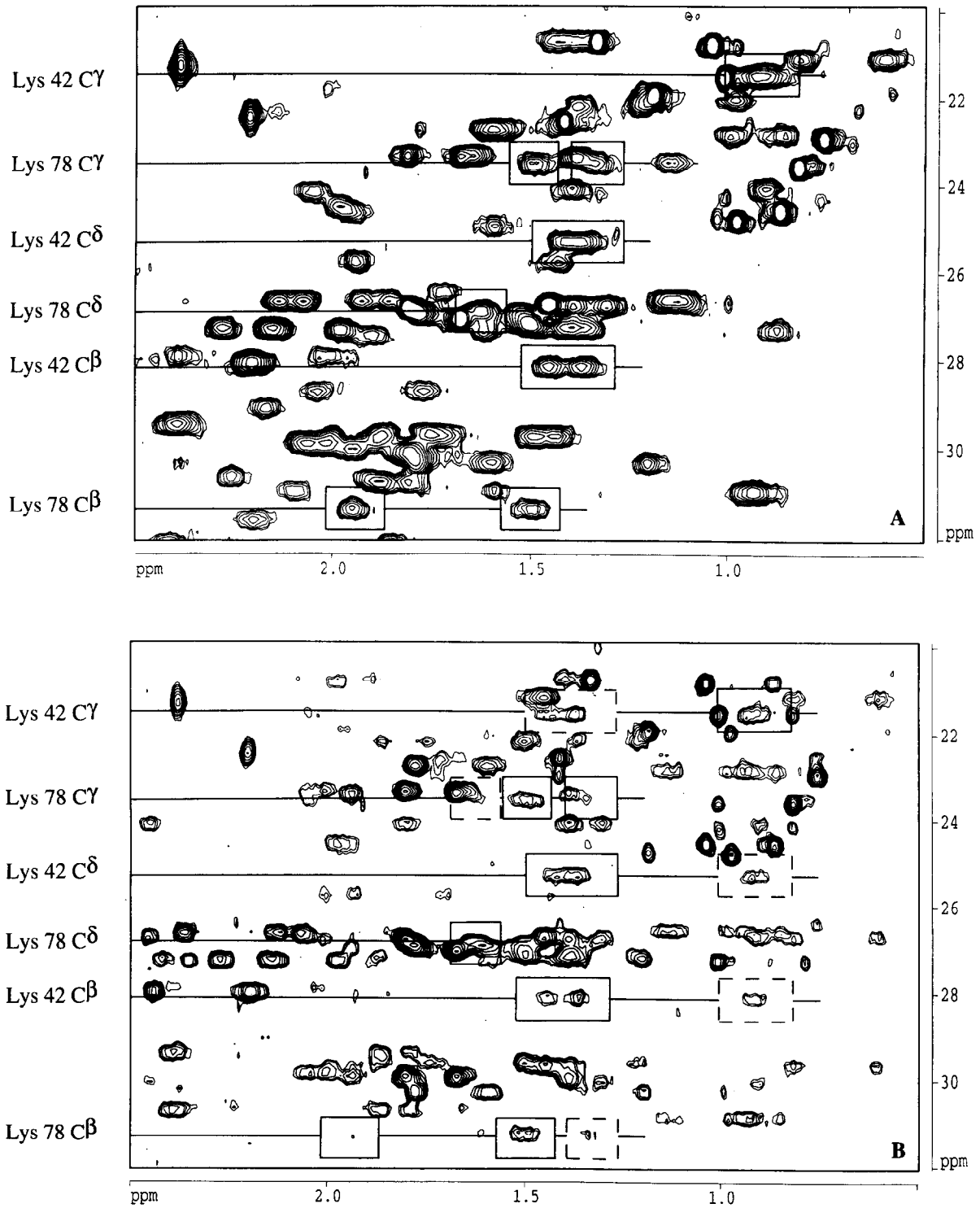

Fig. 2. (A) HSQC spectrum of ferrocytochrome c_{553} from *D. vulgaris* Hildenborough. The side chain of Lys-42 and Lys-78 are labelled. (B) HSQC-HOHAHA spectrum of the same region. Indirect correlations due to the HOHAHA transfer are indicated with dashed lines.

principle, a COSY spectrum should correlate all protons directly coupled by J coupling, but peaks may be missing due to vanishingly small J coupling (for instance, a 3Hz $J_{HC}\alpha_C\beta_H$ may not be detected because of the line width). Furthermore, no definite distinction can be made between ^{2}J and ^{3}J coupling which may have very similar amplitudes. Finally, HOHAHA spectra provide a correlation with the entire side chain; however, one can easily mistake the C^β protons for the C^γ ones, as the intensities of HOHAHA cross-peaks in multispin systems behave in a complex manner. Assignments based on homonuclear spectra frequently rely on the relative intensity of cross-peaks rather than on clear-cut arguments (e.g. absence or presence of peaks) and incomplete and/or incorrect assignments for long side chains (such as Glu, Gln, Arg, Lys or Met) are thus common, leading to imprecise 3D structure.

An illustrative example is given by the Glu-68 side chain which has been previously assigned [7] in an incorrect manner: (2.27 and 1.89 ppm) for the $C^\beta H_2$ and (2.78 and 2.46 ppm) for the $C^\gamma H_2$. This assignment was supported by the following loose argument: the $C^\gamma H_2$ are usually downfield-shifted as compared to the $C^\beta H_2$ (cf. for instance [1]). Because of either overlaps or vanishingly small $J_{HC}\alpha_C\beta_H$, only the $C^\beta H$ at 1.89 ppm correlates with the $C^\alpha H$ and no evidence of a second COSY peak was found. Fig. 1 shows an expansion of the corresponding ^{1}H-^{13}C correlation: two protons at 2.78 and 1.89 ppm are correlated with the same ^{13}C (upper part) as well as to the two protons at 2.27 and 2.46 ppm (lower part). Clearly, the side chain protons of Glu-68 have not been correctly paired, on the basis of homonuclear experiments, which are not able to distinguish ^{2}J from ^{3}J coupling. The second step of the assignment involves the discrimination between the $C^\beta H$ and $C^\gamma H$. As reported in the literature [15] and illustrated in Fig. 1, the ^{1}H chemical shift ranges of Glu $C^\beta H$ and $C^\gamma H$ strongly overlap. As the ^{13}C chemical shift ranges do not overlap (see Fig. 1 and [16]) the more downfield-shifted carbon (δ = 36.1 ppm) is identified as the C^γ, and consequently the protons at 2.27 and 2.46 ppm are identified as C^γ protons. Fig. 1 shows the sharp clustering of the ^{1}H-^{13}C correlations for the $C\beta H_2$ far away from the resonances corresponding to $C^\gamma H_2$ for all Glu and Gln residues.

Ambiguities of this nature are very frequent for other long aliphatic side chains [1]. Fig. 2 illustrates the case of Lys, where major overlaps occur for the chemical shift ranges of C^βH2, $C^\gamma H_2$ and $C^\delta H_2$. According to Groß and Kalbitzer [15], 90% of the protons are found in the following ranges: 2.2–1.2 ppm for C^βH2, 1.8–0.2 ppm for $C^\gamma H_2$ and 1.8–0.9 ppm for $C^\delta H_2$. Furthermore, it is difficult to assign these pairs of protons specifically, even using several HOHAHA spectra recorded at various mixing time: as the matter of fact, the complexity of the J coupling network for Lys makes the prediction of the cross-peak intensity hazardous. Fig. 2A and B show, respectively, an expansion of the HSQC spectrum and of the HSQC-HOHAHA spectrum labelled for Lys-42 and Lys-78. In the published assignment [7], the C^δH2 of Lys-42 has not been identified (as well as the $C^\varepsilon H_2$), and for Lys-78, a signal at 2.92 ppm was erroneously assigned to one C^β proton, and four chemical shifts were reported without specific assignment. Despite its low signal-to-noise ratio, the HSQC-HOHAHA spectrum helps to solve ambiguities and identify protons of the same side chain. In Fig. 2B additional peaks observed in the relayed experiment are indicated with dashed lines. It should be pointed out that the number of visible relayed correlations is smaller for Lys-78 because of the narrow ^{1}H chemical shift dispersion of this residue.

Let us now turn to the use of the ^{13}C chemical shift to identify secondary structure. Figs. 1 and 2 clearly show that a naturally abundant ^{1}H-^{13}C correlation spectrum, with good signal-to-noise ratio, can be recorded on the same sample used for homonuclear experiments. The ^{13}C resonance assignment of ferrocytochrome c_{553} from *D. vulgaris* is reported in Table I. Richarz and Wüthrich [16] have reported the random coil ^{13}C chemical shifts values for linear tetrapeptides and observed significant trends among amino acids. These results were later confirmed by statistical analysis of ^{13}C chemical shifts of proteins [17]. As opposed to ^{1}H, where the ranges strongly overlap, ^{13}C chemical shifts are strongly correlated with the type of carbon and the nature of the amino acid. For instance, glycine's C^α appears near 44

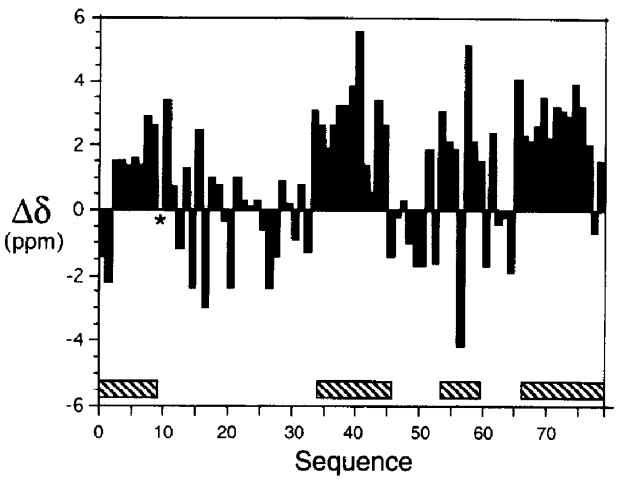

Fig. 3 Secondary chemical shifts for the C^α in *D. vulgaris* ferrocytochrome c_{553}. These values were computed as $\Delta\delta = \delta(\text{obs}) - \delta(\text{random})$, where δ(obs) is the observed chemical shift and δ(random) is the random coil value published by Richarz and Wüthrich [16]. The ^{13}C chemical shifts were referenced with respect to the water resonance calibrated at $\delta(^1\text{H}) = 4.658$ ppm (37°C), using 0.2514-5004 for the ratio of the ^{1}H and ^{13}C frequencies for tetramethylsilane (TMS). Note, however, that the secondary chemical shifts, as relative variations, can be interpreted without reference to this calibration. The location of the α-helical structures, as determined by ^{1}H NMR, is indicated by hatched strips. A star indicates the Cys-10 C^α, which is not identified.

Table I

Chemical shifts of protonated aliphatic carbons in ferrocytochrome c_{553} of *Desulfovibrio vulgaris* Hildenborough (pH 5.9, 37°C)

	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ϵ}
Ala-1	49.3	17.7			
Asp-2	50.4	39.5			
Gly-3	45.3				
Ala-4	52.2	16.5			
Ala-5	52.1	16.3			
Leu-6	55.3	40.6	?	24.6 20.9	
Tyr-7	57.6	37.1			
Lys-8	57.4	29.8	23.3	26.8	39.8
Ser-9	59.1	61.4			
Cys-10	?	33.5			
Ile-11	62.8	36.9	14.5 27.3[b]	11.9	
Gly-12	44.5				
Cys-13	52.6[c]	34.2			
His-14	54.8	?			
Gly-15	41.4				
Ala-16	53.2	15.6			
Asp-17	49.6	38.7			
Gly-18	44.8				
Ser-19	57.3	62.0			
Lys-20	54.3	30.9	22.9	26.6	39.5
Ala-21	48.3	15.1			
Ala-22	51.7	14.9			
Met-23[d]	54.2	28.0	32.5		?
Gly-24	43.9				
Ser-25	56.8	63.8			
Ala-26	50.1	19.1			
Lys-27	52.1	30.3	22.8	27.0	39.8
Pro-28	60.4	?	?	?	
Val-29	61.5	29.1	19.0 16.2		
Lys-30	54.7	30.3	22.1	27.2	39.7
Gly-31	42.9				
Gln-32	54.8	27.9	33.7		
Gly-33	42.5				
Ala-34	53.8	16.0			
Glu-35	57.9	26.7	34.4		
Glu-36	57.2[c]	27.2	34.1		
Leu-37	56.3[c]	41.7	24.3	24.8 22.0	
Tyr-38	60.0	36.5			
Lys-39	57.7	30.0	23.4	27.1	39.6
Lys-40	58.3	30.9	?	?	?
Met-41	59.4[c]	32.0[c]	?		?
Lys-42	55.9	28.1	21.5	25.3?	39.1
Gly-43	44.3				
Tyr-44	59.6[c]	35.2			
Ala-45	53.3	15.5			
Asp-46	51.2	38.6			
Gly-47	43.6				
Ser-48	56.8	61.8			
Tyr-49	55.2[c]	40.3			
Gly-50	42.1				
Gly-51	42.1				
Glu-52	57.2	28.0	34.3		
Arg-53	52.9	28.2	26.7	41.9	
Lys-54	57.6[c]	29.9[c]	20.7	27.3	39.6
Ala-55	52.8	15.8			
Met-56[d]	55.8	30.7	29.4		?
Met-57	49.7	?	?		20.8
Thr-58	65.1	66.5	19.4		
Asn-59	53.5	36.1			
Ala-60	52.2	15.7			
Val-61	58.9	30.6	18.5	15.9	
Lys-62	56.9[c]	29.7	22.2	27.2	39.7
Lys-63	54.1[c]	29.7	21.2	26.7	39.5
Tyr-64	56.0	37.4			
Ser-65	54.6	61.6			
Asp-66	56.7	38.8			
Glu-67	57.6	27.3	34.4		
Glu-68	57.4[c]	27.4	36.1		
Leu-69	56.3[c]	40.2	24.2	23.6 21.6	
Lys-70	58.0	30.1	24.1	26.8	39.5
Ala-71	52.9	16.1			
Leu-72	56.9	41.7	26.4	23.0 22.6	
Ala-73	53.8	16.7			
Asp-74	55.5	39.7			
Tyr-75	60.1	36.8			
Met-76[d]	57.1	33.1	30.6		
Ser-77	58.5	61.3			
Lys-78	53.8	31.3	23.5	26.9	40.1
Leu-79	55.2	40.1	23.5	22.5 19.7	
Heme ring Me:	1 14.8	Me2 22.5		Propionate 7 44.1	
	3 12.8	Me4 21.3		Propionate 6 28.7	
	5 12.6				
	8 12.0				
ϵ-CH_3 of Met[e]					
^{13}C chem. shift	15.7	16.3	20.8	15.4	15.8
^{1}H chem. shift	2.48	2.75	−3.56	2.42	2.41

[a] Question marks indicate missing assignments. Underlined are the chemical shifts of the ^{13}C corresponding to ^{1}H, which were previously either unassigned or misassigned (see [7]).

[b] γ-CH_2 of Ile-11.

[c] This chemical shift is only based on the HSQC spectrum and the ^{1}H chemical shift value. It has not been confirmed by the observation of a relayed cross-peak in the HSQC-HOHAHA spectrum.

[d] The small chemical shift range of the C^{β} and C^{γ} of Met overlaps and the assignment of these carbons can be interchanged.

[e] No specific assignment can be given for the ϵ-CH_3 of methionines on the basis of the here reported experiments (no J coupling with the side chain).

ppm, while the C^{α} of β-branched residues of Val and Ile are downfield-shifted (near 60 ppm). Furthermore, several authors have reported an empirical correlation of the ^{13}C chemical shift with the protein backbone conformation [17,18]. Spera and Bax [18] have shown an average chemical shift increase of 3 ppm in α-helical structures and a decrease of 1.5 ppm in β-sheets. Fig. 3 displays the ^{13}C chemical shift values corrected for the random chemical shift value taken from [16]. A downfield shift is observed for most of the residues which were reported as being part of an α-helical structure [7]: 2 → 8, 34 → 46, 53 → 59 and 67 → 77. A clear exception to this general trend is the C^{α} of Met-57, the sixth ligand of the heme. It is noteworthy that a good correlation between the ^{13}C chemical shift and the secondary structure is found for this cytochrome, despite the large ring current shift of the heme.

4. CONCLUSION

From these data, the following conclusions can be drawn. The sensitivity of the gradient-enhanced ^{1}H-^{13}C HSQC experiment compares favourably with that of the standard homonuclear techniques (NOESY and HOHAHA) used for protein resonance assignment. In homonuclear spectra, the intensity of the cross-peaks is usually less than 10% of the diagonal, in most cases only a few percent. As no technique is available for effective diagonal suppression, dynamical range becomes the major limitation, together with water suppression and t_1 noise. In the gradient enhanced HSQC, no such limitation occurs, as the filtering is performed before the analog-to-digital converter. Thus, it is not surprising that the quality of the heteronuclear data is not far from homonuclear data. The use of the HMQC-HOHAHA experiment was proposed in the past for ^{1}H resonance assignment [19] on ^{15}N-labelled proteins, but almost the same experiment (but based on single quantum coherences) can be performed on proteins with a natural abundance at the ^{13}C level.

In view of our results, we suggest that these experiments should become part of the standard set of experiments used for protein assignment. We have shown that the long side chains can be assigned to a larger extent and with an increased reliability. It is now taken for granted that the qualitity of the NMR-derived structure mainly depends upon the number of distance constraints, and thus on the completeness of the assignment. The assignment of long-chain protons (e.g. Lys or Arg) is thus likely to increase the constraint data set, and hence increase the quality of the NMR-derived structure.

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