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Stereospecific Nuclear Magnetic Resonance Assignments of the Methyl Groups of Valine and Leucine in the DNA-Binding Domain of the 434 Repressor by Biosynthetically Directed Fractional ^{13}C Labeling†

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ABSTRACT: Stereospecific ^{1}H and ^{13}C NMR assignments were made for the two diastereotopic methyl groups of the 14 valyl and leucyl residues in the DNA-binding domain 1-69 of the 434 repressor. These results were obtained with a novel method, biosynthetically directed fractional ^{13}C labeling, which should be quite widely applicable for peptides and proteins. The method is based on the use of a mixture of fully ^{13}C-labeled and unlabeled glucose as the sole carbon source for the biosynthetic production of the protein studied, knowledge of the independently established stereoselectivity of the pathways for valine and leucine biosynthesis, and analysis of the distribution of ^{13}C labels in the valyl and leucyl residues of the product by two-dimensional heteronuclear NMR correlation experiments. Experience gained with the present project and a previous application of the same principles with the cyclic polypeptide cyclosporin A provides a basis for the selection of the optimal NMR experiments to be used in conjunction with biosynthetic fractional ^{13}C labeling of proteins and peptides.

Nuclear magnetic resonance (NMR)[1] spectroscopy in solution is by now quite well established as a method for the determination of the three-dimensional structure of proteins [for recent reviews see, for example, Wemmer and Reid (1985), Clore and Gronenborn (1987), Kaptein et al. (1988), and Wüthrich (1989a,b)], and there is keen interest in additional refinements of the method to further improve the precision of the structure determinations. One avenue toward this goal is the use of stereospecific assignments for diastereotopic groups of protons (Wüthrich, 1986; Kline et al., 1988; Driscoll et al., 1989; Güntert et al., 1989), which are not obtained by the generally used sequential resonance assignment procedure for proteins (Wüthrich et al., 1982; Billeter et al., 1982; Wagner & Wüthrich, 1982; Wider et al., 1982). For structure determinations without stereospecific assignments, a set of pseudoatoms replacing the diastereotopic hydrogen atoms was introduced (Wüthrich et al., 1983). This is inevitably a compromise, since the use of these pseudoatoms reduces the precision of the experimental conformational constraints (Wüthrich, 1986). More recently, systematic manual and automated procedures were introduced for obtaining stereospecific assignments for β-methylene groups (Arseniev et al., 1988; Güntert et al., 1989; Hyberts et al., 1987; Wagner et al., 1987; Weber et al., 1988). For more peripheral side-

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[1] Abbreviations: NMR, nuclear magnetic resonance; COSY, two-dimensional correlated spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; biosynthetic fractional ^{13}C labeling, biosynthetically directed fractional ^{13}C labeling.

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chain protons, however, only few stereospecific assignments were so far obtained, primarily during the final stages of the three-dimensional structure determination by reference to spatially proximate protons (Kline et al., 1988; Senn et al., 1984; Zuiderweg et al., 1985). Among these peripheral groups of protons the isopropyl moieties of Val and Leu have outstandingly large pseudoatom corrections (Wüthrich et al., 1983; Wüthrich, 1986). Furthermore, their methyl resonance lines are prominent features in the ^{1}H NMR spectra of proteins, so that a large number of NOE's with the methyl protons of Val and Leu can usually be observed and identified. Overall, stereospecific assignments of the isopropyl groups in a protein can therefore have an important influence on the precision of the entire structure determination. The present paper describes a novel approach for obtaining such stereospecific NMR assignments in proteins, which uses biosynthetic fractional ^{13}C labeling of the polypeptide chain and heteronuclear NMR experiments, and does not depend on any prior knowledge of the three-dimensional structure. As a practical application, the stereospecific ^{1}H and ^{13}C assignments of the 28 methyl groups of Val and Leu in the DNA-binding domain 1–69 of the 434 repressor are described.

Methods

Stereospecific Assignment of the Methyl Groups of Valine and Leucine by Nonrandom ^{13}C Labeling. This approach for obtaining stereospecific NMR assignments derives from the fact that the biosynthesis of the amino acids valine and leucine from glucose is known to be stereoselective (Crout et al., 1980; Gough & Murray, 1983; Hill & Yan, 1971; Hill et al., 1973, 1979). Thereby, as is shown in Figure 1, the isopropyl group is made up of a two-carbon fragment from one pyruvate unit, while the second methyl groups is transferred from another pyruvate unit. This methyl migration has been shown to be stereoselective, and the migrating methyl group becomes *pro-S* in both valine and leucine; i.e., it is γ^2CH_3 or δ^2CH_3, respectively. Direct proof for this stereoselective biosynthetic pathway was obtained for *Escherichia coli* (Sylvester & Stevens, 1979; Hill et al., 1973), which was also used to express the protein studied in this paper.

Biosynthetic fractionally ^{13}C-labeled proteins can be obtained from microorganisms grown on minimal media containing a mixture of roughly 10% [$^{13}C_6$]glucose and 90% unlabeled glucose as the sole carbon source. The carbon positions in such preparations are uniformly ^{13}C labeled to an extent of about 10%. Disregarding the natural ^{13}C abundance of 1.1% in the unlabeled glucose, the probability that two adjacent carbon positions are labeled in the same molecule is then 1%, unless the two carbon atoms originate from the same carbon source molecule so that this probability becomes 10%. These two distinct situations prevail for the isopropyl group in valine and leucine (Figure 1): The *pro-R* methyl group (γ^1 and δ^1, respectively) and the adjacent >CH– group originate from the same pyruvate molecule and are, in the absence of isotope scrambling, labeled with ^{13}C in the same molecules. On the other hand, the *pro-S* methyl group and the adjacent carbon atom originate from two different pyruvate molecules (Figure 1). Therefore, if the *pro-S* methyl group is enriched with ^{13}C, there is a probability of only 1% that the adjoining >CH– group in the same molecule is also labeled.

The stereospecific distinction between the pairs of isopropyl methyl groups in a fractionally ^{13}C-labeled biosynthetic protein is most clearly evidenced in ^{1}H-decoupled ^{13}C NMR spectra, where the ^{13}C resonance of the *pro-R* methyl group is a doublet with a splitting of about 33 Hz due to the one-bond ^{13}C–^{13}C coupling with the neighboring ^{13}C spin, while the ^{13}C NMR

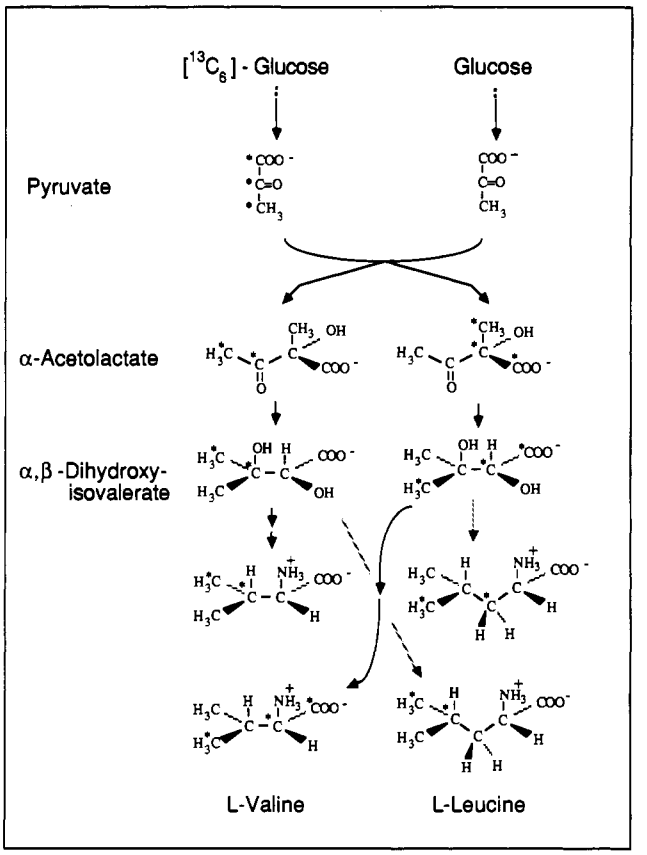

FIGURE 1: Reaction pathways for the biosynthesis of valine and leucine from a mixture of fully ^{13}C-labeled and unlabeled glucose showing the stereochemistry and the principal labeling patterns (an asterisk indicates a ^{13}C label; the absence of the asterisk indicates the natural ^{13}C abundance of 1.1%).

signal of the *pro-S* methyl group is a singlet. For work with proteins, 2D NMR experiments (Ernst et al., 1987) are usually employed to ensure a workable spectral resolution (Wüthrich, 1986). For the present project a two-dimensional ^{1}H-detected heteronuclear correlation experiment, [^{13}C,^{1}H]-COSY, is a good choice. The pulse sequence originally devised by Bodenhausen and Ruben (1980) ensures both ^{1}H–^{1}H and ^{1}H–^{13}C decoupling in the ^{13}C dimension, so that the forementioned differences between the *pro-R* and *pro-S* methyl groups can readily be observed along the ω_1 frequency axis (Figure 2).

Identification of the Pairs of Methyl NMR Lines That Originate from the Same Isopropyl Group. In principle, sequence-specific ^{1}H NMR assignments can be extended to the ^{13}C lines by [^{13}C,^{1}H]-COSY. However, in crowded spectral regions such assignments based entirely on alignment of individual ^{1}H chemical shifts may not all be unambiguous. As an additional assignment criterion TOCSY-relayed [^{13}C, ^{1}H]-COSY at natural abundance of ^{13}C provides particularly clear evidence for identifying the pairs of methyl resonances in the isopropyl groups of Val and Leu (Otting & Wüthrich, 1988). In this experiment the proton magnetization is first transferred to directly bound ^{13}C atoms, from there back to the protons, and finally by a TOCSY pulse sequence to all other protons of the spin system. The resulting unique cross-peak pattern for the isopropyl groups of Val and Leu is shown in Figure 3.

Experimental Procedures

Preparation of Nonrandomly ^{13}C-Labeled 434 Repressor 1–69. The 434 repressor was produced from an overexpression system consisting of NM522 *E. coli* cells (Gough & Murray, 1983) bearing a pRW190 plasmid kindly provided by M.

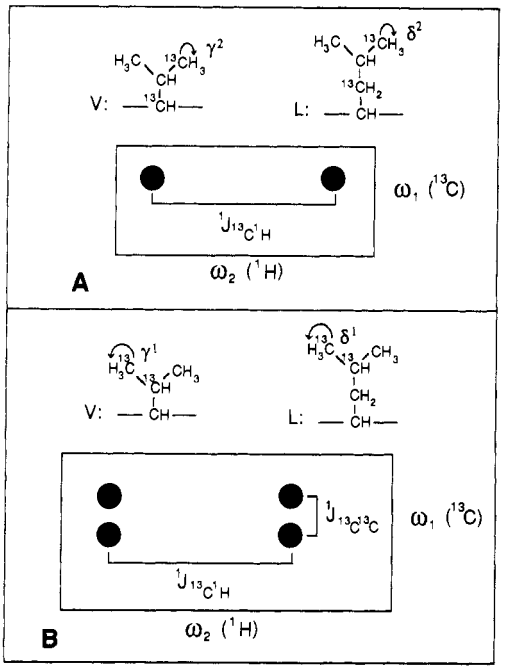

FIGURE 2: Schematic representation of the dominant multiplet fine structures expected for the methyl ^{13}C–^1H cross peaks of Val and Leu in [^{13}C,^1H]-COSY spectra recorded with a protein preparation fractionally labeled with ^{13}C according to Figure 1. The arrows indicate the coherence transfer that is relevant for the present study. (A) For γ^2CH_3 of Val and δ^2CH_3 of Leu the multiplet consists of two components along ω_2 separated by the $^1J_{^{13}C,^1H}$ coupling constant. No splitting is observed along ω_1. This pattern is the same as for methyl groups in a protein with natural abundance of ^{13}C (Figure 4A). (B) In addition to the $^1J_{^{13}C,^1H}$ splitting along ω_2, γ^1CH_3 of Val and δ^1CH_3 of Leu have a splitting of $^1J_{^{13}C,^{13}C}$ along ω_1.

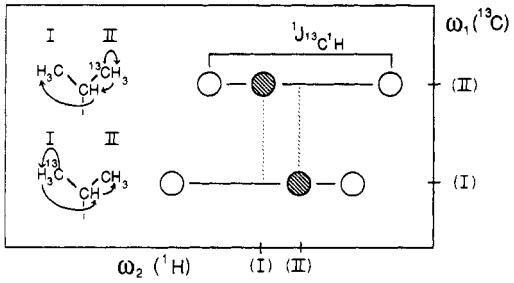

FIGURE 3: Schematic representation of the cross-peak fine structure patterns in TOCSY-relayed [^{13}C,^1H]-COSY at natural ^{13}C abundance that result in the identification of the pairs of diastereotopic methyl resonances belonging to the same isopropyl group of Val or Leu. The two relevant coherence transfer pathways starting from ^{13}C(I) and ^{13}C(II), respectively, are indicated by arrows. In the spectral region displayed in Figure 5, these give rise to two groups of three cross-peak components with identical ^{13}C shifts. At the ^{13}C shift (II) the transfer starting from ^{13}C(II) yields a doublet from the direct [^{13}C,^1H]-COSY cross peak and a singlet from the double relay to the methyl protons I, and vice versa. (Note that the relayed cross peaks from the methyl ^{13}C resonances to the methine proton resonance are outside of the spectral region shown here and in Figure 5.) The two groups of three peaks originating from the same isopropyl group are related by the fact that along ω_2 the singlet with the ^{13}C shift (II) is centrally located relative to the two doublet components at the ^{13}C chemical shift I, and vice versa.

Ptashne and G. Koudelka, Harvard University, Cambridge, MA. The cells were grown at 34 °C with aeration in 8.5 L of a minimal medium containing 10 g of $(NH_4)_2SO_4$, 90 g of K_2HPO_4, 42 g of KH_2PO_4, 50 g of glucose monohydrate, 20 mg of vitamin B1, 10 mg of biotin, 1 g of $MgSO_4 \cdot 7H_2O$, 30 mg of $FeSO_4 \cdot 7H_2O$, and 68 mg of tetracycline hydrochloride. After 150 min from the start of culture growth, 4 g of [$^{13}C_6$]glucose monohydrate (CIL, Cambridge) was added. After another 60 min, when the A_{600} of the culture was approximately 0.6, isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM, and growth was continued for 4 h. The cells were harvested by centrifugation and resuspended in 100 mL of lysis buffer containing 100 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 1.4 mM 2-mercaptoethanol, and 5% glycerol at pH 7.9. To this suspension we added 5 mL of 70–100 mesh glass beads and 5 mg of phenylmethanesulfonyl fluoride in 0.2 mL of dioxane. Cells were then lysed by sonication at 4 °C; the lysate was diluted with 200 mL of lysis buffer and centrifuged for 45 min at 8000 rpm in a GSA rotor of a Sorval centrifuge. The pellet was discarded and 18 mL of 10% poly(ethylenimine) in H_2O was added to the supernatant, which was stirred for 10 min at 4 °C. After centrifugation for 15 min at 7000 rpm with a GSA rotor at 4 °C, the pellet was again discarded and 160 g of ammonium sulfate was added to the supernatant. After being stirred for 45 min at 4 °C, the precipitate was collected by centrifugation at 8000 rpm for 45 min in a GSA rotor. The pellet was then resuspended in 150 mL of buffer A (50 mM Tris-HCl, 0.1 mM EDTA, 1.4 mM 2-mercaptoethanol, 10% glycerol at pH 7.9) plus 50 mM KCl and dialyzed against several changes of the same buffer. Following dialysis this material was loaded onto an S-Sepharose (Pharmacia) column equilibrated with buffer A plus 50 mM KCl. The 434 repressor was bound to the column and was then eluted with a linear gradient from buffer A plus 50 mM KCl to buffer A plus 600 mM KCl. The fractions containing the repressor, which was at this point approximately 70% pure, were passed through an Amicon 8200 ultrafiltration apparatus equipped with a Diaflo YM-10 membrane to obtain a final concentration of 0.4 mg/mL in buffer B (20 mM HEPES, pH 6.9, 0.1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol) plus 60 mM NaCl. The N-terminal domain of the 434 repressor (residues 1–69) was obtained through limited proteolytic digestion of this material (Anderson et al., 1984). Papain (Fluka, 2.2 units/mg activity) was added to the repressor solution under continuous stirring at room temperature to a final protease/protein ratio of 1:600, and the reaction was allowed to proceed for 2 h; antipain (Fluka) was then added in 100-fold excess with respect to papain to stop digestion. The resulting solution was applied to an S-Sepharose (Pharmacia) column equilibrated with buffer B plus 60 mM NaCl. The C-terminal fragment of the 434 repressor flowed through this column, whereas the N-terminal domain was retained. The latter was eluted with a linear gradient from buffer B plus 60 mM NaCl to buffer B plus 400 mM NaCl. The fractions containing the N-terminal domain 1–69 of the 434 repressor were shown to be greater than 90% pure. By use of a YM-2 Amicon Diaflo membrane the buffer was replaced with distilled water, and the protein was lyophilized.

The NMR sample was prepared by dissolving 7 mg of the ^{13}C-labeled N-terminal domain 1–69 of the 434 repressor in 0.45 mL of a ^2H_2O solution containing 25 mM KH_2PO_4 and 100 mM KCl at p^{2}H 6.0. The protein was again lyophilized from this solution and then redissolved in 0.45 mL of ^2H_2O.

NMR Measurements. All NMR measurements were performed at 28 °C. [^{13}C,^1H]-COSY spectra were recorded on a Bruker AM-600 spectrometer with the pulse sequence of Bodenhausen and Ruben (1980) extended by a 2-ms spin lock purge pulse (Otting & Wüthrich, 1988). To improve the suppression of t_1 noise originating from magnetization of ^{12}C-bound protons, which are incompletely relaxed during the preparation time between successive scans, the phase cycle given by Otting and Wüthrich (1988) was extended 2-fold by

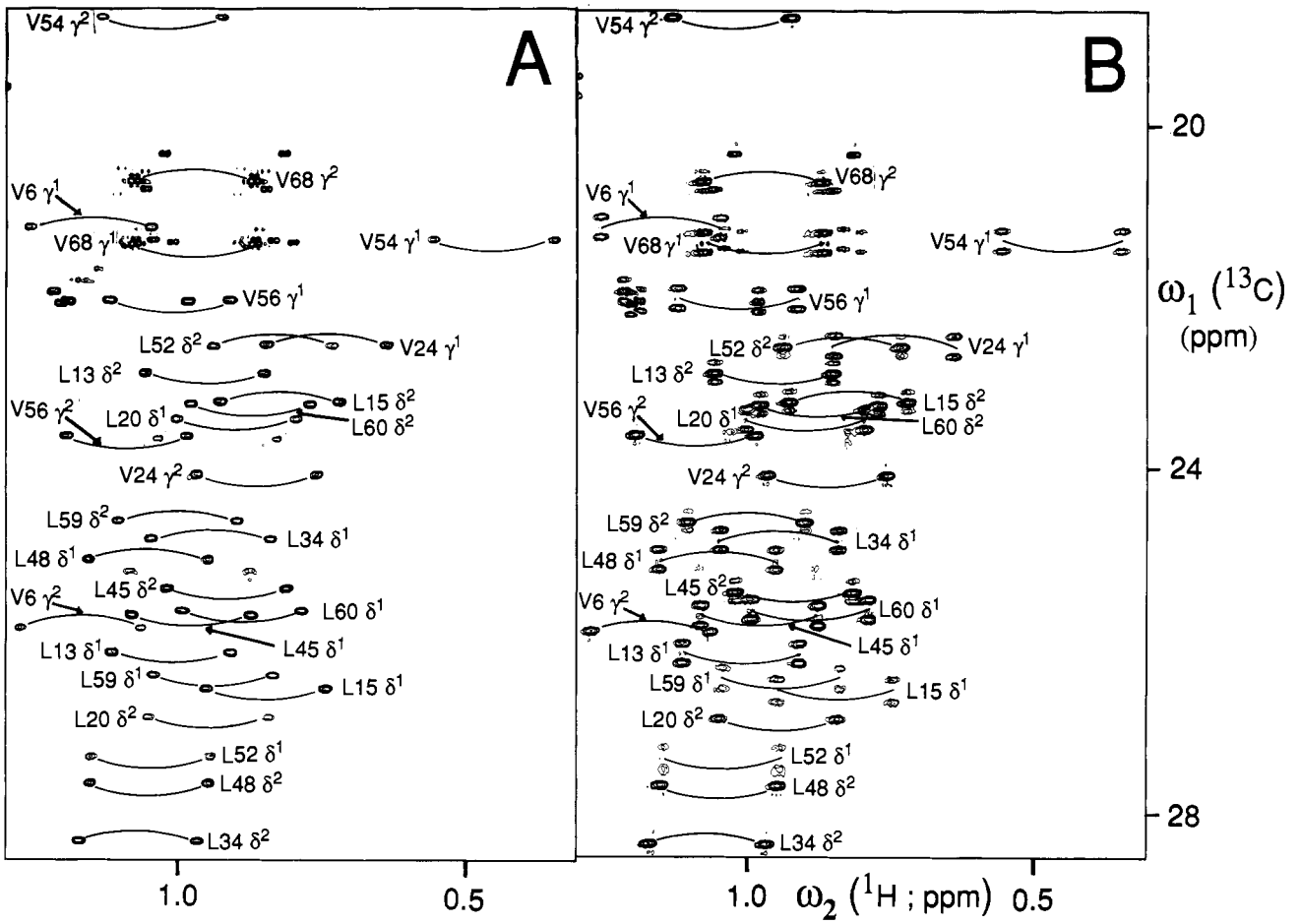

FIGURE 4: Spectral region of two [^{13}C,^{1}H]-COSY spectra of the N-terminal domain 1–69 of the 434 repressor at a proton frequency of 600 MHz containing the cross peaks between the directly bonded carbon and hydrogen atoms of the methyl groups of valine and leucine. (A) Natural abundance of ^{13}C, protein concentration 5 mM. (B) Biosynthetic fractionally ^{13}C labeled in the extent of 15%, protein concentration 2 mM. For each methyl resonance of Val and Leu the fine structure components separated by $^{1}J_{^{13}C,^{1}H}$ along ω_2 are connected by a curved line. The resonance assignments are given by the one-letter code for the amino acid, the sequence position, and the stereospecific identification of the methyl group according to the standard IUB–IUPAC nomenclature; γ^1 and δ^1 are *pro-R* and γ^2 and δ^2 are *pro-S*.

simultaneous permutation of the second 90° (^{13}C) pulse and the receiver phase (Wörgötter et al., 1988). Both spectra shown in Figure 4 were recorded and processed with identical parameters: $t_{1,max}$ = 102 ms; $t_{2,max}$ = 340 ms; total recording time about 60 h. Before Fourier transformation the time domain data were multiplied with sine-bell windows (De Marco & Wüthrich, 1976) along t_1 and t_2, with phase shifts of $\pi/5$ and $\pi/9$, respectively. The digital resolution after zero filling was 2.9 Hz along ω_1 and 1.5 Hz along ω_2.

A TOCSY-relayed [^{13}C,^{1}H]-COSY spectrum (Otting & Wüthrich, 1988) was recorded on a Bruker AM-500 spectrometer. The mixing sequence of the clean-TOCSY experiment (Griesinger et al., 1988) was used: $t_{1,max}$ = 49 ms; $t_{2,max}$ = 246 ms; total recording time about 58 h. Before Fourier transformation the time domain data were multiplied with sine-bell windows along t_1 and t_2, with phase shifts of $\pi/5$ and $\pi/9$, respectively.

Results and Discussion

By comparison of the ^{13}C satellites with the main peaks in a ^{1}H NMR spectrum, the extent of ^{13}C labeling in the 434 repressor 1–69 prepared as described in the preceding section was found to be about 15%. The methyl resonances of the five Val and nine Leu in this protein are all in the spectral region shown in Figure 4. At natural abundance of ^{13}C (Figure 4A), all methyl cross peaks have the fine structure of Figure 2A, as expected. The resonance assignments given in the figure are based on conventional sequential ^{1}H NMR assignments (D. Neri, G. Otting, and K. Wüthrich, unpublished results) and the experiment of Figure 5 below. In the labeled protein (Figure 4B) some of the methyl cross peaks have the four-component fine structure of Figure 2B, which identifies these methyls as γ^1 of Val or δ^1 of Leu. Figure 4 shows that all 28 ^{13}C–^{1}H methyl cross peaks of Val and Leu in 434 repressor 1–69 were resolved in [^{13}C,^{1}H]-COSY, and complete stereospecific assignments were obtained (Table I).

In Figure 4B the two-component multiplet patterns observed for the *pro-S* methyl groups are superimposed by a weak four-component multiplet indicative of the presence of about 2% of $^{13}C^{\delta 2}$–$^{13}C^{\gamma}$. This coincides with the expectations. Similarly, weak two-component signals are superimposed on the strong four-component peaks of the *pro-R* methyl groups. It can also be seen that the methyl ^{13}C chemical shift in $>^{12}CH-^{13}CH_3$ is always about 0.01 ppm downfield from the center of the [^{13}C]methyl doublet in $>^{13}CH-^{13}CH_3$, which is due to the isotope effect on the chemical shift (Hansen, 1983).

Figure 5 shows the methyl region of the TOCSY-relayed [^{13}C,^{1}H]-COSY spectrum of 434 repressor 1–69. The typical isopropyl patterns of Figure 3 are identified for all 14 Val and Leu residues. The resulting identification of the 14 methyl pairs is fully compatible with Figure 4, since in each case a *pro-R* methyl is correlated with a *pro-S* methyl group.

Conclusions

The presently described procedure for obtaining stereospecific assignments of the diastereotopic methyl groups of Val

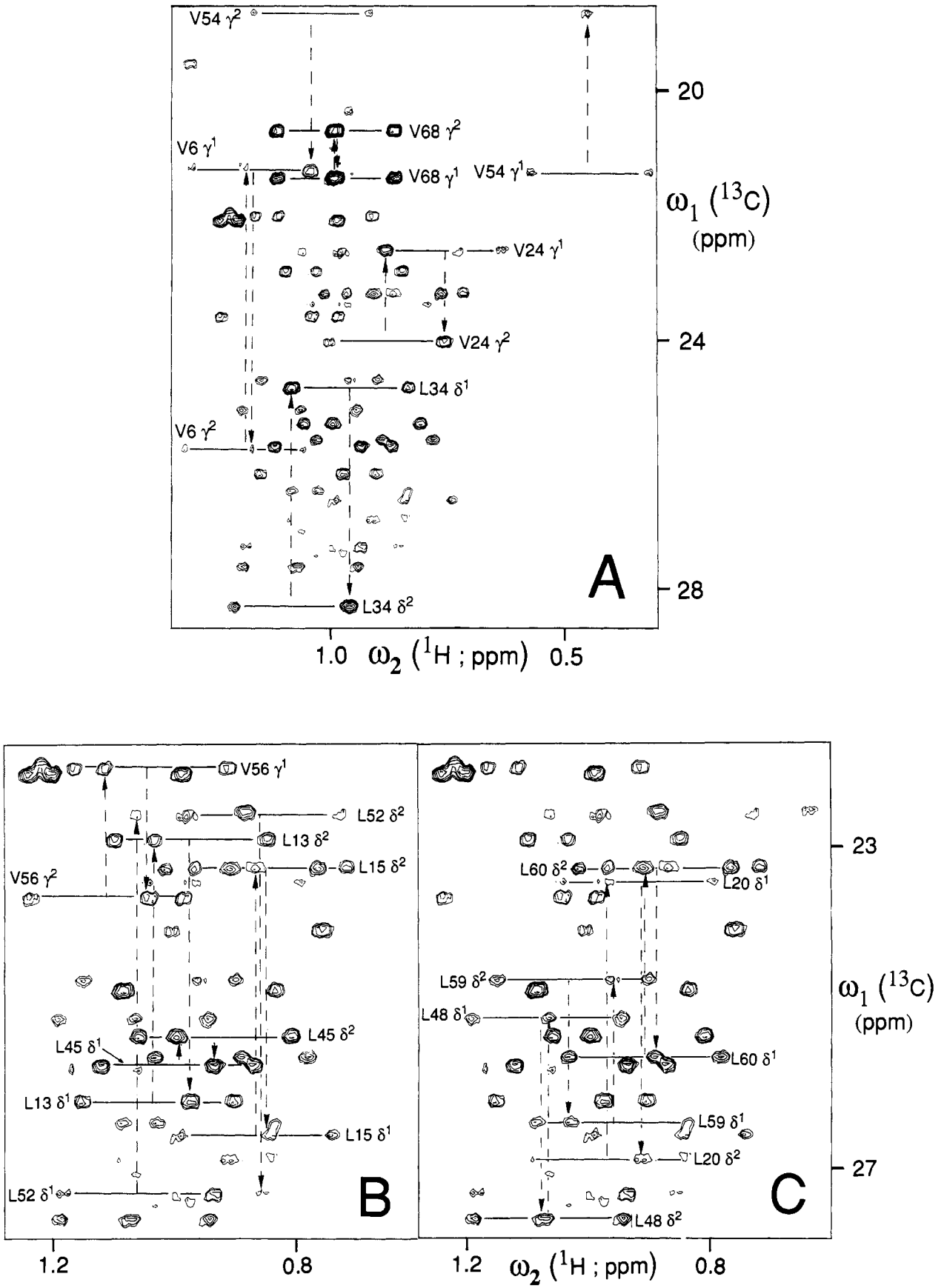

FIGURE 5: TOCSY-relayed [^{13}C,^{1}H]-COSY spectrum of the N-terminal domain 1–69 of the 434 repressor at 500 MHz. The same sample with natural ^{13}C abundance was used as in Figure 4A. (A) Spectral region containing the cross peaks relating the carbon-13 resonance of one methyl group to the proton signal of the other methyl group in the same Val or Leu residue. The solid horizontal and broken vertical lines outline the typical peak patterns for five isopropyl groups, as explained in Figure 3. For improved clarity, arrows pointing at the double-relayed cross peaks have been added to the broken lines. (B and C) The region (ω_1 = 22–28 ppm, ω_2 = 0.6–1.3 ppm) from spectrum A replotted on an expanded scale. The peak patterns leading to the identification of the remaining nine isopropyl groups of Val and Leu in 434 repressor 1–69 are indicated as in (A).

Table I: Chemical Shifts and Stereospecific Assignments Obtained for the ^{13}C and ^{1}H Resonances of the Methyl Groups of Valine and Leucine in the N-Terminal Domain 1–69 of the 434 Repressor

amino acid		chemical shift[a] ^{13}C	chemical shift[a] ^{1}H	amino acid		chemical shift[a] ^{13}C	chemical shift[a] ^{1}H
Val-6	γ^1	21.3	1.15	Leu-20	δ^1	23.5	0.89
	γ^2	25.9	1.16		δ^2	26.9	0.95
Val-24	γ^1	22.6	0.75	Leu-34	δ^1	24.8	0.94
	γ^2	24.1	0.88		δ^2	28.3	1.07
Val-54	γ^1	21.3	0.46	Leu-45	δ^1	25.7	0.98
	γ^2	21.2	1.04		δ^2	25.4	0.92
Val 56	γ^1	22.1	1.02	Leu-48	δ^1	25.1	1.05
	γ^2	23.6	1.09		δ^2	27.7	1.05
Val-68	γ^1	21.4	0.97	Leu-52	δ^1	27.3	1.05
	γ^2	21.7	0.97		δ^2	22.6	0.85
Leu-13	δ^1	26.2	1.02	Leu-59	δ^1	26.4	0.94
	δ^2	22.9	0.96		δ^2	24.6	1.00
Leu-15	δ^1	26.6	0.85	Leu-60	δ^1	25.6	0.89
	δ^2	23.3	0.83		δ^2	23.3	0.87

[a] The stereospecific assignments were obtained by using the nonrandom labeling experiments described in this paper. The *pro-R* methyl groups of Val and Leu are denominated γ^1 and δ^1, according to the standard IUB–IUPAC nomenclature; the *pro-S* methyl groups are denominated γ^2 and δ^2.

and Leu based on biosynthetic fractional ^{13}C labeling (Figure 1) is generally applicable for biosynthetic peptides and proteins. It is a special advantage of this approach that the assignments can be completed before the start of the structure calculations, and hence a reduction of the computation time can be expected to result as well. Since [$^{13}C_6$]glucose is quite expensive, we tried to minimize its use by adding it only 1 h before induction of the overexpression system (see Experimental Procedures). The result obtained indicates that, by careful selection of the time at which the labeled material is added to the cell culture, the technique can probably be further optimized with regard to reducing the amount of labeled glucose used.

In a previous paper the biosynthetically directed fractional labeling approach was used with the cyclic peptide cyclosporin A obtained from *Tolypocladium inflatum* (Senn et al., 1989). The experience gained there and in the present work shows that different NMR experiments will give optimal results with fractionally labeled biosynthetic peptides or proteins, respectively. For example, although in 434 repressor 1–69 the chemical shift dispersion was sufficiently large to enable a complete analysis of the methyl region in [^{13}C,^{1}H]-COSY (Figure 4), lack of conformation-dependent chemical shift dispersion (Wüthrich, 1986) in cyclosporin A prevented a detailed analysis of the same spectral region for this compound. Conversely, TOCSY-relayed [^{13}C,^{1}H]-COSY gave complete sets of cross peaks for the spin systems of all Val and Leu residues in cyclosporin A, so that data corresponding to those obtained here from Figure 4 could be extracted from the cross peaks $\gamma\underline{C}H_3$–$\alpha\underline{H}$ and $\delta\underline{C}H_3$–$\alpha\underline{H}$, respectively (Senn et al., 1989). With the 434 repressor, the sensitivity of this same experiment was sufficient to observe the methyl–methyl correlations within the isopropyl groups (Figure 5), but, presumably because of the shorter spin relaxation time T_2 when compared to cyclosporin A, the cross peaks δCH_3–αH were not observed for all Leu residues.

A comparative analysis of the crystal and solution structures of the protein Tendamistat (Billeter et al., 1989) has shown that, even at 2.0-Å resolution, the orientation of the isopropyl groups of Val and Leu about the C^α–C^β or C^β–C^γ bonds, respetively, is difficult to determine by X-ray crystallography. In contrast, once stereospecific assignments of the two methyls are available, a precise determination of the side-chain conformations of Val and Leu usually results from NMR measurements in solution. This analysis emphasizes further the importance of stereospecific assignments of the isopropyl groups for structural analysis of proteins by NMR spectroscopy.

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Articles

Identification by Proton Nuclear Magnetic Resonance of the Histidines in Cytochrome b_5 Modified by Diethyl Pyrocarbonate†

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ABSTRACT: Diethyl pyrocarbonate (DEP) is an electrophilic reagent that is used to modify reversibly the histidine residues of proteins. Unfortunately, the lability of the acylated histidine adduct usually does not permit the isolation and identification of the modified histidine. By use of 500-MHz proton NMR spectroscopy, it has been possible to identify the C-H resonances of the nonaxial histidines of trypsin-solubilized bovine, rabbit, and porcine cytochrome b_5 and therefore observe the interaction of DEP with specific histidine residues of cytochrome b_5. In addition, the pK_a of the peripheral histidines of bovine and rabbit cytochrome b_5 have been measured in D_2O. In the bovine protein it was found that the histidines are modified sequentially with increasing DEP concentration in the order His-26 > His-15 > His-80. This order is maintained in the rabbit protein with the following additions: His-26 ≈ His-27 > His-15 ≥ His-17 > His-80. The relative reactivity of the peripheral histidines with DEP was rationalized by considering three of their characteristics: (1) the pK_a of the histidine, (2) the fraction of the side chain exposed to the solvent, and (3) the hydrogen-bond interactions of the imidazole ring.

Cytochrome b_5, which exists in both soluble and membrane-bound forms, is a protein that plays an essential role in a variety of electron-transfer reactions including lipid biosynthesis (Oshino, 1978), cytochrome P-450 reduction (Hildebrandt & Estabrook, 1971; Canova-Davis & Waskell, 1984; Pompon & Coon, 1984), and regeneration of ferrous hemoglobin in red blood cells (Hegesh et al., 1986). In the cytochrome P-450 reaction, cytochrome b_5 can provide the second of the two electrons required by the mixed-function oxidase for a complete reaction cycle. The volatile anesthetic methoxyflurane, as well as numerous other compounds, has been shown to require cytochrome b_5 for its metabolism by cytochrome P-450 (Canova-Davis & Waskell, 1984; Noshiro et al., 1979; Kuwahara & Omura, 1980; Sugiyama et al., 1979; Okita et al., 1981; Vatsis et al., 1982; Gruenke et al., 1988; Pompon, 1987; Lipka & Waskell, 1989). In studies designed to delineate the mechanism of the cytochrome b_5 requirement for the oxidation of selected substrates by cytochrome P-450, it was found that treatment of cytochrome b_5 with diethyl pyrocarbonate (DEP), a moderately selective histidine-modifying reagent (Miles, 1977), could reversibly inhibit electron transfer to cytochrome P-450 (Canova-Davis & Waskell, 1984; Konopka & Waskell, 1988a). Potentially, identification of the amino acid residues responsible for the ability of cytochrome b_5 to transfer electrons to cytochrome P-450 could provide information about why certain substrates require cytochrome b_5 for their metabolism. Thus, studies to determine which specific histidine residues were modified were undertaken (Konopka & Waskell, 1988a,b).

One of the major drawbacks of DEP is that the carbethoxy histidine derivative is unstable during the procedures conventionally used to isolate and sequence peptides. As a result, it is frequently impossible to identify modified histidine residues, although with selected proteins the modified histidine has been identified (Hegyi et al., 1974; Igarashi et al., 1985; Cooper et al., 1987; Miles, 1977). The proton NMR spectra of proteins provide detailed structural information and are typically obtained under conditions not expected to hydrolyze the carbethoxy histidine derivative of proteins. In this paper, we demonstrate that 500-MHz high-resolution ^{1}H NMR

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