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STRUCTURE AND DYNAMICS IN PROTEINS OF PHARMACOLOGICAL INTEREST

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Abstract—This paper begins with a brief survey of the standard nuclear magnetic resonance (NMR) method for protein structure determination in solution, which has been applied successfully with numerous globular proteins with molecular weights in the range from 3,000 to 15,000. The results obtained show that for the core of globular proteins, the quality of the structures determined in solution can be comparable to that achieved with diffraction techniques using protein single crystals. In addition, they also indicate that a complete description of proteins in solution may include short-lived, transient structural features that could be of crucial importance for the functional properties. Several supplementary NMR techniques capable of characterizing diverse aspects of flexible polypeptide chains are discussed.

Polypeptide conformations are the result of a multitude of weak, non-bonding interactions among different atoms of the biopolymer chain, and between the biopolymer and the surrounding medium. At temperatures near 300°K, these "secondary bonds" are constantly broken and reformed. As a consequence of the resulting dynamic nature of the spatial molecular structures, these can readily adapt to changes of the surroundings, such as variation of the solvent medium. The packing interactions in protein crystals can also be included among these generalized solvent effects, and therefore it is of fundamental interest that nuclear magnetic resonance (NMR) spectroscopy in solution is now a valid alternative to diffraction measurements in single crystals for the determination of the three-dimensional structure of proteins at atomic resolution [1–5]. Besides the fact that the two methods use, respectively, protein crystals and proteins in solution or other noncrystalline states, the time scales of the two types of measurements are also widely different [1]. Overall, the additional availability of the NMR approach for structure determination thus promises to widen our view of protein molecules with regard to a better grasp of the relations between structure and function.

The introduction of NMR as a second method for protein structure determination at atomic resolution has already increased the number of known protein structures, since compounds can be studied for which no suitable single crystals for diffraction studies are available. This also includes polypeptides of direct pharmacological interest. For the field of Biochemical Pharmacology it is further of interest that the solution conditions used for structure determinations by NMR cannot only be chosen to be closely similar to the physiological environment of the polypeptide studied, but may also be varied over a wide range of conditions [1]. These may include, for example, solvent media used for storage and administration of the molecules of interest.

This article starts with a brief survey of the standard strategy for the use of NMR for protein structure determination [1, 2]. This will be followed by a discussion of supplementary NMR experiments for characterization of flexible portions of polypeptide chains.

Survey of the NMR method for protein structure determination

The foundations of the method are [1, 6]: (i) NMR experiments enabling the use of nuclear Overhauser effects (NOE) for measurements of proton–proton distances in native protein structures in the presence of spin diffusion [7, 8]. Initially, this was achieved with one-dimensional NMR experiments [7, 8], and the same principles were then used in conjunction with two-dimensional (2D) nuclear Overhauser enhancement spectroscopy (NOESY) [9]. In the future, the NOE data collection may benefit from the further improved spectral resolution of 3D NMR experiments [10–12]. (ii) The sequential resonance assignment strategy as an efficient technique for obtaining sequence-specific ^{1}H NMR assignments [13–16]. In principle this assignment strategy can rely entirely on ^{1}H NMR measurements [1], but the combination with isotope labeling and use of heteronuclear NMR experiments [17, 18] may increase significantly the potentialities for studies of more complex systems. (iii) Mathematical algorithms capable of computing three-dimensional protein structures from the individually assigned intramolecular distance constraints obtained from NOE experiments [1, 19]. The initial structure determinations [20–22] all used metric matrix distance geometry calculations [23–25]. Alternative techniques were subsequently added, including a variable target function method [26], the use of molecular dynamics calculations [5, 27], and an ellipsoid algorithm [28].

In present practice, the experimental data for a protein structure determination are collected using two-dimensional NMR [1, 2, 29]. The spectra of prime importance for work with proteins contain an array of diagonal peaks in the two-dimensional frequency plane (Fig. 1), with $\omega_1 = \omega_2$, which display the chemical shift positions of the resonance lines and resemble the conventional one-dimensional spectrum. In addition, there are a large number of cross peaks with $\omega_1 \neq \omega_2$. Through simple geometric

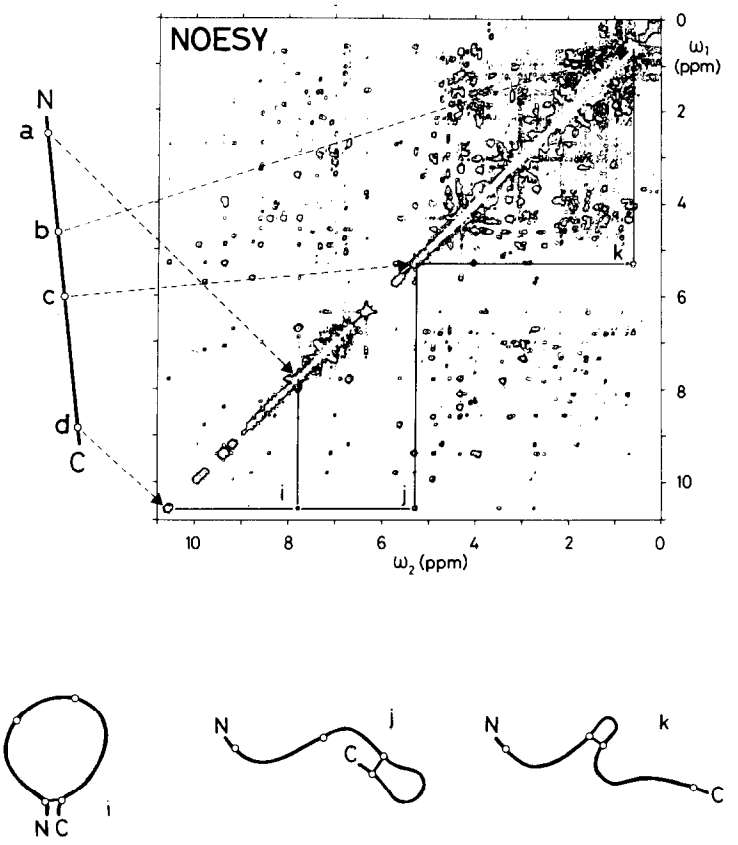

Fig. 1. Proton NOESY spectrum of the protein basic pancreatic trypsin inhibitor (BPTI). The spectrum was recorded at a Larmor frequency of 500 MHz. A contour plot is shown, with the two frequency axes ω_1 and ω_2. Three cross peaks are marked *i*, *j* and *k*, and connected by horizontal and vertical lines with the diagonal positions of the protons connected by the corresponding NOEs. The straight line on the left of the spectrum represents an extended polypeptide chain. N and C identify the chain ends, and four protons are identified by circles and the letters *a* to *d*. The broken arrows connect these protons with their diagonal peaks. Below the spectrum there is a schematic representation of three circular structures formed by the polypeptide chain, which are manifested by the NOESY cross peaks *i*, *j* and *k* (see text).

patterns each cross peak establishes a correlation between two diagonal peaks, as is indicated in Fig. 1 for the cross peaks labeled i, j and k. In NOESY the cross peaks represent NOEs and indicate that the protons corresponding to the two correlated diagonal peaks are separated by only a short distance, say less than 5.0 Å. In 2D correlated spectroscopy (COSY) the cross peaks represent scalar spin–spin couplings. For a protein a COSY cross peak thus indicates that the protons corresponding to the two correlated diagonal peaks are in the same amino acid residue, where they are separated by at most three chemical bonds. A large number of different homonuclear 2D NMR experiments yielding COSY-type through-bond ^{1}H-^{1}H connectivities or NOESY-type through-space ^{1}H-^{1}H connectivities are available [1, 29], and by combined use of a suitable selection of six to ten of these experiments a complete, or nearly complete delineation of the ^{1}H-^{1}H connectivities in a protein with molecular weight up to about 15,000 can usually be obtained. For larger proteins, or for small proteins with particularly complex ^{1}H NMR spectra the additional use of heteronuclear NMR experiments in conjunction with isotope labeling [1, 11, 12, 17, 18, 29] may be required to obtain the corresponding information.

At the outset of the structure determination the ^{1}H-^{1}H connectivities are used to obtain the sequence-specific resonance assignments, i.e. for the protons in the polypeptide chain the corresponding diagonal peaks are identified, as is illustrated in Fig. 1 for the four protons *a*, *b*, *c* and *d*. Once resonance assignments are available for all, or nearly all of the protons, the collection of the data needed as input for the determination of the three-dimensional structure is started. Each NOESY cross peak then tells us that two protons in known locations along the polypeptide chain are separated by a distance of less than approximately 5.0 Å in the folded protein. Since the overall length of the extended polypeptide chain of a protein is several hundred Ångstrøms, the NOEs may thus impose stringent constraints on the polypeptide conformation. This is illustrated at the bottom of Fig. 1 by the information contained in the three NOESY cross peaks *i*, *j* and *k*. The presence of the cross peak *i* in the spectrum shows that the distance between the protons a and d must be short, and hence the chain must form a circular structure.

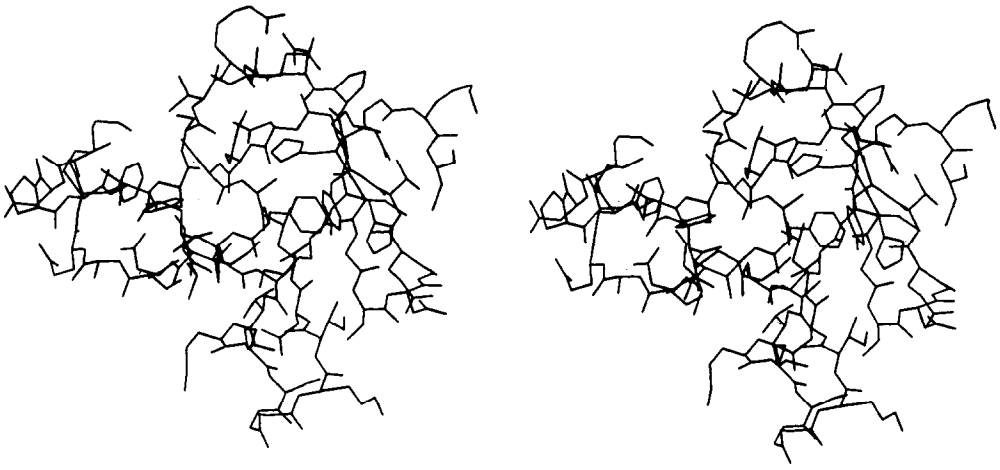

Fig. 2. Stereo view of the three-dimensional structure of the protein bull seminal proteinase inhibitor IIA determined from NMR measurements in aqueous solution [22]. All bonds connecting heavy atoms are drawn.

Similarly, the cross peaks *j* and *k* manifest the formation of smaller circular structures. A partial interpretation of these data can be based on empirical recognition of patterns of such circular structures corresponding to those expected for the common secondary structures in proteins [1, 14, 30], which results in the identification of the regular secondary structures contained in the protein. A more complete interpretation of the NMR data can be achieved with a computer search for protein conformations that are compatible with all experimental measurements and the steric constraints imposed by the covalent polypeptide structure, using one of the previously mentioned mathematical techniques [1, 5, 19, 20, 23–28]. In the preparation of the input for these structure calculations, more precise limits on the allowed distance ranges can be derived from the relative intensities of the NOESY cross peaks [1]. A complete description of the three-dimensional structure can thus be obtained in principle (Fig. 2), and has been obtained with various small globular proteins [e.g. Refs. 22, 31 and 32].

Some initial observations in protein structures determined by NMR in solution

Notwithstanding the fact that in a small number of cases different molecular architectures have been reported for the same polypeptide chain in single crystals and in solution [e.g. Refs. 21 and 33], it appears that many globular proteins have closely similar global molecular architectures in the two environments [e.g. Refs. 22, 32, 34 and 35]. For the α-amylase inhibitor Tendamistat a detailed comparison of the structures in solution and in crystals [35] showed that the spatial orientations of the polypeptide backbone and the interior amino acid side chains are nearly identical, and that for these parts of the molecule the quality of the NMR structure in solution is comparable to the refined crystal structure at 2.0 Å resolution. Significant structural differences occur near the protein surface, and generally the increase of structural disorder near the surface relative to the core of the molecule is more pronounced in the data obtained from the structure determination in solution than in the crystal structure. Similar observations were made with other globular proteins, and in several cases extended parts of the polypeptide chain appear to be "unstructured" and largely flexible in solution [e.g. Refs. 36–39].

The NMR method described in the preceding section, which relies critically on information on intramolecular distances obtained from NOE experiments, may meet its limitations in situations of either static or dynamic structural disorder, where complementary information will, in general, be needed for an unambiguous structural interpretation of the NOEs [1]. On the other hand, more precise characterization of the protein surface in solution by NMR is of special interest, since alternative techniques of investigation have fundamental limitations for this purpose. For example, the potential functions used for energy refinement of protein structures are less reliably calibrated for the molecular surface than for the protein core, and in crystal structures the protein surface is most directly affected by the crystal packing. Among the polypeptides of pharmacological interest there are entire classes of compounds that do not adopt a globular molecular architecture, for the simple reason that their size is too small. In these instances the aforementioned limitations of the NMR method may apply to the entire polypeptide chain in similar ways as they do to the flexible surface areas in globular proteins.

In the following sections, some NMR approaches are discussed that may be used in conjunction with NOE measurements to provide supplementary data to the NOE distance constraints in investigations of flexible segments of polypeptide chains.

NMR studies of the conformation of micelle-bound polypeptide hormones

Hormone functions have been found in a wide variety of polypeptides in the size range 3–35 amino acid residues. Although such molecules in solution may be inherently polymorphous, with rapid

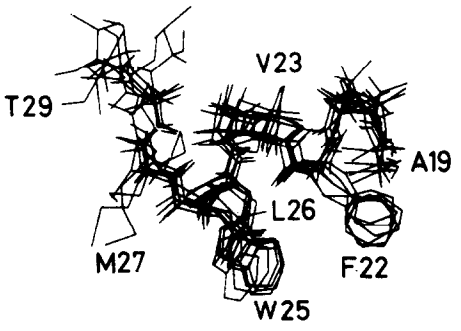

Fig. 3. Three-dimensional structure of the segment consisting of the residues 19–29 in the polypeptide hormone glucagon bound to the lipid–water interphase on the surface of perdeuterated dodecylphosphocholine micelles. Six conformers have been superimposed for minimal root mean square distance, each of which was computed with a metric matrix distance geometry algorithm from the same NMR data but with different starting conditions. The side chains of the residues Gln 20, Asp 21, Gln 24 and Asn 28 are not shown, since they were not constrained by the experimental data [21].

exchange between different limiting spatial structures, modern NMR experiments have in some cases resulted in identifications of preferred conformational species in polypeptides of this size [e.g. Refs. 40 and 41]. In view of the inherent flexibility of these molecules, more detailed structure determinations in solution may neither be feasible nor warranted on the time scales of NMR experiments, and in the rare cases where crystallization was achieved the physiological relevancy of the conformations seen in the crystal lattice may be in doubt. An alternative, physiologically interesting state of these compounds would be when they are bound to the surface of the target cells. However, it appears that polypeptides bound to membraneous preparations, such as lipid vesicles, are not amenable to high resolution NMR studies, presumably because of excessive line broadening. In this situation it is quite interesting that polypeptide hormones can be bound to lipid micelles, which may to some extent mimic the environment on the surface of a cell membrane. Several nonglobular polypeptides have, for example, been found to adopt unique monomolecular conformations when bound to dodecylphosphocholine micelles [21, 42–44]. With the use of perdeuterated micellar lipids or detergents, a detailed NMR structure determination is then feasible in much the same way as for globular proteins [45]. As an illustration, Fig. 3 shows the structure of the C-terminal undecapeptide of micelle-bound glucagon [21].

Identification of hydrogen bonds using pH titration of amide proton chemical shifts

Following the observation of large upfield as well as downfield chemical shifts of individual amide protons in acid-stable proteins when raising the pH value over the range from about 2 to 5 [e.g. Refs. 46 and 47], experiments with a series of oligopeptides established the usefulness of this observation for conformational studies of proteins [48, 49]. Figure 4 illustrates those observations in the model peptides that are of most direct interest for studies with proteins. Figure 4 shows that in the tetrapeptide H-Gly-Gly-Glu-Ala-OH, the amide protons of the residues in positions 3 and 4 both have large titration shifts. These shifts are of opposite sign, which shows that they are caused by different types of interactions with an ionizable group that is deprotonated in this pH range. Furthermore, two different pK_a values are observed for the two amide protons, which shows that the two titration shifts result from interactions with two different deprotonating groups. This is confirmed by the experiments of Fig. 4, b and c, which show that the titration shift of the amide proton of residue 3 is absent when Glu in this position is replaced by Gln, and that the amide proton titration shift of Ala 4 is absent when the C-terminal carboxylate is protected as the methyl ester.

The interpretation of the experiments in Fig. 4 [49] can be summarized as follows. The upfield shift of the amide proton of Ala 4 is the result of an inductive, "through-bond" effect from the deprotonation of the C-terminal carboxylate group. The downfield shift of the amide proton of Glu 3 results from "through-space" hydrogen bonding interactions with the deprotonated γ-carboxylate group of this residue. With regard to the use of such titration shifts for studies of the three-dimensional structure it is of interest that inductive effects arise not only for the amide proton of the C-terminal residue, but generally for nuclei separated by 4 or less covalent bonds from the deprotonated carboxyl group. In particular, such intrinsic titration shifts are observed for the ^1H and ^{13}C NMR lines of the side chain methylene groups of Asp and Glu [50, 51], so that the pK_a values of all carboxylate groups in a protein can be measured independently of the amide proton titrations [e.g. Ref. 52]. By comparison of the pK_a values for the downfield amide proton titration shifts with the intrinsic pK_a values for the individual carboxylates, *both* groups interacting in the NH-to-carboxylate hydrogen bonds can then be identified. Amide proton titration shifts are only the second type of NMR observation, besides nuclear Overhauser effects, for which both interacting groups can be directly identified.

Amide proton titration shifts are an interesting complementation of NOE measurements, since they can be observed also for short-lived transient structures. Thereby the extent of the titration shift, $\Delta\delta$ (Fig. 4), gives an indication of the population of the observed hydrogen-bonded structure [49]. Compared to the peptide in Fig. 4, much larger NH titration shifts of up to 1.4 ppm have been recorded for individual residues in proteins [36, 39, 46, 47], indicating a relatively low equilibrium population of the hydrogen-bonded form of the tetrapeptide. Both in small peptides and in proteins there is also evidence that individual carboxylate groups interact transiently with two or more amide protons [36, 49]. Using amide proton titration shifts, evidence has so far been accumulated for short-lived hydrogen-bonded forms of individual amino acid side chains near the surface, as well as for hydrogen bonding

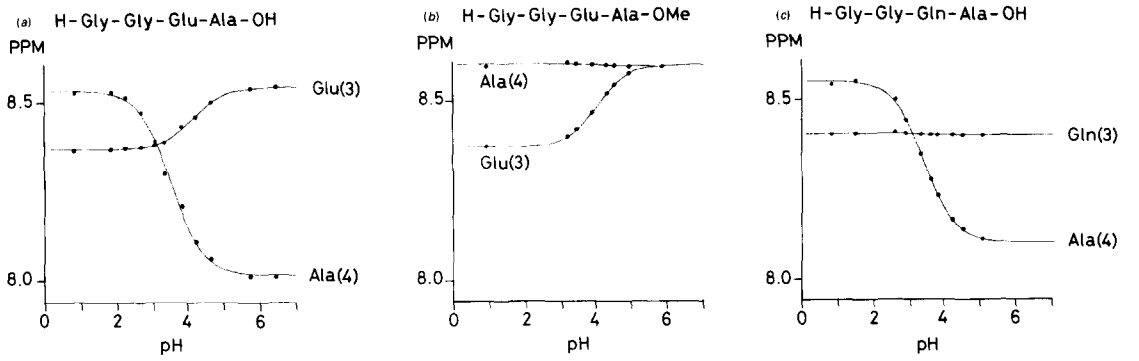

Fig. 4. The pH titration shifts at 25° of the amide protons of the residues in positions 3 and 4 in the three tetrapeptides (a) H-Gly-Gly-Glu-Ala-OH, (b) H-Gly-Gly-Glu-Ala-OCH_3, and (c) H-Gly-Gly-Gln-Ala-OH. The solid lines correspond to nonlinear least-squares fits of one-proton titration curves to the experimental data, which are represented by closed circles. The titration parameters for the residues in positions 3 and 4 are: (a) $\Delta\delta^3 = 0.17$ ppm, $pK_a^3 = 4.1$; $\Delta\delta^4 = -0.51$, $pK_a^4 = 3.5$; (b) $\Delta\delta^3 = 0.23$ ppm, $pK_a^3 = 4.0$; $\Delta\delta^4 = 0.0$; (c) $\Delta\delta^3 = 0.0$; $\Delta\delta^4 = -0.45$; $pK_a^4 = 3.4$ (reproduced from Ref. 49).

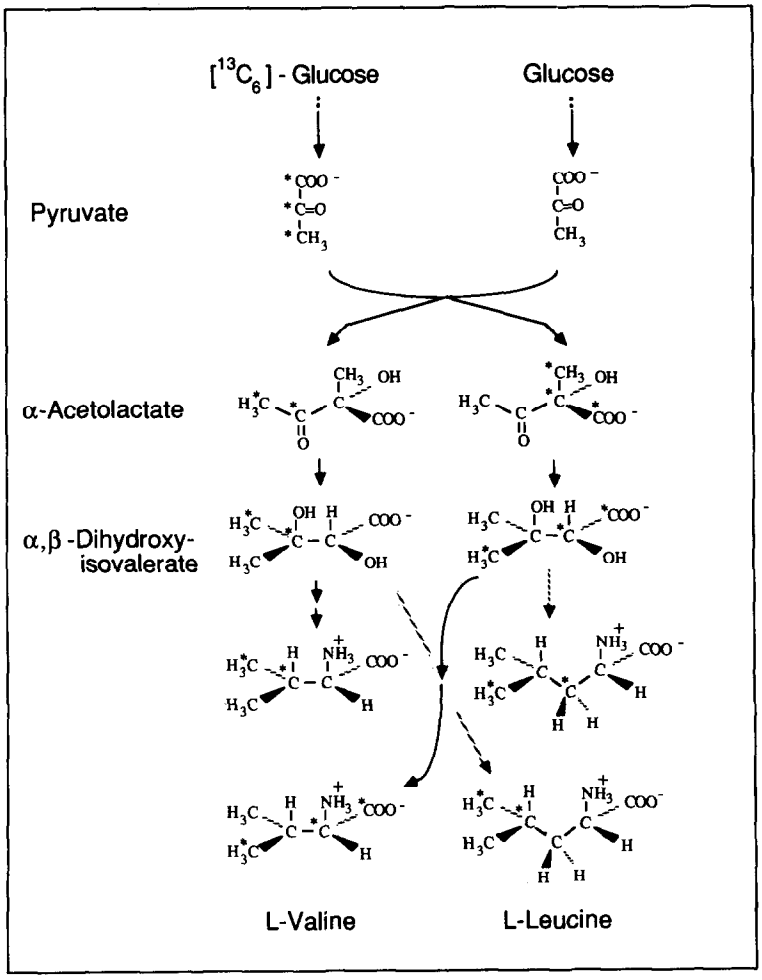

Fig. 5. Reaction pathways for the biosynthesis of valine and leucine from a mixture of fully ^{13}C-labeled and unlabeled glucose, showing the stereochemistry and the principal labeling patterns (an asterisk indicates a ^{13}C label; the absence of the asterisk indicates the natural ^{13}C abundance of 1.1%).

within the well-structured core regions of globular proteins [36, 47, 53].

Stereospecific ^{1}H NMR assignments of the methyl groups of valine and leucine by biosynthetically directed fractional ^{13}C-labeling

The methods available for obtaining sequence-specific ^{1}H NMR assignments in proteins [1, 13–16] do not usually provide stereospecific assignments for diastereotopic groups of protons in the amino acid side chains. To enable the preparation of an input for protein structure calculations [1, 2, 19, 22, 26, 31] in the absence of such stereospecific assignments, pseudoatom structures of the common amino acids have been introduced [54]. The use of these pseudo-structures, however, entails a significant loss of conformational information contained in the NMR data, and it has been clearly established that the availability of stereospecific assignments can improve significantly the quality of a NMR structure determination for a globular protein [55, 56]. Similarly, the situation for conformational characterizations of amino acid side chains on the protein surface or of parts of low molecular weight polypeptides that do not form globular structures may be improved. The following experiment enables individual assignments for the diastereotopic methyl groups of Val and Leu in proteins as well as in "unstructured" peptides, and this information is available prior to the start of a structure calculation.

The method described here 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 [57–59]. Thereby, as shown in Fig. 5, the isopropyl group is made up of a two-carbon fragment from one pyruvate unit, while the second methyl group is transferred from another pyruvate unit. This methyl migration has been shown to be stereoselective, and the migrating methyl group is pro-S in both valine and leucine, i.e. it is γ^2CH_3 or δ^2CH_3 respectively. In the process of biosynthetically directed fractional ^{13}C-labeling, microorganisms are grown on a minimal medium 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, whence this probability becomes 10%. These two distinct situations prevail for the isopropyl group in valine and leucine (Fig. 5). The pro-R methyl group (γ^1 and δ^1 respectively) and the adjacent >CH— group originate from the same pyruvate molecule so that, in the absence of isotope scrambling, there is a probability of 10% that both sites are ^{13}C-labeled in the same molecule. On the other hand, the pro-S methyl group and the adjacent carbon atom originate from two different pyruvate molecules (Fig. 5). Therefore, if the pro-S methyl group is enriched with ^{13}C, there is a probability of only 1% that the adoining >CH— group in the same molecule is also labeled.

The NMR distinction between the individual diastereotopic isopropyl methyl groups in a fractionally ^{13}C-labeled protein is readily achieved with 1D or 2D ^{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 neighbouring ^{13}C spin, while the ^{13}C NMR signal of the pro-S methyl group is a singlet. From ^{13}C the assignments are then readily transferred to ^{1}H by heteronuclear correlation experiments. Experiments that have been used for NMR studies of proteins with biosynthetic fractional ^{13}C-labeling include homonuclear [^{13}C, ^{13}C]-COSY [60], TOCSY-relayed [^{1}H, ^{13}C]-COSY [60, 61], and heteronuclear [^{1}H, ^{13}C]-COSY [62].

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