Overcoming the Overlap Problem in the Assignment of \(^1\)H NMR Spectra of Larger Proteins by Use of Three-Dimensional Heteronuclear \(^1\)H–\(^{15}\)N Hartmann–Hahn–Multiple Quantum Coherence and Nuclear Overhauser–Multiple Quantum Coherence Spectroscopy: Application to Interleukin 1\(\beta\)†

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ABSTRACT: The application of three-dimensional (3D) heteronuclear NMR spectroscopy to the sequential assignment of the \(^1\)H NMR spectra of larger proteins is presented, using uniformly labeled (\(\sim 95\%\)) \(^{15}\)N-interleukin 1\(\beta\), a protein of 153 residues and molecular mass of 17.4 kDa, as an example. The two-dimensional (2D) 600-MHz spectra of interleukin 1\(\beta\) are too complex for complete analysis, owing to extensive cross-peak overlap and chemical shift degeneracy. We show that the combined use of 3D \(^1\)H–\(^{15}\)N Hartmann–Hahn–multiple quantum coherence (HOHAHA–HMQC) and nuclear Overhauser–multiple quantum coherence (NOESY–HMQC) spectroscopy, designed to provide the necessary through-bond and through-space correlations for sequential assignment, provides a practical general-purpose method for resolving ambiguities which severely limit the analysis of conventional 2D NMR spectra. The absence of overlapping cross-peaks in these 3D spectra allows the unambiguous identification of \(\mathrm{C}^\alpha\mathrm{H}(i)–\mathrm{NH}(i+1)\) and \(\mathrm{NH}(i)–\text{NH}(i+1)\) through-space nuclear Overhauser connectivities necessary for connecting a particular \(\mathrm{C}^\alpha\mathrm{H}(i)–\text{NH}(i)\) through-bond correlation with its associated through-space sequential cross-peak. The problem of amide NH chemical shift degeneracy in the \(^1\)H NMR spectrum is therefore effectively removed, and the assignment procedure simply involves inspecting a series of 2D \(^1\)H–\(^1\)H slices edited by the chemical shift of the directly bonded \(^{15}\)N atom. Connections between residues can be identified almost without any knowledge of the spin system types involved, though this type of information is clearly required for the eventual placement of the connected residues within the primary sequence. Strategies for obtaining identification of spin system types include traditional analysis of the spectrum of nonlabile protons, site-directed mutagenesis, and specific labeling of selected amino acids. It is envisaged that the intrinsic simplicity of the 3D heteronuclear spectra, even for proteins of 150–200 residues, will permit the development of efficient computer-assisted or automated sequential assignment methods.

The extension of nuclear magnetic resonance (NMR)\(^1\) experiments from one to two dimensions [see Ernst et al. (1987) for a review] has played a key role in the development of methods for the determination of three-dimensional structures of macromolecules in solution. The first step of such structure determinations is the sequential assignment of the \(^1\)H NMR spectrum by means of experiments that identify through-bond and sequential through-space connectivities. By spreading the resonances into two frequency dimensions, the concomitant increase in both resolution and information content enables these connectivities to be readily analyzed. To date, the spectra of a substantial number of small proteins (<100 residues) have
been completely assigned, and in some cases their three-dimensional structures have been determined [see Wüthrich et al. (1986, 1989), Clore and Gronenborn (1987, 1989), and Bax (1989) for reviews].

Despite these tremendous advances, the limitations in the application of purely 2D homonuclear experiments to larger proteins containing more than about 100 residues become rapidly apparent. First, the increase in the number of resonances leads to severe crowding and chemical shift degeneracy in the two-dimensional spectra, resulting in unresolvable ambiguities in the identification of both sequential and long-range NOEs. Second, the accompanying increase in the rotational correlation time leads to short $T_2$ and $T_1$, relaxation times and to an increase in line widths. As all scalar correlation experiments (e.g., COSY, multiple quantum, and HOHAHA) are limited to cases where the line widths are not significantly greater than the size of the $J$ couplings, the identification of complete amino acid proton spin systems becomes increasingly difficult as the size of the molecule increases.

In the last few years a number of experiments involving isotope labeling have been proposed to partially resolve these problems. Thus, for example, proton line widths can to some extent be reduced by the random incorporation of deuterium at about the 80% level (LeMaster, 1987; LeMaster & Richards, 1988; Torchia et al., 1988a). An alternative procedure which also results in spectral simplification makes use of $^{15}$N-detected heteronuclear multiple quantum correlation (HMQC) experiments to establish one-bond correlations between protons and a directly bonded $^{15}$N or $^{13}$C atom (Mueller, 1979; Bax et al., 1983; Redfield, 1983; Markley et al., 1984; Roy et al., 1984; Sklenar & Bax, 1987a; Glushka & Cowburn, 1987), combined with isotope-edited NOE experiments (Wilde et al., 1986; Griffey & Redfield, 1987; Rance et al., 1987; Bax & Weiss, 1987; Senn et al., 1987; Fesik et al., 1987). These experiments rely on the fact that the $^1$H–$^{15}$N and $^1$H–$^{13}$C couplings are large (90 and 125–160 Hz, respectively) and have met with considerable success in the case of both T4 lysozyme (McIntosh et al., 1987a,b) and staphylococcal nuclease (Torchia et al., 1988b, 1989). Unfortunately, specific labeling is both expensive and technically difficult as it may require the use of axotrophic strains and further necessitates numerous sample preparations as well as the recording of a very large number of spectra. For this reason, efforts have been made to develop more general-purpose strategies.

One such approach involves completely labeling the protein with $^{15}$N and recording 2D relayed $^{15}$N–$^1$H HMQC–COSY, HMQC–HOHAHA, and HMQC–NOE spectra (Gronenborn et al., 1989a,b; Shon & Opella, 1989). In this manner, $^1$H–$^1$H correlations involving NH protons are related to the chemical shift of the directly bonded $^{15}$N atom. For smaller proteins it is relatively rare to find that both the $^1$H and $^{15}$N chemical shifts of two NH groups are degenerate, and hence, these heteronuclear experiments can be used in combination with the conventional homonuclear ones to resolve ambiguities. Recently, this has been applied to the sequential assignment of the helical DNA binding protein NER where it proved indispensable for resolving ambiguities arising from severe overlap and limited spectral dispersion (Gronenborn et al., 1989b). The power of these experiments lies in overcoming uncertainties in the assignment caused, in particular, by overlap of the NH proton resonances. For proteins larger than about 130 residues, however, it is exactly a region comprising the NH proton resonances, the NH–$^1$H fingerprint region, where one again not all correlations can be recognized because of severe multiple cross-peak overlap.

In contrast, when 2D NMR is extended into a third dimension, cross-peak overlap is largely removed. Any 3D NMR experiment can be conceived by simply combining two 2D NMR experiments, leaving out the detection period of the first and the preparation period of the second (Griesinger et al., 1987). The first applications to proteins involved homonuclear 3D experiments of the HOHAHA–NOESY type (Oschkinat et al., 1988, 1989; Vuister et al., 1988). While elegant, the applicability of the homonuclear 3D experiment to large proteins is limited as the efficiency of the scalar correlation step is severely reduced with increasing line widths. A more useful set of 3D experiments suggests itself by simply converting the 2D relayed HMQC experiments into corresponding 3D experiments. To date, the only 3D heteronuclear experiments reported for proteins have been the HMQC–NOESY (Fesik & Zuiderweg, 1988) and NOESY–HMQC (Bax et al., 1988; Marion et al., 1989; Zuiderweg & Fesik, 1989) ones. The application of the second experiment to staphylococcal nuclease (Marion et al., 1989) and the anaphylatoxin C5a (Zuiderweg & Fesik, 1989) clearly demonstrated their potential power for spectral simplification as they effectively generate a series of two-dimensional NOESY spectra edited by the chemical shift of the directly bonded $^{15}$N atoms.

With respect to sequence-specific assignment of protein $^1$H NMR spectra, no report has yet been published of the application of 3D NMR to systems where 2D spectroscopic methods have not already proved successful. The 3D HMQC–NOESY or NOESY–HMQC experiment on its own is insufficient for carrying out sequence-specific assignments as there is no simple means of separating out intra- from interresidue effects. Moreover, distinguishing these two effects by reference to a 2D COSY or HOHAHA experiment is difficult in the presence of severe spectral overlap of the NH resonances. Consequently, in those cases that necessitate the use of 3D heteronuclear experiments, a 3D HOHAHA–HMQC or COSY–HMQC experiment is also essential.

In this paper we show for the first time that the combination of three-dimensional $^{1}$H–$^{15}$N HOHAHA–HMQC and NOESY–HMQC experiments, designed to provide the through-bond and through-space connectivities necessary for the sequential assignment procedure, greatly facilitates the sequence-specific assignment of the $^1$H NMR spectrum of interleukin 1$\beta$, a protein of 153 residues. This particular protein is an important component of the immune and inflammatory response systems and, in addition, is directly cytotoxic for certain tumor target cells [see Oppenheim et al. (1986) for a review]. Although there have been a large number of functional studies on interleukin 1$\beta$, little is known about its detailed mechanism of action. Our own interest is aimed at solving its three-dimensional structure in solution as a first step toward obtaining a better understanding of structure/activity relationships of interleukin 1$\beta$.

**Materials and Methods**

**Interleukin 1$\beta$ Preparation and $^{15}$N Labeling.** Interleukin 1$\beta$ was purified from *Escherichia coli* harboring the expression vector derived from pPL24 (Buell et al., 1985) in which interleukin 1$\beta$ is expressed under control of the bacteriophage $\lambda$ promoter and the ribosome binding site of the bacteriophage Mu mer gene (Wingfield et al., 1986). Uniformly ($\sim$95%) $^{15}$N-labeled protein was prepared from bacteria grown in minimal medium with $^{15}$NH$_4$Cl as the sole nitrogen source and purified as described previously (Wingfield et al., 1986).

The spectra were recorded on a 2 mM sample dissolved in 150 mM sodium phosphate buffer, 90% H$_2$O/10%D$_2$O, and 0.5 mM sodium azide, pH 7.5, and at a temperature of 27 °C.
Under these conditions at least 120 amide NH peaks can be detected in a regular 2D $^{15}$N–H HMQC experiment.

**NMR Spectroscopy.** The 3D NOESY–HMOC and HOHAHA–HMOC spectra were recorded on Nicolet NT-500 and Bruker AM600 spectrometers, respectively. Processing of the 3D spectra was carried out on a SUN-4 computer using a simple in-house written routine for the $F_2$ Fourier transformation (Kay et al., 1989) combined with commercial 2D software (NMR2 from New Methods Research Inc., Syracuse, NY) for the $F_1$–$F_2$ 2D Fourier transformation.

In addition to the 3D spectra, conventional homonuclear 2D HOHAHA (Bruinscweiler & Ernst, 1983; Davis & Bax, 1985) and $^{15}$N-edited NOESY (Mcintosh et al., 1987a; Senn et al., 1987; Fesik et al., 1988) spectra were also recorded on a Bruker AM600 spectrometer. Water suppression in the $^{15}$N-edited NOESY spectrum was achieved with a 1–1 echo read sequence (Sklenar & Bax, 1987b). Water suppression in the HOHAHA spectrum was achieved with a 90° flip back pulse, a fixed recovery delay set to half the mixing time to compensate for rotating-frame Overhauser effects, and the “jump-return” sequence placed after the WALTZ-17 mixing sequence (Bax et al., 1987). $^{15}$N decoupling in $F_2$ was achieved with a WALTZ-16 decoupling sequence (Shaka et al., 1983) during the acquisition time $t_2$.

**RESULTS AND DISCUSSION**

**3D HOHAHA–HMOC and NOESY–HMOC Experiments.** In this section we first describe the two $^1$H–$^{15}$N 3D experiments and highlight those practical features that are important for obtaining high-quality spectra.

The general form of the 3D heteronuclear experiment is illustrated along with the HOHAHA–HMOC and NOESY–HMOC pulse sequences in Figure 1. The principles of the NOESY–HMOC experiment have been described in detail (Kay et al., 1989) and are for the most part directly transferable to the HOHAHA–HMOC experiment. The two sequences have in common a DANTE-type off-resonance saturation of the $H_2O$ signal, a nonselective $^1$H 90° preparation pulse, a $t_1$ evolution period, respective mixing period schemes for $^1$H–$^1$H NOE or HOHAHA transfer, the $^1$H–$^{15}$N HMOC filter which incorporates the $t_2$ evolution time, and finally the detection period during which the signal is recorded as a function of $t_3$. In both these schemes all $^1$H and $^{15}$N resonances are recorded in $F_1$ and $F_2$, respectively, while the NH region is recorded in $F_3$. The $F_1$($^1$H–$^1$H) projection corresponds to the $F_1$($^1$H–$^{15}$N) region of a regular $^1$H–$^{15}$N NOESY or HOHAHA spectrum and thus ensures that NH–C$^4$H connectivities can be easily observed very close to the water resonance. In the $F_1$(NH–$^{15}$N) region of a 2D-NOESY spectrum, a width of at least 0.5 ppm centered about the water is unusable due to a large band of $t_1$ noise. The $F_1$($^1$H–$^{15}$N) projection corresponds to the 2D NOESY–HMOC spectrum (which can only be recorded with $^1$H in $F_2$ and $^{15}$N in $F_3$) and the $F_1$($^{15}$N–$^1$H) projection to the 2D HMOC spectrum.

The minimal phase cycling for both 3D experiments involves four steps, two for axial peak suppression in $F_1$ and two for the $^{15}$N filter. Using twice the minimum number of scans (eight), we find it is convenient to record each 3D spectrum over a total period of 3–5 days.

The HOHAHA mixing period comprises a 1.5-ms trim pulse along the $y$ axis of the rotating frame, followed immediately by a $^1$H 90° pulse that returns the magnetization to the $z$ axis. The WALTZ-16 sequence (Shaka et al., 1983) causes isotropic mixing of this magnetization, and the final $^1$H "read" 90° pulse returns the magnetization to the transverse plane. The incorporation of a fixed delay immediately before and after the WALTZ-16 pulse train is used to remove rotating-frame NOE effects in a manner analogous to that described by Griesinger et al. (1988). These delays additionally allow time to switch the transmitter offset for the mixing sequence to a frequency that maximizes the efficiency of the HOHAHA magnetization transfer between the amide NH and C$^4$H protons (~1.3 ppm downfield of the $H_2O$ resonance at 6.0 ppm).

One significant difference in the application of these two experiments is the use in the NOESY–HMOC sequence of an off-resonance 1–1 type read pulse (Clore et al., 1983). This is used to help suppress the residual $H_2O$ magnetization present at the end of the mixing time and allows the power of the presaturation pulse train to be minimized. In the case of the HOHAHA–HMOC experiment, mild preirradiation together with the dephasing of the water signal that occurs during the
The only difference with regard to the 3D experiments is that connections between one residue and the next must be made not only between different sets of peaks but between different planes of the spectrum as well. The major advantage, however, is that resolution of overlapping cross-peaks into three-dimensions removes most of the ambiguities present in the 2D spectra. Moreover, the 3D experiments are recorded as a set of 2D $^1$H-$^1$H ($t_1$, $t_2$) experiments, with the $t_2$ delay incremented after the acquisition of each 2D data set. This makes for straightforward programming of the sequence and subsequent data processing.

**Application to Interleukin 1β**. Interleukin 1β, the focus of the current study, is a protein with 153 amino acids and a molecular mass of ~17.4 kDa. A low-resolution X-ray structure (3 Å) has recently been published and has shown that it is entirely composed of $\beta$-sheets connected by loops (Priestle et al., 1988). As a subject for NMR studies, interleukin 1β is at the top end of the molecular weight range for which it appeared possible to obtain sequence-specific assignments by conventional 2D homonuclear techniques. Figure 3A, however, shows that the fingerprint region of the 2D $^{15}$N-edited NOESY and $^{15}$N-decoupled HOHAHA spectra of this protein is very complex, with many overlapping cross-peaks. The spectrum does not yield easily to the traditional 2D sequential assignment strategy. For example, we encounter limiting temperature (<40 °C) and pH (pH >4.3) conditions suitable for the study of the protein. In addition, the width of individual resonances appears to be rather large when compared to those of some proteins of similar size (e.g., hen egg white lysozyme and flavodoxin). This factor has limited our ability to identify relayed correlations from the NH protons with HOHAHA spectroscopy and hence has hindered spin system identification.

Figure 3B shows two corresponding slices at $\delta(F_2) = 123.7$ (15$^N$) ppm taken from the 3D NOESY–HMQC and 3D HOHAHA–HMQC spectra of a 2 mM sample of uniformly labeled (~95%) 15$^N$interleukin 1β at pH 7.5 and 27 °C. Alongside each slice of the 3D experiments, the corresponding regions of the corresponding homonuclear 2D 15$^N$-edited NOESY and 15$^N$-decoupled HOHAHA experiments are plotted. Note that the slices from the 3D experiment exhibit very good sensitivity and are essentially devoid of cross-peak overlap. More importantly, the combination of the 3D NOESY–HMQC and HOHAHA–HMQC experiments allows unambiguous identification of the NOEs that originate from a given amide NH and their connection with the C$^\alpha$H proton of that residue, as indicated by the dashed lines in Figure 3B for four well-resolved cross-peaks arising from the amides of Val-41, Leu-73, Glu-83, and Glu-149. These lines connect the intraresidue NH(i)--$^1$H(i) HOHAHA cross-peak with the interresidue sequential NH(i)--$^1$H(i−1) NOE cross-peak. Knowledge of the through-bond NH(i)--$^1$H(i) connectivity from the HOHAHA–HMQC experiment eliminates the difficulty of obtaining its location in the 3D NOE–HMQC spectrum by reference to 2D HOHAHA and HMQC–HOHAHA spectra. This is especially important for the case of $\beta$-sheet structure since the intraresidue C$^\alpha$H(i)--NH(i) NOE is often weak or absent. The almost complete absence of overlap in the whole of the 3D spectra of interleukin 1β means that any degeneracy of the $^1$H shifts for the amide NH protons is essentially eliminated: in this particular case, virtually no NH groups with degenerate $^1$H and 15$^N$ chemical shifts are found. In principle, problems of chemical shift degeneracy can potentially be overcome in 2D spectroscopy by varying the experimental conditions of pH and temperature.

trim pulse is sufficient. (Note that no H$_2$O recovery occurs during the WALTZ-16 mixing period.)

In recording and presenting the 3D experiments, an effort has been made to retain the characteristics of the corresponding 2D experiments. Thus the spectra obtained can be regarded as a "stretched-out" 15$^N$-edited spectrum with each $^1$H--$^1$H plane representing the identical region covered by the 2D experiment, only containing many fewer cross-peaks (Figure 2). In this way the normal rules for making the sequence-specific assignment of residues can be readily applied.
In the case of large proteins, however, this may simply result in shifting NH degeneracies from one set of spin systems in the 2D spectrum to another set. At the same time different sets of peaks become lost in the ridge created by the residual solvent resonance at the $F_1 = 0$ frequency. This can be very difficult to keep track of though it may be facilitated by computer bookkeeping algorithms (Billeter et al., 1988; Weber & Mueller, 1989). With 3D spectroscopy, NH degeneracies and the corresponding scalar and NOE connectivities can be resolved by inspection of a single 3D spectrum. For example, two spin systems in the spectrum of interleukin 1β which have identical NH and C=H chemical shifts and therefore directly overlapping (NH, C=H) cross-peaks in HOHAHA and COSY spectra are readily resolved in two widely separated planes of the 3D spectra. In particular, the NH(i)--C=H(i) intraresidue cross-peaks of Val-72 and Thr-79 are exactly superimposed in the 2D experiments at $\delta = 8.75$ (NH) and 4.60 (C=H) ppm under the experimental conditions used here. Raising the temperature causes these peaks to disappear into the water ridge line at the $F_1 = 0$ frequency, and prior to recording the 3D spectrum, they could not be resolved at all in the 2D spectra. The corresponding $^{15}$N chemical shifts are 116.8 and 119.7 ppm, respectively, five planes apart in the 3D experiments.

Given the tremendous simplicity of the slices of the 3D spectra, it is a relatively straightforward process to make the sequential assignment by hopping from one pair of HOHAHA/NOESY planes to another pair, connecting them via either C=H(i)--NH(i+1) or NH(i)--NH(i+1) NOEs exactly as for the analysis of 2D spectra. This hopping is illustrated for the stretch of residues from Ser-45 to Gln-39 in Figure 4.

Consecutive stretches of sequential NH(i)--NH(i+1) NOE connectivities in interleukin 1β are readily interpreted from the 3D NOESY–HMQC spectrum, as has been already documented for the anaphylatoxin C5a (Zuidervog & Fesik, 1989). In a 2D spectrum the NH(i)--NH(i+1) NOE cross-peaks are located symmetrically about the diagonal in a 2D plane. In the 3D spectrum, on the other hand, they are located symmetrically about the diagonal in 3D space. As the spectra are plotted here as planes perpendicular to the $F_2$ ($^{15}$N) coordinate, each NH(i)--NH(i+1) NOE connection appears in two planes, one peak on each side of the diagonal. Unambiguous identification of a consecutive stretch of NH(i)--NH-
(i+1) connectivities, particularly in a helical protein, depends less on a knowledge of the corresponding \textsuperscript{13}C chemical shifts, and hence is less dependent on the availability of a 3D HOHAHA–HMQC spectrum, than that for the predominant \textsuperscript{13}C(i)–NH(i+1) connections found for a \(\beta\)-sheet protein such as interleukin 1\(\beta\). Clearly, at the interface of stretches of NH(i)–NH(i+1) and \textsuperscript{13}C(i)–NH(i+1) connected spin systems the 3D HOHAHA–HMQC experiment is invaluable.

Because resonance overlap of both scalar and NOE NH–C\textsuperscript{13}C connectivities is also removed by the 3D \textsuperscript{13}C–\textsuperscript{1}H heteronuclear experiments, \textsuperscript{13}C\textsuperscript{1}H chemical shift determination can be achieved more accurately by 3D than by 2D, so that ambiguities arising from nearly degenerate \textsuperscript{13}C\textsuperscript{1}H resonances can still be overcome. One limitation, however, of the 3D heteronuclear assignment strategy presented in this paper is the presence of absolute \textsuperscript{13}C\textsuperscript{1}H chemical shift degeneracy. In general \textsuperscript{13}C\textsuperscript{1}H chemical shifts are less sensitive to experimental conditions such as temperature and pH than are amide NH shifts. In the case of 2D spectroscopy the ambiguity arising out of completely degenerate \textsuperscript{13}C\textsuperscript{1}H chemical shifts is addressed by the application of additional criteria to the connection of two spin systems, usually by reference to a knowledge of the spin systems types and their agreement with the protein sequence, or by the presence of \textsuperscript{13}C(i)–NH(i+1) NOEs. Unfortunately, for many larger proteins the identification of spin system types or the position of \textsuperscript{13}C\textsuperscript{1}H chemical shifts is rendered difficult for all but a small number of residues. In the case of interleukin 1\(\beta\), \textsuperscript{13}C\textsuperscript{1}H chemical shift identification has proved quite difficult. However, through the unambiguous identification of a small number of assignments from site-directed mutagenesis (Gronenborn et al., 1986; P. C. Driscoll, G. M. Clore, P. T. Wingfield, and A. M. Gronenborn, unpublished data), the identification of a few residues with characteristic side-chain coupling patterns (Gly, Ala, Ser, Thr, Val), and the unequivocal connections obtained from the analysis of the 3D spectra, the assignment of this “large” protein is now tractable.

Additional strategies for completing the assignment include the production of a selected number of specifically labeled samples (e.g., \([15\text{N}]\)Leu) interleukin 1\(\beta\) to resolve ambiguities still present in the 3D spectra. Thus, for example, there are 15 Leu residues widely dispersed in the sequence of interleukin 1\(\beta\) so that a sample of \([15\text{N}]\)Leu-labeled protein would provide 15 unambiguous assignments of residue type.

**CONCLUDING REMARKS**

We have shown in this paper that 3D heteronuclear NMR spectroscopy can be applied successfully to the assignment of the spectrum of a 153-residue protein. These 3D techniques are comparatively sensitive and can thus be applied to quite dilute samples by NMR standards (less than 2 mM and possibly as little as 0.5 mM). One can record 3D spectra in a reasonable amount of spectrometer time; the 3D HOHAHA–HMQC and NOESY–HMQC experiments together required less than 1 week of measuring time. The overlap of cross-peaks that makes the corresponding 2D spectra impossible to interpret is essentially completely removed. Though identification of spin system types is intrinsically difficult for this size of protein, the 3D spectra can be used as part of an overall assignment strategy, based on selective labeling, site-directed mutagenesis, or conventional NMR spectral analysis. The relative simplicity of the 3D spectra, along with the almost complete absence of overlap in the spectrum, will make them a good starting point for the application of computer-based approaches to automated assignment, even for quite large proteins.

**REFERENCES**


