A ^{1}H NMR Study on the Interaction of Aminoxyl Paramagnetic Probes with Unfolded Peptides

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The use of soluble spin labels to filter out the cross peaks of outer proton nuclei in 2D NMR spectra has been proposed as a general method to obtain structural information for complex molecules. Here the paramagnetic effects observed on backbone protons of an unfolded 27 amino acid peptide are discussed. The lack of any differential intensity change of the NH–Hα cross-peaks in TOCSY spectra is suggested as an additional general criterion for the identification of unfolded structures.

Structural information of complex molecules such as proteins are now routinely obtained *via* multidimensional NMR spectroscopy. The typical extent of chemical shift dispersion and scalar coupling values and the type of patterns on the amino acid spin systems are the basis for the first stage analysis of protein NMR spectra. Then, nuclear Overhauser effects are measured and, from the calculated cross-relaxation rates, a wealth of conformational restraints are obtained for subsequent model building procedures. Proton relaxation itself, in diamagnetic protein solution, is not very informative from a structural point of view, since for most nuclei it encodes an overall similar averaged contribution from surrounding protons. This contribution can very seldom be disentangled without the aid of selective 1D or multidimensional experiments.

It has been shown that solvent spin-labelling can remove to some extent the degeneracy in the proton relaxation process and that the paramagnetic effects induced on the longitudinal relaxation rates by the presence in solution of soluble aminoxyls are governed by the molecular structure.[1] More recently, the use of aminoxyls for solvent spin-labelling has been proposed as a paramagnetic relaxation filter of signal intensities of multidimensional NMR spectra of peptides[2] and proteins.[3,4] In the absence of specific interactions between these molecules and the paramagnetic probe, a random approach model can be suggested, since the observed spectral simplifications are strongly related to the solution conformations. In fact, the diagonal and cross-peak intensities of surface exposed nuclei have intensities which are much more reduced than the inner ones.

In the present study, the paramagnetic filtering effects observed on polypeptides which do not have defined conformations in solution are explored to delineate typical patterns of the aminoxyl induced perturbations on NH–Hα cross-peak intensities.

Camel β-endorphin has been used as a model system for unfolded polypeptides. Its molecular conformation in pure water is completely disordered and only in the presence of micelles[5,6] or in some organic solvents[7,8] can a α helical structure be observed.

The peptide, which contains 16 different amino acid residues, is also a remarkable molecular test for the occurrence of some preferential interaction of the paramagnetic probe with chemically different side chains.

Experimental

The 1–27 fragment of camel β-endorphin, YGGFMTSEKSQTPLVTLFKNAIIKNAH, was obtained from Peninsula Laboratories Inc. and used without further purification. The sample was 2 mmol dm^{-3} in H_2O:D_2O 95:5, at pH 6.3.

Experimental conditions for lysozyme and gramicidin S studies are described in refs. 2 and 4 respectively. The NMR spectra of the endorphin fragment, recorded in the presence of the spin label, were obtained by adding few cm^3 of a 2.0 mol dm^{-3} solution of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL, Sigma) to the 500 cm^3 peptide solution directly in the NMR tube, in order to obtain the desired 10:1 TEMPOL:peptide ratio.

^{1}H NMR spectra were acquired at 298 K on a Bruker AM 500 spectrometer, operating at 500.13 MHz, and the data were processed using the UXNMR software for the X32 workstation and FELIX from Biosym for the personal IRIS 4D-30. No internal standard for chemical shift measurements was used, to avoid corrections for differential effects of the free radical on the solute and the reference compound.[9] Chemical shifts were always referred to the computer memory location corresponding to the methyl resonance of Met-5 at 2.08 ppm[10] for β-endorphin.

For the Clean-TOCSY experiments,[11] a sweep width of 5000 Hz was used in both dimensions, 64 transients were collected for each of the 512 experiments, acquired with 2048 data points in the t_2 dimension. The solvent was suppressed with a 2.5 s presaturation for the period of the relaxation delay. A 17 ms spin-lock mixing train was employed, preceded and followed by a 1.5 ms trim pulse. The experimental data were weighted in both dimension with a cosine-bell function and zero-filled to 2 K in t_1 before Fourier transformation. All measurements in the presence and in the absence of the spin-label were performed in the same experimental conditions and the data were all processed with the same parameters to allow for quantitative comparison of peak intensities. Peak volumes were calculated with UXNMR and FELIX softwares. In Fig. 2, the attenuations are reported as variances with respect to the normalised mean value.

Results and Discussion

As already pointed out,[2,4] the analysis of paramagnetic relaxation effects is best performed using TOCSY type data, as

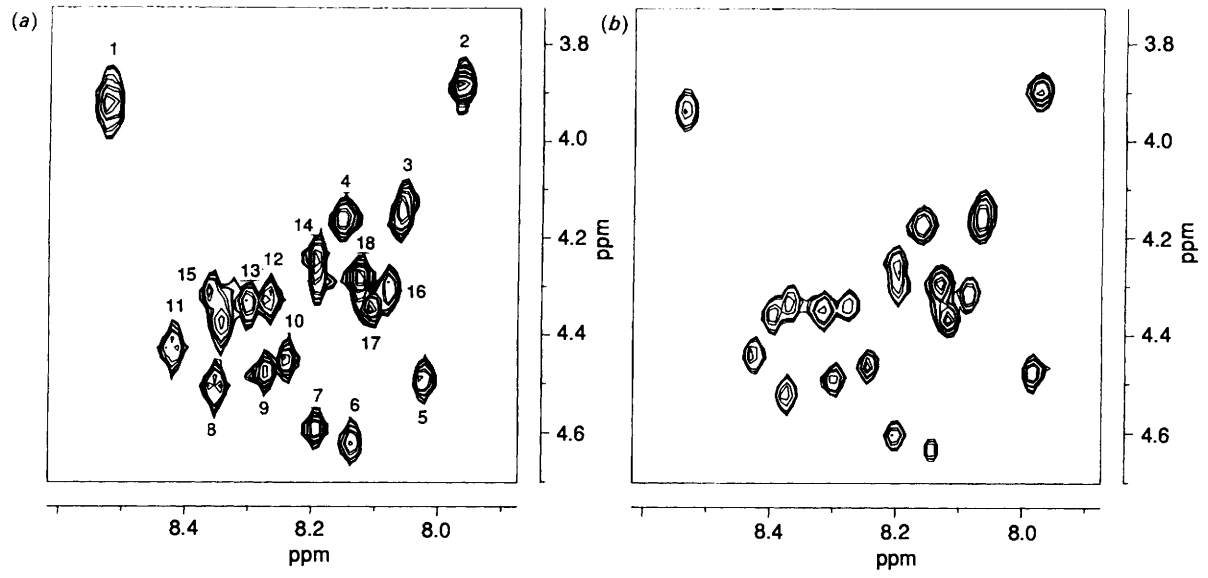

Fig. 1 The fingerprint region of the clean TOCSY 2D spectrum of the 1–27 β-endorphin fragment measured: (*a*) in the diamagnetic water solution and (*b*) in the presence of TEMPOL. Single NH–Hα correlations are attributed to signals labelled as 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 16. Two NH–Hα correlations are attributed to signals labelled as 4, 14, 15, 17 and 18. Three correlations overlap in the frequency positions of the signal number 3.

the in phase structure of the TOCSY cross peaks reduces possible J modulations of signal intensities, maintaining also a more favourable T_2 character of the data in respect to the NOESY ones. In the present study, we employed 2D clean TOCSY spectra to avoid possible spurious dipolar contributions.

The investigated 1–27 β-endorphin fragment contains a good variety of different amino acids: polar, apolar, aromatic, negatively and positively charged residues are all found along the peptide sequence.

In principle, various chemical interactions between the peptide and the aminoxyl could be explored, favoured also by the experimental conditions, as a relatively high spin label concentration was chosen.

Therefore, any differential peak attenuation induced by TEMPOL on β-endorphin in aqueous solution, could only be explained in terms of specific chemical interaction, as long as no conformational contribution is expected from the disordered peptide chain.

Experimentally, it was still possible to observe good quality 2D spectra from the paramagnetic solution, as shown in Fig. 1(*b*). The NMR spectrum of the diamagnetic fragment, Fig. 1(*a*), is consistent with the data previously reported for the neuropeptide.[10] Owing to the exchange of the amino terminus and the presence of one P residue, 25 cross-peaks are expected in the NH–Hα fingerprint region of the 2D spectrum, but spectral overlapping reduces the number of the resolved connectivities to 18. This lack of resolution is typical for polypeptides with unordered conformations and the grouping of the cross-peaks is reported in the Fig. 1 caption. A direct comparison of the fingerprint region of the two clean-TOCSY spectra of Fig. 1(*a*) and 1(*b*) clearly indicates that all the cross-peak intensities are equally reduced by the paramagnetic probe. Furthermore, in agreement with previous studies,[1–4] the presence of the spin label causes very small chemical shift variations for all the 18 cross-peaks (*viz.* average $\Delta\delta = 0.02$, with a maximum 0.04 ppm shift for peak 15).

In general, the paramagnetic perturbation, *i.e.* the dipolar interaction between the nucleus and the unpaired electron, is effective also at relatively long distances: in large molecules, with well defined structures,[4] even buried protons are sensibly affected. These long range effects should exhibit a direct and inverse dependence on the experimental aminoxyl concentration and the average molecular radius respectively, prompting for specific preliminary optimisation work to prevent levelling effects of a strong resonance attenuation which could mask the fine details of the conformationally driven interaction.

A quantitative evaluation of the paramagnetic filter effects can be performed by measuring the cross-peak volumes of the NH–Hα correlations obtained in the diamagnetic, V_d, and paramagnetic, V_p, solutions.

The spin label induced attenuations, A_ps, defined as

$$A_p = 1 - V_p/V_d \qquad (1)$$

can be autoscaled, using their mean value as a scaling factor. Thus, the relative fluctuations of the A_ps, *i.e.* the variances, can be discusssed for a structural interpretation of the spectrum changes rather than the absolute extents of the signal attenuations which may vary depending on the particular experimental conditions. In diagrams such as the ones shown in Fig. 2, the variance around the mean attenuation, for each resolved cross peak, is reported.

All the A_p parameters, calculated for β-endorphin, are essentially identical, as shown in Fig. 2(*c*). They primarily reflect the unhindered interaction between the probe and the biopolymer undergoing statistical collisions.

Absence of preferential interactions between the paramagnetic probe and the peptide can be inferred from these data and a similar pattern of peak attenuation should be always expected for molecules with unordered structure.

In fact, when dealing with structured molecules, such as lysozyme and gramicidin S, the data reported in ref. 2 and 4 yield attenuation diagrams like the ones shown in Fig. 2(*a*) and 2(*b*), respectively. Regions of the lysozyme diagram, where the autoscaled attenuations, A_{pi}, are lower than 0.5, correspond to protein sequences involved in defined secondary structures. The long and buried α helix between residues 25 and 35 and the β pleated sheet involving the residues 50–60 are easily recognised. Other secondary structure elements which exhibit high surface exposure, such as the loops found in lysozyme and centred at

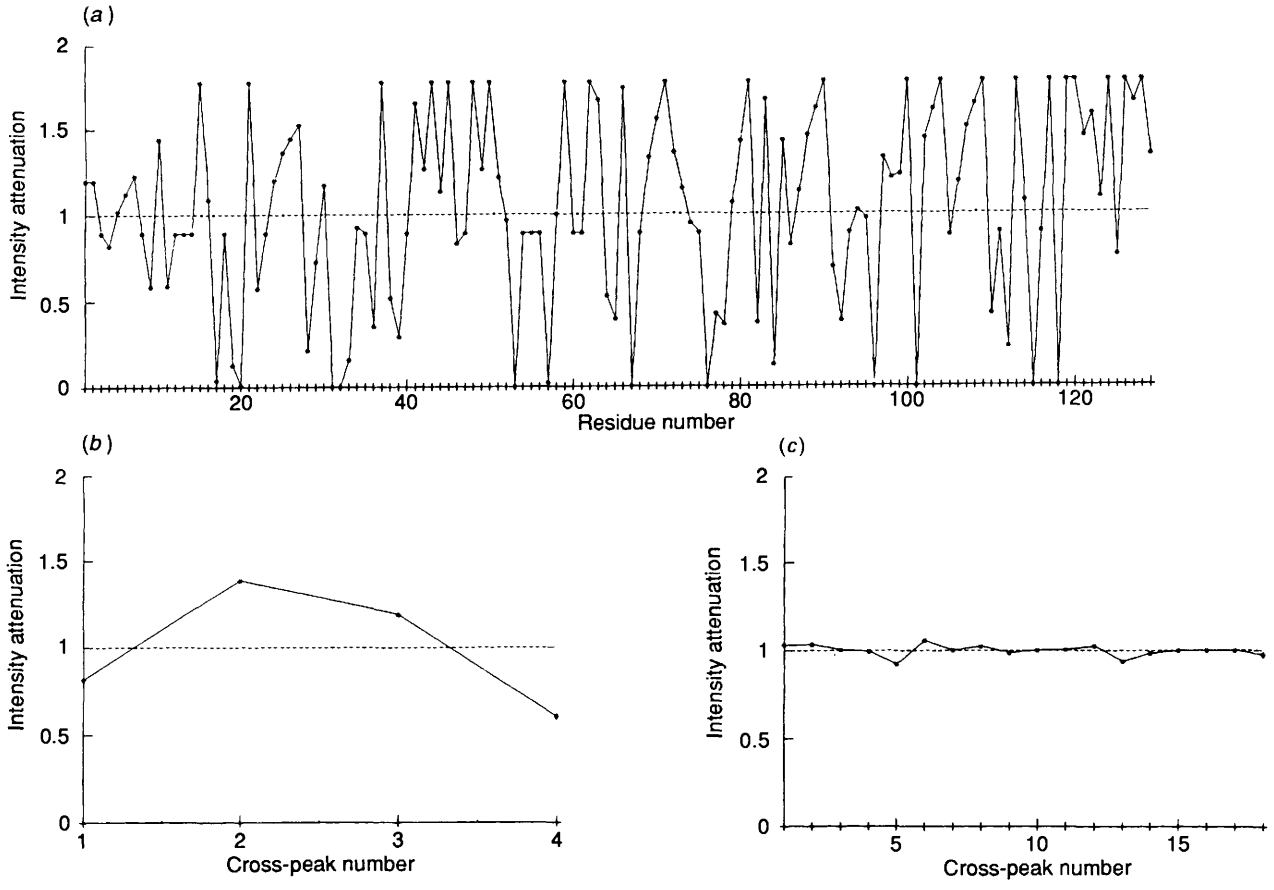

Fig. 2 The cross peak attenuation, reported as the auto-scaled value A_{pi} of eqn. (1), induced by the presence of TEMPOL for the NH–Hα correlations of (*a*) lysozyme, (*b*) gramicidin S and (*c*) 1–27 β-endorphin fragment. Numbers in abscissas refer to (*a*) residue sequence position for lysozyme, (*b*) valine, phenilalanine, ornithine and leucine respectively for gramicidin S and (*c*) the cross peak labelling of Fig. 1 for the β-endorphin fragment.

position 21, 71, 81, 104 and 117, invariably correlate with autoscaled attenuation values higher than 1.5.

The paramagnetic attenuations obtained for gramicidin S, see Fig. 2(*b*), again unambiguously indicate the different surface accessibility of the phenylalanine and ornithine amide protons, $A_{pi} > 1$, versus the hydrogen bonded valine and leucine NH hydrogens,[2] $A_{pi} < 1$.

Conclusions

Here we have presented an extension of the paramagnetic perturbation approach to polypeptides devoid of secondary structure elements. In conjunction with the data previously obtained with structured molecules such as lysozyme and gramicidin S, these further results suggest the use of soluble aminoxyls as an alternative tool for preliminary structural assessment of small peptides, isolated protein fragments or even entire proteins when some departure from native structure may be conceived because of the experimental conditions.

The fluctuation range of the paramagnetic perturbation affecting NH–Hα cross-peaks is diagnostic of the presence of stable conformations. Whenever a molecule exhibits similar auto-scaled attenuation values as those measured for the β-endorphin fragment, there is no need to perform a complete time consuming spectral assignment and to evaluate J_ϕ couplings and NOEs, since these parameters will not be consistent with any secondary structure. In addition, when compared with other methodologies such as temperature or pH catalysed H/D isotope exchange kinetics, or temperature dependence of chemical shifts, aminoxyl based structural conclusions are much more reliable because they are not inferred from moving targets, but are restricted to the conformational state as determined by a specific set of experimental conditions. This feature should prove valuable when attempting to delineate structural dynamics of denaturing proteins or, in general, of molecules interacting with some ligands.

A different application of structural probing by paramagnetic perturbations can be envisaged when the polypeptide under study is only poorly structured. This is the case with some flexible polypeptide chains, exhibiting non-vanishing extents of local conformational order also in aqueous media, as extensively reported by Dyson *et al.*[12,13] Provided that no specific interactions occur, the small transient differential accessibility of the peptide surface should be quenched by statistical probe sampling, especially in view of the long range character of the paramagnetic perturbation. However, by decreasing the frequency of the sampling, *i.e.* by decreasing the concentration of the probe, differential accessibility should in principle still show up as differential resonance attenuation. This expectation relies, of course, on a statistical collision model that appears confirmed by our observations in different solvents. In fact, the extent of resonance attenuation measured in DMSO and water solutions reflects the product of the solute and probe molar fractions, since comparable attenuation figures are obtained using TEMPOL:solute ratios of 1:2 and 6:1, in DMSO and water, respectively.

Acknowledgements

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