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Lanthanide induced residual dipolar couplings for the conformational investigation of peripheral $^{15}NH_2$ moieties

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Abstract

The Ca_2 calbindin protein in which one calcium has been substituted with Ce(III), Yb(III) and Dy(III) displays substantial alignment in high magnetic fields due to the high anisotropy of the metal magnetic susceptibility. This property has allowed the measurement of residual dipolar coupling contributions to $^1J_{HN}$ and $^2J_{HH}$ couplings of asparagine and glutamine NH_2 moieties. Such data have been used to aid structural characterization of these groups. The exploitation of auto-orientation of magnetic anisotropic metalloproteins represents a step ahead in the investigation of the conformational space of peripheral residues that are not fixed by the protein folding.

Abbreviations: Asn, asparagine; Gln, glutamine; CaLnCb, Ln(III) substituted calbindin D_{9k} with Ln = Ce, Yb, Dy; DQ, double quantum; ZQ, zero quantum; Δrdc's, residual dipolar couplings; RMSD, root mean square deviation.

Introduction

^{1}H-^{15}N residual dipolar couplings (Lohman and Maclean, 1978; Tolman et al., 1995; Bothner-By, 1996; Tjandra et al., 1996) arising from partial orientation of molecules in solution were recently shown to provide excellent structural information on macromolecules (Tjandra and Bax, 1997; Banci et al., 1998b; Clore et al., 1998; Arnesano et al., 2000; Bertini et al., 2000) and their complexes (Tjandra et al., 1997; Clore and Gronenborn, 1998; Bayer et al., 1999; Koenig et al., 1999) as well as on relative domain orientations (Tolman et al., 1997; Biekofsky et al., 1999; Fischer et al., 1999). The least perturbative device to obtain the necessary degree of alignment of macromolecules in solution is to exploit their natural magnetic anisotropy, responsible for partial orientation in high magnetic fields.

Paramagnetic metal ions characterized by anisotropic magnetic susceptibility can provide large contributions to the overall molecular magnetic susceptibility tensor (Bertini and Luchinat, 1996). Several solution structures of paramagnetic proteins were indeed obtained, or further refined, with constraints deriving from backbone ^{1}H-^{15}N residual dipolar couplings (Banci et al., 1998b; Arnesano et al., 2000; Bertini et al., 2000). Substitution with paramagnetic metal ions can be used on purpose to favor molecular alignment and measure residual dipolar couplings with increasing accuracy (Bertini et al., 2000).

In principle, residual dipolar couplings between any pair of nuclei can provide excellent structural information if methods are developed for their accurate measurement. In particular, the magnitude of the dipolar interaction increases with the product of the gyromagnetic ratio of the two nuclei involved, making ^{1}H-^{1}H residual dipolar coupling particularly promising. Only recently a few reports in which residual dipolar couplings of ^{1}H-^{1}H (Bolon et al., 1999; Cai et al., 1999; Tian et al., 2000; Tjandra et al., 2000a, b) or ^{1}H-X in XH_2 systems (Ottiger et al., 1998; Otting et al., 1999) or both at the same time (Carlomagno

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et al., 2000; Otting et al., 2000; Peti and Griesinger, 2000) were measured on bicelle or phage oriented systems and proposed as possible structural constraints have appeared in the literature.

We concentrate here on the ^{1}H-^{1}H dipolar interaction in the NH_2 group of asparagine (Asn) and glutamine (Gln) side chains to show (i) the feasibility of the experiment, and (ii) the type of structural information that can be obtained on external, solvent exposed residues. The NMR approach proposed, in addition to the ^{1}H-^{1}H residual dipolar coupling, gives also the two NH residual dipolar couplings of NH_2 groups. In this way the three residual dipolar couplings ($\Delta rdc\,(H_1N)$, $\Delta rdc(H_2N)$, $\Delta rdc(H_1H_2)$) that characterize the NH_2 group can be measured simultaneously.

The system used to test the approach is calbindin, a calcium binding protein in which one of the two calcium binding sites has been replaced with a lanthanide metal ion (Ce(III), Yb(III), Dy(III)) (Allegrozzi et al., 2000). Lanthanide metal ions are expected to be excellent tools to trigger at will the paramagnetic effects on nuclear relaxation and shift as well as partial orientation in a magnetic field. In particular, Ce(III), Yb(III) and Dy(III) are characterized by increasing anisotropic magnetic susceptibility tensors that provide an increasing degree of molecular alignment in high magnetic fields. This research explores the possibility of using one or more lanthanide probes to investigate the behavior of NH_2 containing peripheral residues.

Experimental part

NMR samples of 2.0 mM, pH 6.0, Ln(III) substituted calbindin D_{9k} (termed hereafter CaLnCb, with Ln = Ce, Yb, Dy) was prepared as previously described (Brodin et al., 1986; Johansson et al., 1990; Allegrozzi et al., 2000). All NMR experiments were carried out at 298 K on Bruker AVANCE 800 MHz and AVANCE 400 MHz spectrometers operating at 18.8 T and 9.4 T, respectively. Acquisition parameters were: $1024(t_1)\cdot 2048^*(t_2)$ with acquisition times of 104 ms (t_1) and 85 ms (t_2) at 800 MHz and of 209 ms (t_1) and 170 ms (t_2) at 400 MHz. Twenty-four scans were collected for each transient for a total duration of about 7 h per experiment. Experiments were repeated three times at each magnetic field, changing the ^{1}H (4.81 ppm and 6.41 ppm) and ^{15}N (115.40 and 120.08 ppm) transmitters in order to have more independent measurements. The ^{1}H-^{15}N transfer delay was set to 5.2 ms. Data matrices were apodized with a Lorentz-to-Gaussian window function, zero filled to 8192 (t_1)·4096 (t_2) and only the relevant part of the spectra was retained.

The PSEUDYANA (Banci et al., 1998a) module of the DYANA program (Güntert et al., 1997) was used to include the residual dipolar couplings involving NH_2 groups in structure calculations. A weight of $1(\mathrm{Hz/\AA})^2$ for residual dipolar couplings was used. The family of structures of CaCeCb recently obtained in our laboratory (Bertini et al., 2000) was used as the starting point to include residual dipolar coupling constraints for NH_2 groups of Asn and Gln residues. A modified amino acid was added in the library used by PSEUDYANA in order to include ^{1}H-^{1}H residual dipolar couplings in calculations.

Method

NMR experiments

The three coupling constants that characterize the NH_2 group of Asn and Gln side chains were determined using an HMQC experiment in which ZQ and DQ are separately evolved in the indirect dimension (Bax et al., 1983). Quadrature detection in the indirect dimension is achieved as described by Permi et al. (1999). For each of the two protons that constitute the NH_2 group (H_1 and H_2), two cross peaks are observed in this experiment. Taking H_1 as an example, one cross peak corresponding to ZQ at (ω_{H_1}, $\omega_N - \omega_{H_1}$) and one to DQ at (ω_{H_1}, $\omega_N + \omega_{H_1}$) are observed. The couplings of N and H_1 to the third spin H_2 are resolved in the indirect dimension, which means that, in such a moiety, a splitting corresponding to $(J_{H_2N} - J_{H_1H_2})$ is observed on the ZQ peak (Δ_{ZQ}) whereas on the DQ peak the splitting (Δ_{DQ}) is $(J_{H_2N} + J_{H_1H_2})$ (Ernst et al., 1987). Therefore the three coupling constants characterizing the NH_2 system can be determined through the following relationships:

$$J_{NH_2} = 0.5\left(\Delta_{DQ}(H_1N) + \Delta_{ZQ}(H_1N)\right) \quad (1)$$

$$J_{NH_1} = 0.5\left(\Delta_{DQ}(H_2N) + \Delta_{ZQ}(H_2N)\right) \quad (2)$$

$$J_{H_1H_2} = 0.5\left(\Delta_{DQ}(H_1N) - \Delta_{ZQ}(H_1N)\right) = 0.5\left(\Delta_{DQ}(H_2N) - \Delta_{ZQ}(H_2N)\right) \quad (3)$$

Note that $J_{H_1H_2}$ is determined from two independent data.

The observed J_{HX} couplings (with $X = N$ or H) are a sum of several contributions:

$$J_{HX}(B_0) = J_{HX}(0) + rdc(\theta, \phi, B_0) + \delta_{DFS}(B_0) \quad (4)$$

where J_{HX} (0) is the field independent scalar coupling constant, $rdc\,(\theta, \phi, B_0)$ is the residual dipolar coupling and $\delta_{DFS}\,(B_0)$ is the dynamic frequency shift. The field dependence of the dynamic frequency shift is expected to be very small and was neglected. The residual dipolar coupling (in Hz) is given by (in SI units) (Gayathri et al., 1982; Bothner-By, 1996; Banci et al., 1998b):

$$rdc(\theta, \phi, B_0) = -\frac{1}{4\pi}\frac{B_0^2}{15kT}\frac{\gamma_H\gamma_X h}{4\pi^2 r_{HX}^3} \left[\Delta\chi_{ax}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{rh}(\sin^2\theta\cos 2\phi)\right] \quad (5)$$

where $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic anisotropies of the molecular magnetic susceptibility tensor (χ^{mol}), and θ and ϕ are the polar coordinates describing the orientation of the X-H bond vector in the tensor axis system.

The difference between the coupling measured at 800 MHz and at 400 MHz gives

$$\Delta rdc(\theta, \phi) = \left[(J_{800MHz} - J_{400MHz})\right] \quad (6)$$

which has the same expression as in Equation 5, with the only variation that B_0^2 is substituted by $\Delta B_0^2 = (18.8\text{T})^2 - (9.4\text{T})^2$, the difference between the two magnetic fields used.

Structure calculations

Residual dipolar couplings for NH backbone amides were recently used to refine the solution structure of CaCeCb (Allegrozzi et al., 2000; Bertini et al., 2000). The molecular magnetic anisotropy tensor was available from backbone NH residual dipolar couplings and was used as input together with all other structural constraints available. In order to be consistent with the experimental data available for CaCeCb on amide NH residual dipolar couplings, obtained at 800 MHz and at 500 MHz (instead of 800 MHz and 400 MHz), a scaling factor $(800^2 - 500^2)/(800^2 - 400^2)$ was applied to the H_1N and H_2N residual dipolar couplings of NH_2 groups. For what concerns H_1H_2 residual dipolar couplings, in addition to the above mentioned scaling factor, the data were also multiplied by (Momany et al., 1975; Némethy et al., 1983): $(\gamma_N r_{HH}^3/(\gamma_H r_{HN}^3)$.

In this way, a set of 'corrected' residual dipolar couplings (to which we will refer in the following discussion) were obtained and then used in calculations.

Table 1. The experimental sets of three residual dipolar couplings (Δrdc's) scaled as described in the Experimental section

Residue[a]		Ce(III)	Yb(III)	Dy(III)
Asn 21	$\Delta rdc\,(H_1N)$	−0.61		
(1.88–1.72)	$\Delta rdc\,(H_2N)$	0.30		
	$\Delta rdc\,(H_1H_2)$	−0.14		
Gln 33	$\Delta rdc\,(H_1N)$	0.04	0.28	−6.16
(1.07–0.89)	$\Delta rdc\,(H_2N)$	−0.96	2.64	−14.77
	$\Delta rdc\,(H_1H_2)$	−0.35	2.04	−9.39
Gln 67	$\Delta rdc\,(H_1N)$	−0.45		
(1.17–1.14)	$\Delta rdc\,(H_2N)$	0.02		
	$\Delta rdc\,(H_1H_2)$	−0.11		
Gln 75	$\Delta rdc\,(H_1N)$	−0.02	−0.12	0.01
	$\Delta rdc\,(H_2N)$	0.07	−0.26	−0.60
	$\Delta rdc\,(H_1H_2)$	0.04	0.03	−0.09

The error on the measurements is of the order of 0.1–0.2 Hz for Ce(III) substitution and increases to 0.1–0.4 and to 0.2–1.0 Hz for Yb(III) and Dy(III), respectively. The error on Gln 33 in the Dy(III) case can be up to 2.5 Hz due to the large linewidths at 800 MHz. All the measured residual dipolar couplings lie in the range determined for backbone NH groups in Ln(III) substituted calbindin (−1.26–0.6 Hz for Ce(III) (Bertini et al., 2000), 2.95−−2.88 for Yb(III) and −15.8–11.2 Hz for Dy(III) (in preparation)).

[a]In brackets the starting and final pairwise RMSD (Å) of the residue calculated for a family of 30 conformers (except for the last residue in the protein).

Results and discussion

The measured NH_2 Δrdc's ($\Delta rdc(H_1N)$, $\Delta rdc(H_2N)$, $\Delta rdc(H_1H_2)$) are reported in Table 1. Determination of three Δrdc's characterizing the NH_2 system requires accurate measurement of the couplings at the different magnetic field strengths. This could be achieved with enough accuracy in case of Ce(III) substitution, for four out of six residues: Asn 21 and Gln 33, 67, 75 (Table 1). The remaining two residues, Gln 22 and Asn 56, are very close to the lanthanide binding site; the distance between the nitrogen atom belonging to the NH_2 group and the Ln(III) site in the solution structure (Bertini et al., 2000) ranges from 5.4–7.1 Å and 2.4–4.0 Å for Gln 22 and Asn 56, respectively. The resonances belonging to these two residues can be detected through tailored NMR experiments (Banci et al., 1994; Bertini and Luchinat, 1996). The large linewidths and low signal-to-noise ratio prevent accurate measurement of the couplings.

When Ce(III) is replaced by Yb(III), the higher paramagnetism of the latter broadens the lines of Gln 22 and Asn 56 beyond detection. The distances of the nitrogen atom in the NH_2 group of Asn 21 and 67 from

the Ln(III) site in the solution structure of the protein (Bertini et al., 2000) are in the ranges 12.1–14.2 Å and 13.3–14.6 Å, respectively. These two residues can still be observed through optimized experiments, but the increasing linewidth, especially at 800 MHz, and very low signal-to-noise ratio introduce a large error on the measurement of the Δrdc's.

For the Dy(III) substitution, as already noted earlier (Allegrozzi et al., 2000), resonances of nuclei at a distance of less than 15 Å from the lanthanide metal ion cannot be detected. In the present case, only Gln 33 and 75, at more than 20 Å from the paramagnetic center, can be observed. Asn 21 and Gln 67 lie at the border of detection and cannot yield reliable measurements of couplings. Asn 22 and Gln 56 are irremediably lost.

Therefore, despite the fact that detection and assignment can be pushed further through tailored NMR experiments, only residues giving rise to well resolved multiplet components with sufficient signal-to-noise ratio at the two magnetic field strengths considered were used in the following analysis.

Before moving to the analysis of the measured residual dipolar couplings, a comment is due about the possible influence of dynamics, which is a crucial point for solvent exposed, peripheral side chains that can, in principle, be characterized by large amplitude motions due to their position in the protein frame. In case of complete conformational averaging, residual dipolar couplings are averaged to zero. However, the protein matrix still imposes steric constraints restricting the conformational space available even for side chains facing the protein surface. Calculations were thus carried out in which the four investigated side chains were selectively rotated in order to perform a rough but significant sampling of the available conformational space. Results show that residual dipolar couplings calculated for each conformer still average to zero. Therefore, when at least one of the three Δrdc's is large in absolute value, extensive conformational averaging can be excluded and the residue must sample a much more restricted conformational space. This is the case for Asn 21, Gln 33 and Gln 67 (Table 1).

In particular, in the case of Gln 33, residual dipolar couplings could be measured for the three different lanthanide substitutions and clearly confirm that the effects observed are dipolar in nature, since: (i) the measured couplings depend on the square of the magnetic field strength, (ii) the magnitude of the Δrdc's increases with increasing anisotropy of the lanthanide metal ion, and (iii) the signs of the observed Δrdc's change when Yb(III) is substituted, in good qualitative agreement with the opposite sign of the anisotropy (Allegrozzi et al., 2000). For comparison, the ranges of residual dipolar couplings determined for backbone amide systems are reported in the caption of Table 1, showing that the values determined for Gln 33 lie in the same ranges.

A set of three very small residual dipolar couplings is instead observed for Gln 75. This could also be a result of a specific conformation, but the fact that the residual dipolar couplings are not increasing proportionally with the extent of partial alignment induced by the paramagnetic metal ion seems to be most easily explained by assuming extensive conformational averaging. This is not surprising, as Gln 75 is the last residue in the polypeptide chain.

Ce(III) substituted calbindin and structure calculations

The aim of the measurements is to evaluate the capability of the set of three residual dipolar couplings that can be measured for NH_2 systems of providing structural constraints and/or information on possible averaging processes. For this purpose we chose to analyze in detail the Ce(III) substituted calbindin because of the extensive amount of experimental NMR data (NOEs, couplings, pseudocontact shifts) (Allegrozzi et al., 2000) that were recently obtained on it to determine the solution structure. This was then also refined through backbone NH residual dipolar couplings (Bertini et al., 2000) and thus also the magnetic molecular susceptibility tensor is already available.

The four sets of Δrdc's were included in structure calculations, as described in the Experimental section, obtaining a new family of structures. The target function (Güntert et al., 1997) does not appreciably increase (from a range of 0.86–1.35 $Å^2$ to 0.83–1.39 $Å^2$); the average RMSD remains basically unaltered whereas a slight local decrease is observed for the residues under analysis (Table 1). The agreement between experimental and calculated Δrdc's improves as shown, residue by residue, in Figure 1. This means that conformations are selected in such a way that they also agree with Δrdc's in addition to the available constraints. A reasonable tolerance of 0.15 Hz was given to the constraints, as mobility may undermine their use. Likewise, in the case of conformational mobility, interresidual NOEs provide average values ($1/r^6$) and therefore wrong average r

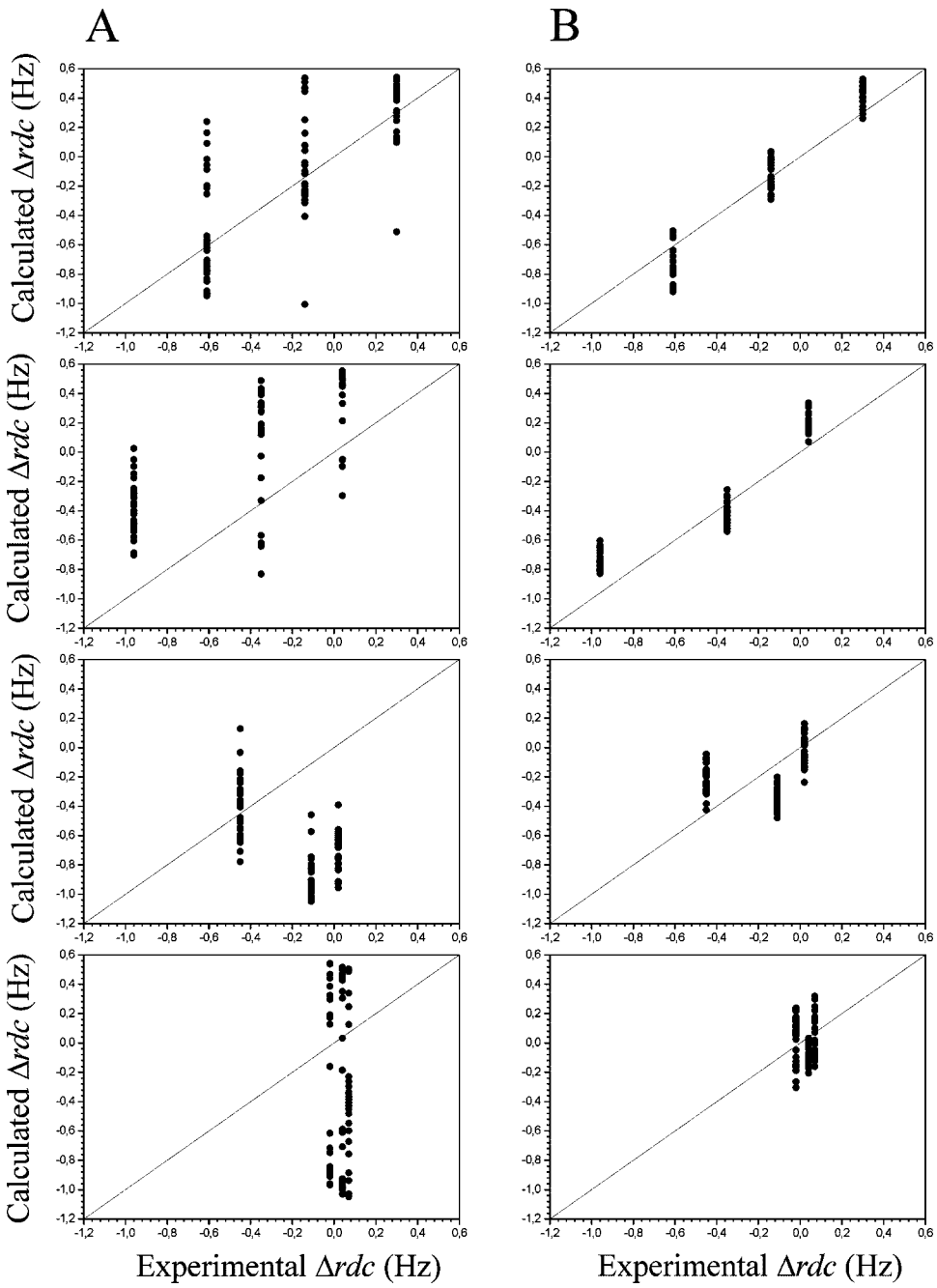

Figure 1. Calculated versus experimental residual dipolar couplings on the starting family of structures (Allegrozzi et al., 2000; Bertini et al., 2000) (A) and after the inclusion of NH_2 residual dipolar couplings as structural constraints (B). One graph is shown for each residue (from top to bottom: Asn 21, Gln 33, Gln 67, Gln 75).

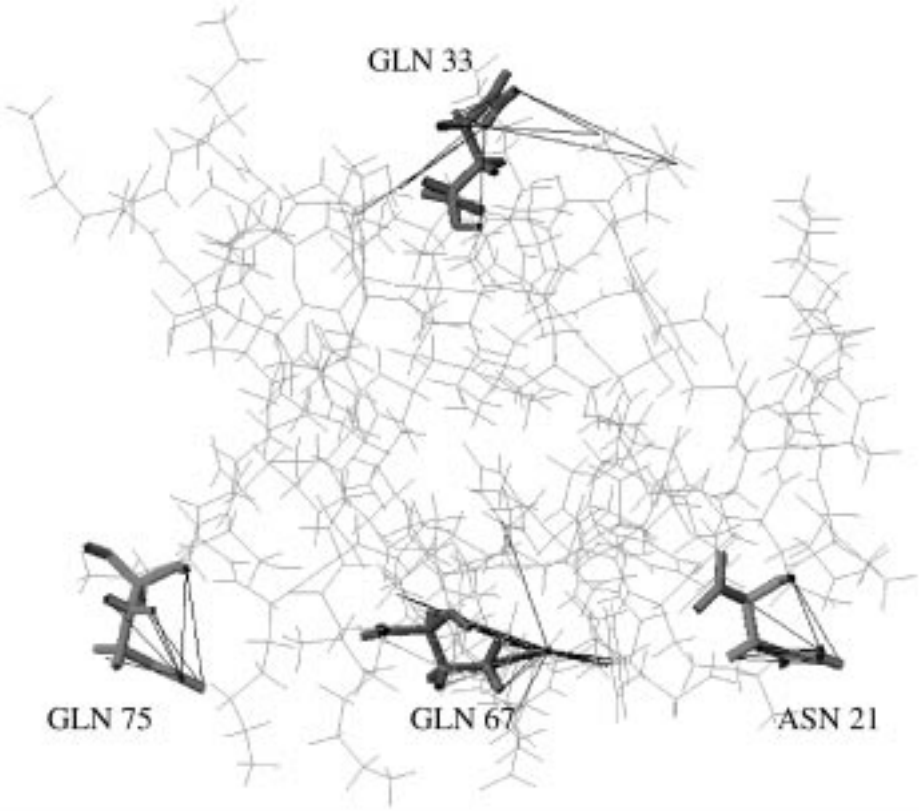

Figure 2. Schematic representation of the distance constraints available for the NH_2 groups of Gln 33, Gln 67, Gln 75. Distance constraints are depicted as lines in the frame of the 3D CaCeCb structure. In particular, the number of connectivities identified for the two protons of the NH_2 group were: 7 intra-residue for Asn 21, 5 intra- and 11 inter-residue for Gln 33, 10 intra- and 8 inter-residue for Gln 67, 7 intra- and 1 inter-residue (sequential) for Gln 75.

values. Researchers have learned to live with it. However, if both NOEs and residual dipolar couplings are consistent with a single conformation the structural result is sound. The validation of the consistency is made through back calculation of NOEs using the program CORMA (Borgias et al., 1989, 1990) and of residual dipolar couplings (Banci et al., 1998b).

Among the four residues studied, Gln 33 is the least disordered one in the original family of structures because a few inter-residue NOEs are observed (Figure 2). Inclusion of NH_2 Δrdc's selects one main conformation (with a population of 90%) in agreement with upper distance limits, quite similar to the original one even if not identical and more defined (Figure 3). A second conformation, which differs from the main one only in the orientation of the $CONH_2$ plane, is found in only 3 out of 30 conformers. Back calculation of NOE intensities with neighboring residues (methyl groups of Leu 30 and 40, Hα of Pro 37, $H\gamma_1$ of Lys 29) and of Δrdc's for two structures representative of the two different local conformations indicate that the most populated conformation is in better agreement with experimental data than the one present in a very small percentage. This finding is reliable on the ground that, in the presence of significant conformational exchange, NOEs and residual dipolar couplings would average to different structures. From the chemical point of view, the conformation of Gln 33 is stabilized by hydrophobic interactions with neighboring residues and by the NH_2 group that points towards the backbone CO group of Lys 29.

For Asn 21 only intra residue NOEs are observed for the two protons of the NH_2 group (Figure 2). Asn 21 is very disordered in the starting family of structures, characterized by more than three conformers (not shown). The use of NH_2 Δrdc's pins down the conformers to two main ones that differ in the χ_1 dihedral angle and still agree with upper distance limits used in structure calculations. Back calculation of NOE intensities confirms that no additional cross

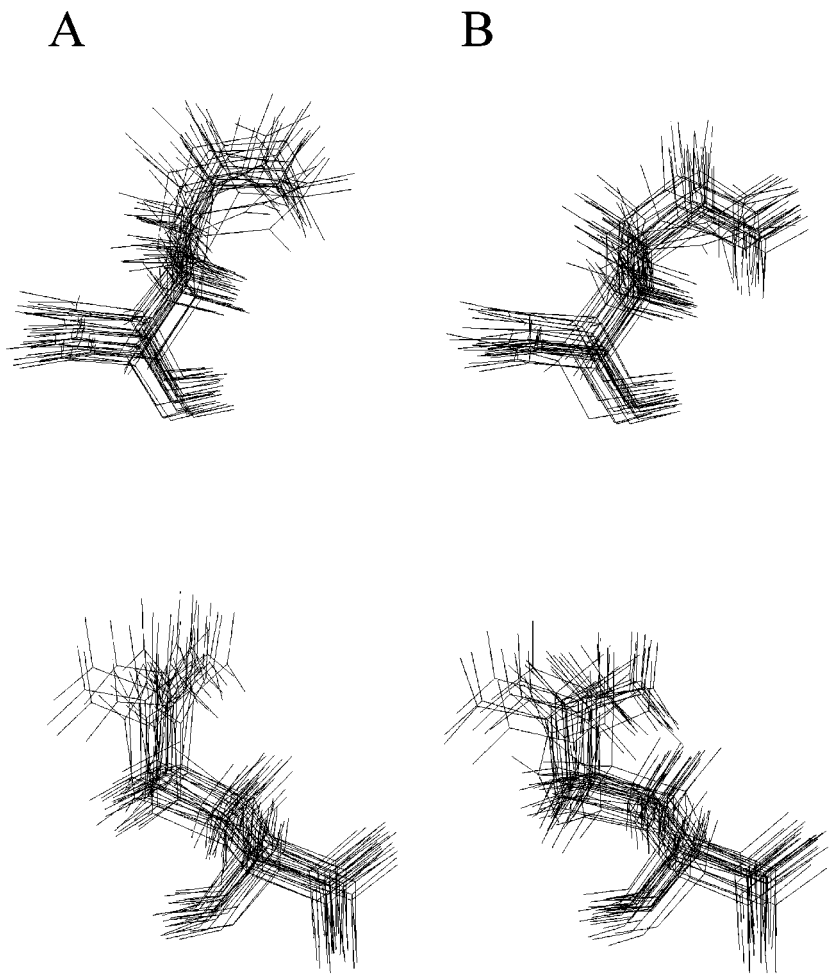

Figure 3. The side chains of Gln 33 (top) and of Gln 67 (bottom) are shown before (A) and after (B) inclusion of the set of three Δrdc's as structural constraints.

peaks are predicted due to the external position of this residue. Close inspection of the well resolved NOEs shows that one of the two conformers (the more populated in the family of structures) is the more probable because it is in better agreement with the relative intensities of the available intra-residue NOEs (the NOE between NH_1 and $H\beta_2$ that is much larger than all the others). This conformer appears stabilized by the interaction of the NH_2 group with the terminal part of Asp 19.

In the case of Gln 67, which is well defined up to Cβ, but becomes increasingly disordered going to Cγ, Cδ and Nε, the use of NH_2 Δrdc's selects two conformations that are very similar and differ only in the orientation of the $CONH_2$ plane (Figure 3). Both conformations are in agreement with upper distance limits. The NH_2 protons give well resolved NOEs with the methyl groups of Leu 6 and Val 70 and with the aromatic ring of Phe 63 for which only three resonances have been identified, showing that the aromatic ring is in fast rotation. Interestingly H_1, on average, gives more intense NOEs with respect to H_2. H_1 gives NOEs with residues on opposite sides and this would be in best agreement with the presence of both conformations in rapid interconversion (rapid with respect to the chemical shift difference, since only one set of resonances is observed for Gln 67). Gln 67 is sandwiched between the hydrophobic side chains of Val 70 and Phe 63 and thus hydrophobic interactions could stabilize each of the two conformations and at the same time allow easy interconversion between the two.

Finally, residue 75, the last in the polypeptide chain, shows only intra residue and sequential NOEs (Figure 2). It is found completely disordered in the

starting family of structures and after inclusion of NH_2 Δrdc's the situation does not improve much, even if some conformations that agree with experimental data (within the error) are found. However, observation of NOEs smaller than those observed for the other side chains in combination with the very small values of NH_2 Δrdc's indicates a flexible residue undergoing conformational averaging, in agreement with its position in the polypeptide chain.

A comment on lanthanide substitution

The three lanthanide metal ions, Ce(III), Yb(III) and Dy(III), were selected to sample from the least to the most anisotropic metal ion. Yb(III), as an intermediate one, was chosen because its anisotropy is of opposite sign with respect to that of Ce(III) and Dy(III). The couplings measured on Gln 33 of Ln(III) substituted calbindin clearly show dependence on the square of the magnetic field and on the anisotropy of the Ln(III) metal ion, confirming that their variation arises from residual dipolar couplings. This supports the validity of the experimental approach proposed. The effects observed lie in a 1:5:20 proportion for Ce(III):Yb(III):Dy(III). When Dy(III) is substituted, proton–proton Δrdc's greater than 20 Hz are observed. Therefore, as far as the degree of alignment is concerned, Dy(III) is the most suitable lanthanide metal ion to substitute, since the larger the effects, the easier they can be quantified. Orientation effects have the advantage that they do not depend on the distance of the two nuclei with respect to the lanthanide metal ion. Paradoxically, if a lanthanide binding site were constructed on purpose in a particular macromolecule, it would be best placed as far as possible from the region investigated in order to take advantage of orientational effects. However, a compromise between large orientation effects and observability should be found. For residues too close to the lanthanide binding site that escape detection because of heavy relaxation rate enhancements, other lanthanide metal ions can be substituted that, even if less effective in aligning the molecule, still yield measurable contributions. In the present study the number of residues that could be investigated increases on passing from Dy(III) to Ce(III) substitution.

The Ce(III) substituted protein was selected to evaluate the impact that NH_2 Δrdc's can have in structure determination and to check if they are consistent with the other kinds of constraint available (NOEs, psudocontact shifts). Note that easily measurable ^3J coupling constants are not available to constrain NH_2 groups. Results show that the use of Δrdc's can contribute to better define the conformation of side chains, especially for those residues that lie on the surface of the protein, for which generally only a few experimental constraints are available.

Conclusions

We have shown that residual dipolar couplings involving the three nuclei that constitute NH_2 groups can be measured exploiting lanthanide-induced magnetic alignment. The sets of three Δrdc's provide information on the conformational variability of Asn and Gln side chains especially when, as often happens, they are solvent exposed on the surface of the protein. In the case of rotation of the entire residue the Δrdc's approach zero; when sizeable Δrdc's are observed, the rotational freedom is much restricted. The combined analysis of Δrdc's and NOEs provides significant insight in defining the conformational space of the residue.

Additional constraints for protons in the solvent exposed regions are very important to improve the definition of the surface of solution structures. Indeed, it could also turn into an advantage of NMR versus X-ray methods, since it is clear that upon crystallization, the interphase region will not reflect the exact situation that the molecule experiences in solution and thus in vivo. Often surface residues are the key ones driving protein–protein (or protein–DNA/RNA) interactions and thus macromolecular recognition. Therefore constraints to better define the surface of the protein are very important.

Substitution of different lanthanide metal ions allows modulation of the extent of magnetic alignment. Dy(III) provides roughly a 20-fold larger contribution to orientation compared to Ce(III). In principle a larger degree of molecular alignment renders Δrdc's easier to measure. However, also line broadening increases on passing from Ce(III) to Dy(III) and thus a compromise should be found between relaxation rate enhancements and increasing anisotropy. In the present case of a protein of 75 amino acids Ce(III) represents the best compromise, as it introduces sufficient alignment with moderate line broadening. The advantage of residual dipolar couplings is that they do not depend on the distance from the metal ion. Therefore the use of metal ions such as Dy(III) can become a powerful tool to study large proteins or protein complexes in which statistically, a larger percentage of the

residues will be at a suitable distance from the metal center to be detected.

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