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Structure-independent cross-validation between residual dipolar couplings originating from internal and external orienting media

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Abstract

Lanthanide-substituted calcium binding proteins are known to partially orient in high magnetic fields. Orientation provides residual dipolar couplings (rdc's). Two of these systems, Tm^{3+}- and Dy^{3+}-substituted calbindin D_{9k}, dissolved in an external orienting medium (nonionic liquid crystalline phase) provide rdc values which are the sum of those induced by the lanthanides and by the liquid crystalline phase on the native calcium binding protein. This structure-independent check shows the innocence of the orienting medium with respect to the structure of the protein in solution. Furthermore, the simultaneous use of lanthanide substitution and external orienting media provides a further effective tool to control and tune the orientation tensor.

NMR-based structure determination still mostly relies on the possibility of obtaining a large number of short-range geometrical constraints, typically dihedral angles and NOEs. Completely independent sets of structural constraints can be derived from high-resolution NMR experiments carried out on soluble macromolecules partially oriented in the magnetic field: when molecules are partially oriented, the dipolar interactions within pairs of magnetic nuclei no longer average zero (Saupe and Englert, 1963; Bastiaan et al., 1987). This causes additional splittings which are called residual dipolar couplings (rdc's) (Tolman et al., 1995; Tjandra and Bax, 1997). From rdc values, angular information on the orientation of the internuclear vector can be obtained, and the corresponding constraints can be used for structure determination (Tjandra et al., 1997, 2000a, b; Banci et al., 1998; Clore and Gronenborn, 1998; Ottiger et al., 1998; Bayer et al., 1999; Fischer et al., 1999; Zhou et al., 1999; Arnesano et al., 2000; Bertini et al., 2000a, 2001a; Chou et al., 2000; Delaglio et al., 2000; Fowler et al., 2000; Huang et al., 2000; Hus et al., 2000; Meiler et al., 2000; Mollova et al., 2000; Al-Hashimi et al., 2001; Choy et al., 2001; Luy and Marino, 2001; Schwalbe et al., 2001; Warren and Moore, 2001). Partial orientation can be typically obtained through the addition of orienting devices in solution (Bax and Tjandra, 1997; Clore et al., 1998; Hansen et al., 1998; Ramirez and Bax, 1998; Wang et al., 1998; Cavagnero et al., 1999; Koenig et al., 1999; Ottiger and Bax, 1999; Sass et al., 1999; Barrientos et al., 2000; Bertini et al., 2000b; Fleming et al., 2000; Rückert and Otting, 2000; Sass et al., 2000; Tycko et al., 2000; Desvaux et al., 2001; Zweckstetter and Bax, 2001) or by exploiting the magnetic susceptibility anisotropy of paramagnetic metalloproteins (Tolman et al., 1995; Banci et al., 1998; Biekofsky et al., 1999; Contreras et al., 1999; Volkman et al., 1999; Arnesano et al., 2000; Bertini et al., 2000a, 2001a; Déméné et al., 2000; Ma and Opella, 2000; Veglia and Opella, 2000; Feeney et al., 2001).

The rdc's in the presence of an external orienting device are expressed as a function of the so-called alignment tensor, A, according to Equation 1:

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$$rdc^{\text{ext}}(\theta,\phi) = -\frac{1}{\pi}\frac{\mu_0}{4\pi}\frac{\gamma_H\gamma_N h}{2\pi r^3}\frac{A_{zz}}{2}S \left[\left(3\ \cos^2\theta - 1\right) + \eta\left(\sin^2\theta\ \cos\ 2\phi\right)\right] \quad (1)$$

where A_{zz} is the zz-component of the (diagonal) alignment tensor and η, the asymmetry parameter, is given by $(A_{xx} - A_{yy})/A_{zz}$. θ and ϕ are the cylindrical coordinates describing the orientation of the dipole-coupled nuclear pair (e.g., of the N-H bond vector) within the principal axis system of the A tensor, S is an order parameter, and r is the effective internuclear distance. All other symbols have their usual meaning.

If an internal metal ion with magnetic susceptibility anisotropy is present, its contribution to the alignment tensor of Equation 1 is given by:

$$A_{zz} = \frac{\Delta\chi_{ax}^{\text{para}} B_0^2}{15\mu_0 kT} \quad (2)$$

and

$$\eta = \frac{3\Delta\chi_{rh}^{\text{para}}}{2\Delta\chi_{ax}^{\text{para}}}, \quad (3)$$

where $\Delta\chi_{ax}^{\text{para}}$ and $\Delta\chi_{rh}^{\text{para}}$ are the axial and rhombic components of the paramagnetic susceptibility tensor, χ^{para}, and all other symbols have their usual meaning. The paramagnetic contribution to the experimental rdc can be directly obtained by subtracting the ^1J coupling of the ^{15}N-^{1}H amide moiety of a diamagnetic metalloprotein from the value of the analogous paramagnetic metalloprotein at the same field. Calcium binding proteins are ideal systems for the latter experiments, because diamagnetic calcium(II) can be substituted by paramagnetic lanthanide(III) ions, generally without alteration of the protein structure (Campbell et al., 1973; Lee and Sykes, 1983).

If an external orienting device is added to a solution of a paramagnetic lanthanide-substituted calcium protein, then a new resulting alignment tensor is obtained which is the tensorial sum of the two A tensors. As a consequence, the experimental rdc's measured under these conditions are the sum of the experimental rdc's measured in the presence of either the external or the internal orienting devices, provided the structure remains the same. Therefore, if this self-consistency is fulfilled, the 'innocence' of the external orienting device with respect to structural alterations of the protein solute is directly demonstrated in a structure-independent way.

The approach consists of recording three sets of, e.g., J-modulated ^{15}N-^{1}H experiments on a ^{15}N enriched protein sample: (1) paramagnetic protein in isotropic medium (internal orienting device only), (2) diamagnetic protein in anisotropic medium (external orienting device only), (3) paramagnetic protein in the same anisotropic medium (internal and external orienting devices simultaneously present), plus (4) diamagnetic protein in isotropic medium as the blank. We have used here the mutant P43M of calbindin D_{9k} (Cb hereafter), a 75 residue protein that binds two calcium ions through EF-hand motifs (Kretsinger, 1980; Linse et al., 1987), one of which (the C-terminal) can be selectively substituted by a lanthanide ion (Vogel et al., 1985; Akke et al., 1991; Allegrozzi et al., 2000). The paramagnetic lanthanide ion chosen for this work is Tm^{3+}, which provides strong self-orientation and whose line broadening effects are also strong but still allow the accurate measurement of J-splittings for 38 out of the 72 peptide NH's of the protein at 700 MHz. The external orienting device was a binary mixture of $C_{12}E_5$ (penta-ethyleneglycol dodecyl ether, Fluka) and neat n-hexanol (Fluka), which forms a stable liquid crystalline phase made of neutral aggregates in the temperature range 295–312 K (Rückert and Otting, 2000). To corroborate the analysis, another set of data was recorded at 500 MHz by using Dy^{3+} as the orienting metal. The number of measurable rdc is more limited in this case, as dysprosium(III) has the highest orienting capability but also the highest line broadening effect among lanthanides in macromolecules at high fields (Bertini et al., 2001b).

A series of J-modulated HSQC experiments (Tjandra et al., 1996) at 700 MHz was thus carried out on the paramagnetic Tm^{3+}-substituted calbindin D_{9k} (CaTmCb) in isotropic solution (*iso* hereafter) and in liquid crystalline solutions (*lc* hereafter), as well as on its diamagnetic analogue Lu^{3+}-substituted calbindin D_{9k} (CaLuCb) in both media. The rdc values ranged from -18 to $+15$ Hz for CaTmCb(*iso*), from -10 to $+11$ Hz for CaLuCb(*lc*), and from -15 to $+12$ Hz for CaTmCb(*lc*). Figure 1A shows the rdc values measured for CaTmCb(*lc*) *vs* the sum of the rdc values measured for CaLuCb(*lc*) and for CaTmCb(*iso*). The correlation is excellent, the small scatter being consistent with what expected from proper propagation of the estimated error on each set of measurements, which includes both uncertainty from fitted parameters and non-perfect reproducibility of the liquid crystalline dispersion. Similar results are obtained for the CaDyCb derivative (Figure 1B), where the

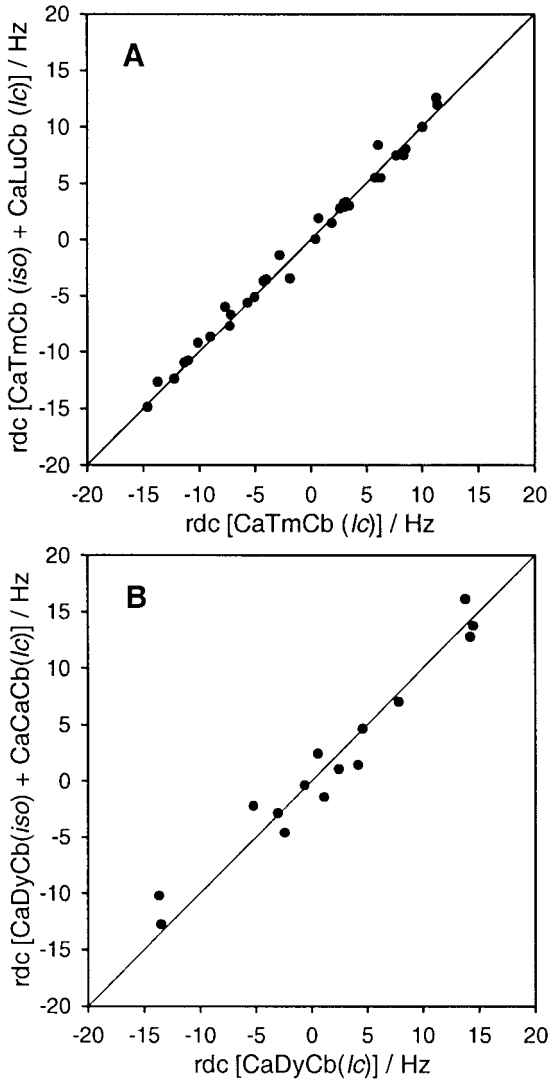

Figure 1. (A) Correlation plot of the sum of the rdc values for CaTmCb(*iso*) and CaLuCb(*lc*) *vs* the rdc values for CaTmCb(*lc*), measured at 700 MHz. (B) Correlation plot of the sum of the rdc values for CaDyCb(*iso*) and CaCaCb(*lc*) *vs* the rdc values for CaDyCb(*lc*), measured at 500 MHz.

somewhat larger scatter is accounted for by the larger paramagnetic line broadening.

The self-consistency (within the experimental uncertainty) of the three sets of rdc's obtained after subtraction of the blank is a demonstration of the innocence of the external orienting device in the present system (and, incidentally, of the internal orienting device as well, if this were an issue). We stress again that such demonstration has been obtained in a structure-independent way, i.e., irrespectively of how well the three sets of experimental rdc's agree with a pre-existing protein solution structure. Furthermore, the alignment tensors for the two orienting media are highly non collinear in this case, and therefore provide independent sets of geometrical constraints which can be used for structural refinement. This feature is likely to be general, because the external and internal orienting media are totally unrelated. Although only two of the three sets of rdc values are linearly independent, they arise from three independent experiments, and therefore all three sets can be used in structure calculation programs to further reduce the experimental uncertainty.

Moreover, all members of the lanthanides series can be substituted in protein calcium binding sites. Six of them (from Tb^{3+} to Yb^{3+}) have sufficiently large magnetic susceptibility anisotropy to provide a contribution to the alignment tensor of the same order as that provided by the commonly used external orienting devices (Bertini et al., 2001a). Although different lanthanides substituted in the same calcium binding site provide magnetic susceptibility anisotropy tensors which show some degree of collinearity (Bertini et al., 2001a), the differences may be still large enough to make the use of several lanthanides meaningful. Work is in progress to evaluate the impact on the structure quality of the inclusion of several sets of rdc from different lanthanide-substituted calbindin derivatives as well as from diamagnetic and paramagnetic calbindin in external orienting media.

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References

Akke, M., Forsén, S. and Chazin, W.J. (1991) *J. Mol. Biol.*, **220**, 173–189.

Al-Hashimi, H.M., Majumdar, A., Gorin, A., Kettani, A., Skripkin, E. and Patel, D.J. (2001) *J. Am. Chem. Soc.*, **123**, 633–640.

Allegrozzi, M., Bertini, I., Janik, M.B.L., Lee, Y.-M., Liu, G. and Luchinat, C. (2000) *J. Am. Chem. Soc.*, **122**, 4154–4161.

Arnesano, F., Banci, L., Bertini, I., van der Wetering, K., Czisch, M. and Kaptein, R. (2000) *J. Biomol. NMR*, **17**, 295–304.

Banci, L., Bertini, I., Huber, J.G., Luchinat, C. and Rosato, A. (1998) *J. Am. Chem. Soc.*, **120**, 12903–12909.

Barrientos L.G., Dolan, C. and Gronenborn, A.M. (2000) *J. Biomol. NMR*, **16**, 329–337.

Bastiaan, E.W., Maclean, C., Van Zijl, P.C.M. and Bothner-By, A.A. (1987) *Annu. Rep. NMR Spectrosc.*, **19**, 35–77.
Bax, A. and Tjandra, N. (1997) *J. Biomol. NMR*, **10**, 289–292.
Bayer, P., Varani, L. and Varani, G. (1999) *J. Biomol. NMR*, **14**, 149–155.
Bertini, I., Felli, I.C. and Luchinat, C. (2000a) *J. Biomol. NMR*, **18**, 347–355.
Bertini, I., Castellani, F., Luchinat, C., Martini, G., Parigi, G. and Ristori, S. (2000b) *J. Phys. Chem.*, **104**, 10653–10658.
Bertini, I., Janik, M.B.L., Lee, Y.-M., Luchinat, C. and Rosato, A. (2001a) *J. Am. Chem. Soc.*, **123**, 4181–4188.
Bertini, I., Luchinat, C. and Parigi, G. (2001b) In *Solution NMR of Paramagnetic Molecules*, Elsevier, Amsterdam.
Biekofsky, R.R., Muskett, F.W., Schmidt, J.M., Martin, S.R., Browne, J.P., Bayley, P.M. and Feeney, J. (1999) *FEBS Lett.*, **460**, 519–526.
Campbell, I.D., Dobson, C.M., Williams, R.J.P. and Xavier, A.V. (1973) *Ann. N.Y. Acad. Sci.*, **222**, 163.
Cavagnero, S., Dyson, H.J. and Wright, P.E. (1999) *J. Biomol. NMR*, **13**, 387–391.
Chou, J.J., Li, S. and Bax, A. (2000) *J. Biomol. NMR*, **18**, 217–227.
Choy, W.-Y., Tollinger, M., Mueller, G.A. and Kay, L.E. (2001) *J. Biomol. NMR*, **21**, 31–40.
Clore, G.M. and Gronenborn, A.M. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 5891–5898.
Clore, G.M., Starich, M.R. and Gronenborn, A.M. (1998) *J. Am. Chem. Soc.*, **120**, 10571–10572.
Contreras, M.A., Ubach, J., Millet, O., Rizo, J. and Pons, M. (1999) *J. Am. Chem. Soc.*, **121**, 8947–8948.
Delaglio, F., Kontaxis, G. and Bax, A. (2000) *J. Am. Chem. Soc.*, **122**, 2142–2143.
Déméné, H., Tsan, P., Gans, P. and Marion, D. (2000) *J. Phys. Chem.*, **B104**, 2559–2569.
Desvaux, H., Gabriel, J.C.P., Berthault, P. and Camerel, F. (2001) *Angew. Chem. Int. Edit.*, **40**, 373–376.
Feeney, J., Birdsall, B., Bradbury, A.F., Biekofsky, R.R. and Bayley, P.M. (2001) *J. Biomol. NMR*, **21**, 41–48.
Fischer, M.W., Losonczi, J.A., Weaver, J.L. and Prestegard, J.H. (1999) *Biochemistry*, **38**, 9013–9022.
Fleming, K., Gray, D., Prasannan, S. and Matthews, S. (2000) *J. Am. Chem. Soc.*, **122**, 5224–5225.
Fowler, B.A., Tian, F., Al-Hashimi, H.M. and Prestegard, J.H. (2000) *J. Mol. Biol.*, **304**, 447–460.
Hansen, M.R., Mueller, L. and Pardi, A. (1998) *Nat. Struct. Biol.*, **5**, 1065–1074.
Huang, X., Moy, F. and Powers, R. (2000) *Biochemistry*, **39**, 13365–13375.
Hus, J.C., Marion, D. and Blackledge, M. (2000) *J. Mol. Biol.*, **298**, 927–936.
Koenig, B.W., Jin-Shan, H., Ottiger, M., Bose, S., Hendler, R.W. and Bax, A. (1999) *J. Am. Chem. Soc.*, **121**, 1385–1386.
Kretsinger, R.H. (1980) *CRC Crit. Rev. Biochem.*, **8**, 119–174.
Lee, L. and Sykes, B.D. (1983) *Biochemistry*, **22**, 4366–4373.
Linse, S., Brodin, P., Drakenberg, T., Thulin, E., Sellers, P., Elmden, K., Grundstrom, T. and Forsén, S. (1987) *Biochemistry*, **26**, 6723–6735.
Luy, B. and Marino, J.P. (2001) *J. Biomol. NMR*, **20**, 39–47.
Ma, C. and Opella, S.J. (2000) *J. Magn. Reson.*, **146**, 381–384.
Meiler, J., Blomberg, N., Nilges, M. and Griesinger, C. (2000) *J. Biomol. NMR*, **16**, 245–252.
Mollova, E.T., Hansen, M.R. and Pardi, A. (2000) *J. Am. Chem. Soc*, **122**, 11561–11562.
Ottiger, M. and Bax, A. (1999) *J. Biomol. NMR*, **13**, 187–191.
Ottiger, M., Delaglio, F., Marquardt, J.L., Tjandra, N. and Bax, A. (1998) *J. Magn. Reson.*, **134**, 365–369.
Ramirez, B.E. and Bax, A. (1998) *J. Am. Chem. Soc.*, **120**, 9106–9107.
Rückert, M. and Otting, G. (2000) *J. Am. Chem. Soc.*, **122**, 7793–7797.
Sass, H.J., Musco, G., Stahl, S.J., Wingfield, P.T. and Grzesiek, S. (2000) *J. Biomol. NMR*, **18**, 303–309.
Sass, J., Cordier, F., Hoffmann, A., Rogowski, M., Cousin, A., Omichinski, J.G., Löwen, H. and Grzesiek, S. (1999) *J. Am. Chem. Soc.*, **121**, 2047–2055.
Saupe, A. and Englert, G. (1963) *Phys. Rev. Lett.*, **11**, 462.
Schwalbe, H., Grimshaw, S.B., Spencer, A., Buck, M., Boyd, J., Dobson, C.M., Redfield, C. and Smith, L.J. (2001) *Protein Sci.*, **10**, 677–688.
Tjandra, N. and Bax, A. (1997) *Science*, **278**, 1111–1114.
Tjandra, N., Grzesiek, S. and Bax, A. (1996) *J. Am. Chem. Soc.*, **118**, 6264–6272.
Tjandra, N., Omichinski, J.G., Gronenborn, A.M., Clore, G.M. and Bax, A. (1997) *Nat. Struct. Biol.*, **4**, 732–738.
Tjandra, N., Marquardt, J. and Clore, G.M. (2000a) *J. Magn. Reson.*, **142**, 393–396.
Tjandra, N., Tate, S., Ono, A., Kainosho, M. and Bax, A. (2000b) *J. Am. Chem. Soc.*, **122**, 6190–6200.
Tolman, J.R., Flanagan, J.M., Kennedy, M.A. and Prestegard, J.H. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 9279–9283.
Tycko, R., Blanco, F.J. and Ishii, Y. (2000) *J. Am. Chem. Soc.*, **122**, 9341.
Veglia, G. and Opella, S.J. (2000) *J. Am. Chem. Soc.*, **122**, 11733–11734.
Vogel, H.J., Drakenberg, T., Forsén, S., O'Neil, J.D. and Hofmann, T. (1985) *Biochemistry*, **24**, 3870–3876.
Volkman, B.F., Wilkens, S.J., Lee, A.L., Xia, B., Westler, W.M., Berger, R. and Markley, J.L. (1999) *J. Am. Chem. Soc.*, **121**, 4677–4683.
Wang, H., Eberstadt, M., Olejniczak, E.T., Meadows, R.P. and Fesik, S.W. (1998) *J. Biomol. NMR*, **12**, 443–446.
Warren, J.J. and Moore, P.B. (2001) *J. Biomol. NMR*, **20**, 311–323.
Zhou, H., Vermeulen, A., Jucker, F.M. and Pardi, A. (1999) *Biopolymers*, **52**, 168–180.
Zweckstetter, M. and Bax, A. (2001) *J. Biomol. NMR*, **20**, 365–377.