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Are proteins even floppier than we thought?

Ad Bax[1] and Nico Tjandra[2]

With ever stronger magnetic fields, magnetic alignment of proteins and nucleic acids with the magnetic field can now be observed. In such magnetically aligned systems, dipolar couplings no longer average to zero and the residual splittings contain unique information on molecular conformation and possibly mobility.

Structure determination by solution NMR so far has been based entirely on the very local information contained in NOEs, *J*-couplings and chemical shifts[1–4]. NOEs provide the all-important interproton distance information, but for a variety of technical reasons these are in practice only interpreted as semi-quantitative measures for the time-averaged interproton distance. Three-bond *J*-couplings intrinsically are highly accurate reporters on the intervening dihedral angle[5], but their measurement is often fraught with difficulty, particularly in larger systems where the natural resonance line width exceeds that of the small *J*-couplings. Chemical shifts, in contrast, can be easily measured with high accuracy, and are exquisitely sensitive to local geometry[6]. Unfortunately, at present our knowledge of the various factors determining chemical shifts, in particular those of ^{13}C and ^{15}N, remains incomplete. Therefore, these shifts too can only be interpreted as a qualitative indicator for backbone geometry and very local packing. Thus, it may be considered remarkable, and perhaps surprising, that macromolecular structures can be determined at all by NMR spectroscopy. The reason why the method works for globular structures is that 'long range' NOEs, corresponding to short interproton distances between residues far apart in the polymer sequence, are conformationally highly restrictive. However, it is clear that the NMR method has been weak at determining structures of molecules that lack such long-range constraints. For example, it is difficult to distinguish straight double-stranded DNA from slightly curved DNA on the basis of NMR data. Similarly, the method has been ineffective at determining relative positions of protein domains in cases where few interdomain NOEs can be identified.

A common assumption made in solution NMR is that, over time, a given protein will sample all orientations with respect to the static magnetic field with equal probability, and consequently that all dipolar interactions average to zero. In the absence of such averaging, that is, in the solid state, a dipolar interaction between atoms I and S is proportionate to $\gamma_I\gamma_S r_{IS}^{-3}$, where γ is the magnetogyric ratio and r_{IS} the internuclear distance. This interaction results in a splitting in the I-spin and S-spin spectra proportionate to $\gamma_I\gamma_S r_{IS}^{-3}$ $(3\cos^2\theta-1)$, where θ is the angle between the I–S vector and the direction of the static magnetic field. For directly bonded pairs of atoms such as N–H or C–H pairs, where the internuclear distance is known, the dipolar interaction therefore provides a measure for the angle θ. Many years ago, Bothner-By and coworkers demonstrated that in solution the dipolar couplings in molecules with a significantly anisotropic susceptibility tensor no longer average to exactly zero but exhibit a minute residual splitting, which scales with the square of the magnetic field[7–9]. More recently, Prestegard and coworkers demonstrated that such residual dipolar couplings, on the order of a few Hz, can also be observed in paramagnetic cyanometmyoglobin[10], and we measured such splittings in human ubiquitin[11], a diamagnetic protein with a ~10-fold smaller susceptibility anisotropy and proportionately smaller dipolar couplings. These dipolar couplings carry information on the orientation of a bond vector relative to the protein's magnetic susceptibility tensor, that is, they can orient bond vectors all relative to the same frame, regardless of their location in the molecule. This distinguishes these constraints from all other constraints used during NMR structure calculation, such as NOEs, *J*-couplings and chemical shifts, which are strictly local in nature. By their ability to define such long range order, dipolar couplings offer a potential cure to one of the principal remaining weaknesses of the NMR structure determination method.

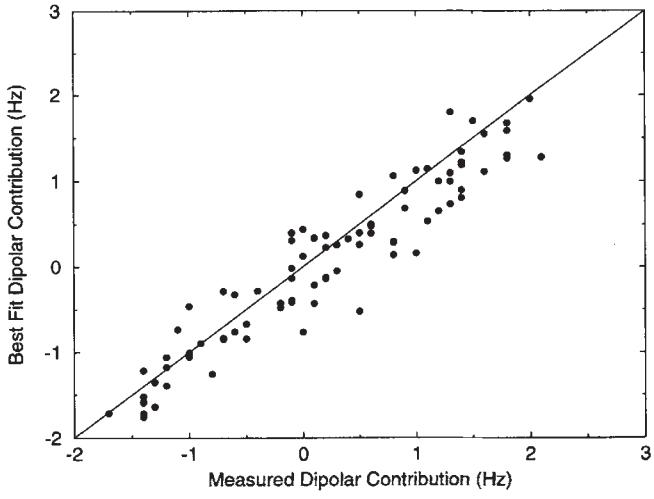

Fig. 1 Plot of the myoglobin dipolar couplings, kindly provided to us by Tolman *et al*[12], versus those predicted by a 'best-fit' magnetic susceptibility tensor with $\Delta\chi$ =1069 VVk mol^{-1}, $\delta\chi$ = –415 VVk mol^{-1} and α= –19°, β= –18°, γ =73°.

Determination of the susceptibility tensor

The magnetic susceptibility of macromolecules is generally assumed to correspond to the sum of the susceptibility tensors of its components. Whereas peptide groups contribute small amounts, contributions from aromatic rings are about an order of magnitude larger. The susceptibility tensor of paramagnetic sites or certain prosthetic groups such as haems is yet another order of magnitude larger. Therefore, although all proteins in principle have a non-zero magnetic susceptibility tensor, paramagnetic proteins frequently have considerably larger susceptibility anisotropies than diamagnetic proteins. If

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the structure of a protein is known, its susceptibility tensor can be predicted from the sum of its individual components. Alternatively, the susceptibility tensor can be determined experimentally by searching for the tensor that minimizes the difference between the individually measured dipolar couplings and those predicted by the susceptibility tensor, using the known relative positions of the dipolar coupled pairs of atoms, I and S[11]. This amounts to a three- or five-dimensional search problem in the case of an axially symmetric or a fully asymmetric susceptibility tensor, which can readily be solved provided the number of dipolar couplings measured is much greater than this number of unknowns[11].

If the protein structure were static, that is, in the absence of internal motions, one would expect good agreement between the calculated and experimentally derived magnetic susceptibility tensors, provided the susceptibilities of the individual groups are known accurately and the protein is fully monomeric. Small amplitude internal motions about the average positions, however, will scale down the dipolar couplings by a factor that corresponds to the generalized order parameter S[11–13]. Except for flexible loop regions, extensive measurement of ^{15}N relaxation times indicates that, at least for ^{15}N-^{1}H pairs, S^2 values are relatively uniform and have values of $S^2 = 0.85 \pm 0.04$, or $S = 0.92 \pm 0.02$ (ref. 14). However, it should also be emphasized that ^{15}N relaxation measurements, and thereby S^2 values, only reflect motions faster than the overall tumbling of the protein, that is, faster than ~10 ns. Dipolar couplings measured for cyanometmyoglobin by Tolman *et al.*[12] are about 27% smaller than values predicted by its crystal structures; these authors interpret this difference as evidence for very low order parameters, that is, very large amplitude motions.

Floppy myoglobin?

The two major contributions to the magnetic susceptibility tensor of myoglobin have opposite sign and stem from the paramagnetism of the metal and the diamagnetism of the haem. The paramagnetic term has been determined by LaMar and co-workers[16] on the basis of paramagnetic shifts of protons close to the metal centre, assuming the myoglobin structure to be static and identical to the crystal structure. The diamagnetic susceptibilities of several porphyrins have been measured[8] and their values are expected to be representative for the myoglobin haem. As mentioned above, fitting of the N–H dipolar couplings measured by Tolman *et al.*[12] to either the X-ray or neutron diffraction crystal structure of cyanometmyoglobin yields a susceptibility tensor which is 27% smaller than the one predicted on the basis of the sum of the paramagnetic and diamagnetic components. Systematic errors in the measurement of the dipolar couplings presumably can be excluded because very similar values were measured with different pulse schemes. Tolman *et al.* interpret the difference between the predicted and measured dipolar couplings as evidence for large amplitude dynamics. However, as amide hydrogen exchange rates are slow, hydrogen bonds must remain intact. This therefore suggests that it is not the individual amide N–H bond vectors that are undergoing the large amplitude angular excursions, but that myoglobin's α-helices move as intact units relative to one another. Indeed, this conclusion finds some support in the so-called 'essential dynamics' description of protein dynamics by the Berendsen group[17]. There are also numerous sources of other experimental evidence that such motions indeed can occur: experimentally observed hydrogen exchange requires local unfolding and solvent access, even in the core of the protein[18]; access of small ligands into the core of a protein is relatively fast[19]; rapid flipping (typically on a ms time scale) of the aromatic Phe and Tyr rings requires motions by several Ångstroms of nearby groups[20]; and even the haem itself can flip up-side down, although the latter is a very slow process[21].

The difference in the conclusions by Tolman *et al.*[12] relative to these other experimental findings is that the large amplitude deviations from the average structure do not occur just occasionally, but are present all the time: on average, the axis orientations of several of myoglobin's helices differs by 25° or more from their time-averaged positions. As ^{15}N relaxation times do not show any evidence for such large amplitude motions, they must occur on a time scale slower than that of rotational diffusion (~10 ns). However, they also must be faster than milliseconds because slower motions would result in very extensive line-broadening in the NMR spectrum.

On the other hand, there are also strong experimental arguments against such an extremely dynamic 'jello-like' myoglobin model. First, if myoglobin indeed were as dynamic as concluded by Tolman *et al.*, the measurement of the paramagnetic term of the susceptibility tensor by LaMar and co-workers[16] on which Tolman's conclusions are based, would be in error as the LaMar study assumed a static myoglobin model. Second, the very good correlation between experimentally observed ring current shifts, which are exquisitely sensitive to small local changes in structure, and those predicted by a static crystal structure[22] suggests that the structure must remain close to that observed in the crystalline state for a large fraction of time. Third, because in the jello-myoglobin model interhelical proton pairs would only be present for a very short fraction of the time, one would expect intra-helical NOEs to be much stronger than interhelical NOEs. Fourth, one would expect numerous weak interhelical NOEs that cannot be satisfied simultaneously, reflecting the distribution of interhelical NOE contacts a given proton samples over time. Although some evidence for the latter exists in a limited number of cases, usually they can be explained by small local rearrangements.

The conclusion of large amplitude collective motions in myoglobin is based on the difference between the measured and the predicted dipolar couplings in myoglobin. This conclusion conflicts with the widely held view of proteins as having rather well-defined backbones, with very rapid (sub-ns), relatively small amplitude oscillations about this average. The large amplitude motions invoked in the Tolman study would require most of the interactions which stabilize the protein's tertiary structure to be present for only a small fraction of the time. It is important to note, however, that Tolman's dipolar couplings themselves are in reasonable agreement with the static crystal structure, provided the magnitude and orientation of the magnetic susceptibility tensor are allowed to float (Fig. 1). This latter approach yields a $\Delta\chi$ which is about 27% smaller and an orientation of the $\Delta\chi$ symmetry axis which differs by ~9° from the predicted one. The answer to the question of whether the collective, intermediate time scale motions in myoglobin are real or not hinges on how reasonable a 27% difference is between the best-fit and the tensor predicted for static myoglobin. This amounts to a difference of 19% relative to the tensor predicted for myoglobin with $S^2 = 0.85$. Tolman *et al.* do not believe such a large difference is credible. In our opinion, however, it is conceivable that there are other factors, not accounted for in the Tolman study, which could influence the magnetic susceptibility ten-

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sor. For example, very weak intermolecular interaction at the high protein concentration used in the myoglobin study could alter the degree of magnetic alignment. It also would be interesting to measure the dipolar couplings in diamagnetic carbonmonoxymyoglobin, which would permit separation of the diamagnetic and paramagnetic contributions, and could rule out significant errors in the $\Delta\chi$ used for the haem.

Ubiquitin is the only other protien for which the predicted and experimentally determined magnetic susceptibility tensors have been compared[11]. Two sets of measurements, intially at 360 and 600 MHz[11] and subsequently repeated at 360 and 750 MHz, show quite good agreement with one another and yield magnetic susceptibilty anisotrophy tensors that are only sightly smaller than predicted from the sum of the group susceptibilities. This suggests that large amplitude motions are not present in ubiquitin.

Because dipolar couplings are relatively insensitive to motion (they scale with S and not with S^2), Tolman *et al.* needed to invoke rather extreme degrees of mobility to explain the discrepancy between observed and predicted dipolar couplings. If the susceptibility tensor is derived from the dipolar couplings themselves, no large amplitude dynamics are needed to explain the data and good agreement with the myoglobin crystal structure is obtained. In our opinion, therefore, because dipolar couplings are exquisitely sensitive to structure but only weakly to dynamics, they hold most promise for structure determination[10], not for the study of dynamics.

[1]*Laboratory of Chemical Physics, Building 5, National Institute of Diabetes and Digestive and Kidney Diseases and* [2]*Laboratory of Biophysical Chemistry, Building 3, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, USA Email:* bax@nih.gov

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How Src exercises self-restraint

Jack T. Nguyen[1] and Wendell A. Lim[1–3]

Two recent landmark structures of Src family kinases reveal how a sensitive conformational switch can be built with SH2 and SH3 domains.

The c-Src kinase and its family of homologues have long been known to function as molecular switches, playing a critical role regulating cell growth and differentiation[1,2]. In the oncogene v-Src this switch is broken; mutations result in a constitutively active kinase, and consequently transformation. The precise mechanism by which this switch works has remained elusive after many years of research, although it has been clear for some time that the well-studied Src Homology 2 and 3 (SH2 and SH3) domains play a central role in the process. Sicheri *et al.* and Xu *et al.* have now solved the crystal structures of the nearly intact Hck and Src kinases, respectively, in their repressed states[3,4]. These structures reveal a remarkable, conserved network of intramolecular interactions involving the SH2 and SH3 domains, which together appear to stabilize the inactive conformation of the kinase. The structures show that Src kinase repression is built upon subtle layers of interdependent interactions which could be disrupted by multiple activating inputs.

Src Family Kinases and Src Homology Domains

To date, nine members of the Src kinase family have been identified: Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk, and Yrk[1,2]. While the expression of Src is ubiquitous, expression of other family mem-

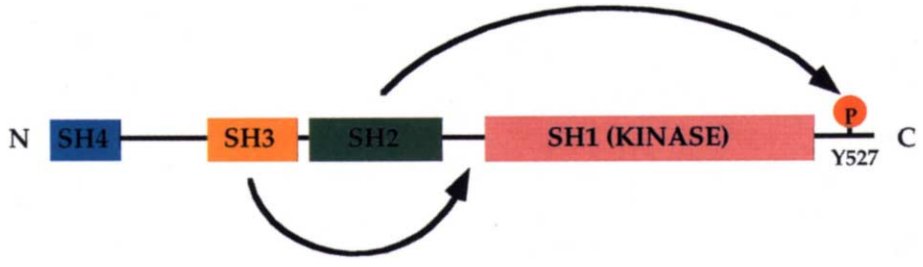

Fig. 1 Domain organization of Src-family kinases. The SH4 domain contains a myristoylation site and serves to anchor the protein to the cell membrane. SH2 and SH3 domains mediate protein–protein complexes. Phosphorylation of Tyr 527 in the C terminus by Csk is necessary for the regulation of kinase activity. Arrows represent intramolecular interactions observed in the repressed state crystal structures of Src and Hck.