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Paramagnetic NMR restraints for the characterization of protein structural rearrangements

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Abstract

Mobility is a common feature of biomacromolecules, often fundamental for their function. Thus, in many cases, biomacromolecules cannot be described by a single conformation, but rather by a conformational ensemble. NMR paramagnetic data demonstrated quite informative to monitor this conformational variability, especially when used in conjunction with data from different sources. Due to their long-range nature, paramagnetic data can, for instance, i) clearly demonstrate the occurrence of conformational rearrangements, ii) reveal the presence of minor conformational states, sampled only for a short time, iii) indicate the most representative conformations within the conformational ensemble sampled by the molecule, iv) provide an upper limit to the weight of each conformation.

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Introduction

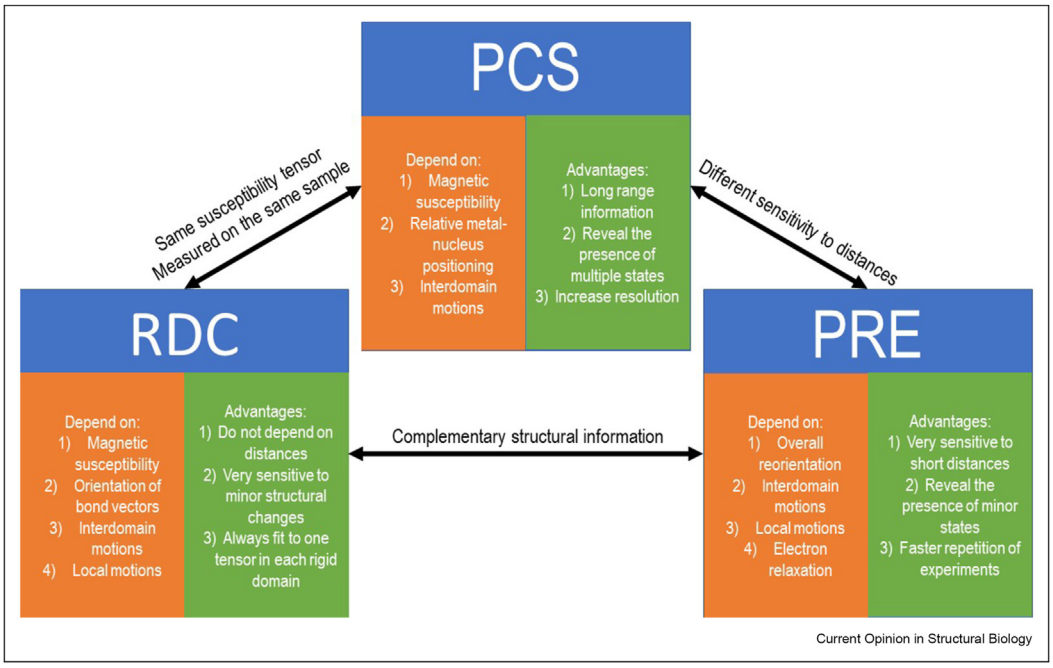
Conformational rearrangements in proteins are often key steps of functional regulation. These rearrangements often require the system to visit high-energy (i.e.: transient and scarcely populated) states. The characterization of such transient states can rely only upon very few methodologies. Among those, we can count NMR, which is an outstanding tool for the atomic-resolution characterization of dynamic processes in biomolecules. Even more so, when aided by the use of paramagnetism-based restraints, like paramagnetic relaxation enhancements (PREs), pseudocontact shifts (PCSs) and paramagnetic residual dipolar couplings (RDCs) [1], NMR turns out to be very informative to shed light on protein dynamics. Paramagnetic effects are very sensitive to even modest structural rearrangements, not observable through the more conventional (e.g. NOE) detection [2]. These NMR data can be measured in metalloproteins by replacing the diamagnetic metal ion with a paramagnetic ion (or *vice versa*), switching between paramagnetic and diamagnetic states of the same metal, and in all biomolecules upon attachment of a paramagnetic tag [3–7]. They are in fact obtained from the difference in relaxation rates (PREs), NMR shifts (PCSs), and ^{1}J-couplings (RDCs) between the paramagnetic and the diamagnetic forms of the protein [8]. When paramagnetic tags are used, it is very important that these tags are rigidly attached to the proteins, because tag mobility can largely reduce the possibility of retrieving information on the occurring protein mobility.

The potentialities of paramagnetic NMR data to shed light on conformational rearrangements can be even more profitably exploited when complemented by other experimental techniques, including X-ray crystallography and cryo-electron microscopy, or by computational studies [9]. Single-molecule Förster resonance energy transfer (FRET) [10], small-angle X-ray scattering (SAXS) and double electron–electron resonance (DEER) [11] can also result useful complementary techniques.

Experimental approaches

In the presence of a paramagnetic metal ion, the relaxation rate of a protein nucleus increases because of the additional contribution to relaxation (the PRE) from the dipole–dipole interaction between the nuclear magnetic moment and the magnetic moment of the paramagnetic metal ion [12]. This contribution mainly depends on the distance between nucleus and paramagnetic metal, besides on other terms which in most

Figure 1



Dependences and advantages as structural restraints of PCSs, RDCs and PREs measured for systems with interdomain mobility.

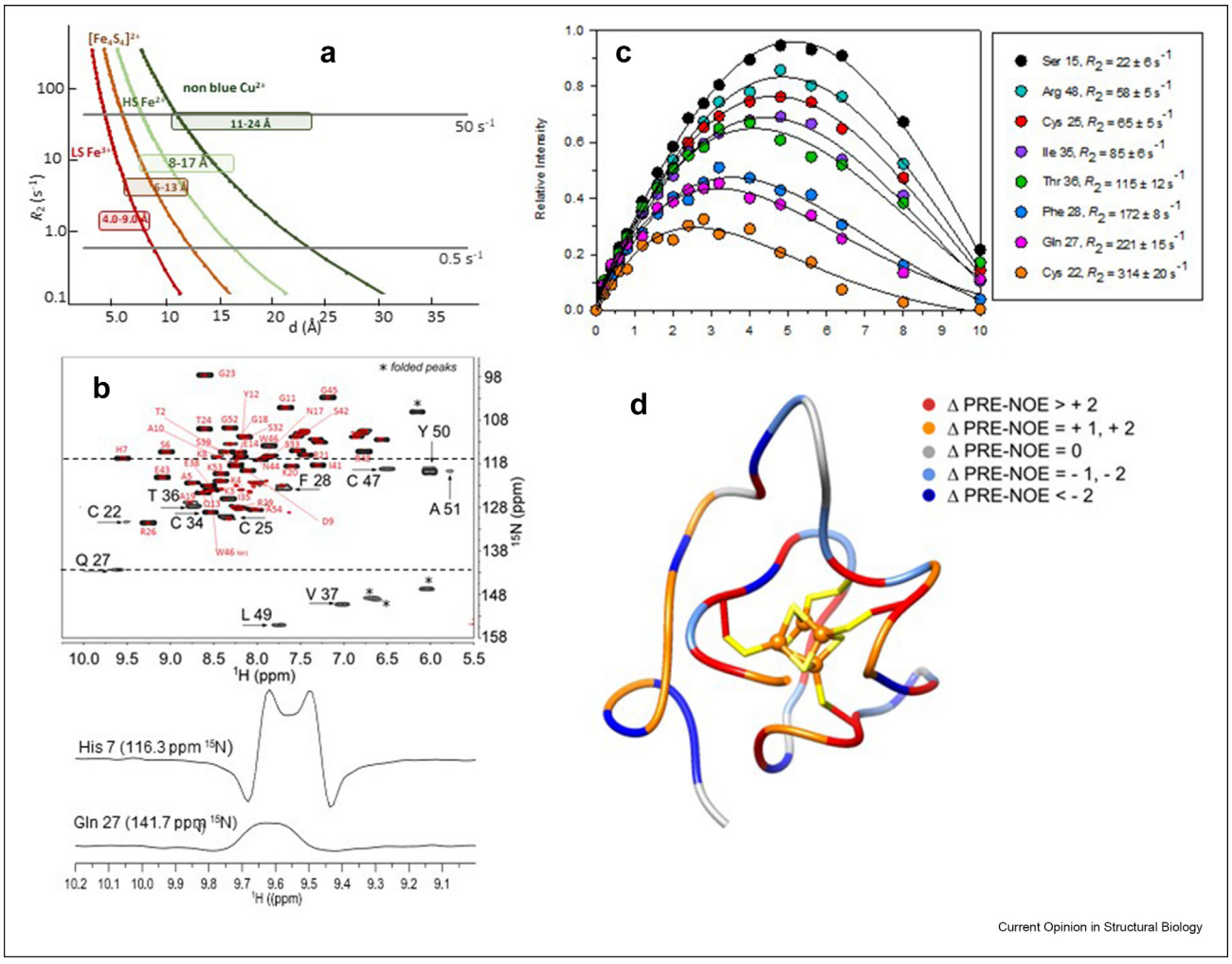
cases can be assumed constant for all nuclei (unless anisotropic effects, like the static zero-field splitting, should be considered [12,13], or Fermi-contact relaxation is effective [14]). Due to their inverse sixth-power dependence on the nucleus-metal distance, PREs are very sensitive to changes in the nuclear positions. The PCS experienced by a protein nucleus depends on the position of the nucleus in a common frame defined by the magnetic susceptibility tensor of the paramagnetic metal ion [12,15]. Therefore, from PCS measurements it is possible to obtain information on the positions of all protein nuclei in this frame. Also paramagnetic RDCs are linked by the same paramagnetic susceptibility tensor, but, at variance with PCSs, they do not change with the distance of the paired nuclei from the metal, and rather depend on the orientations of the paired nuclei in the common frame of the magnetic susceptibility tensor [12,16] (Figure 1).

The quantification and exploitation of these effects is not trivial because paramagnetic relaxation limits the efficiency of coherence transfer pathways, which is essential for signals assignment, prevents the accurate measurement of relaxation rates, and even signal identification. For each NMR observable quantity, experimental approaches provide lower and upper limit values[1]; these values, depending on the property of the paramagnetic center (electronic correlation time, spin quantum number, excited states of the electron spin ladder …), on the magnetic field and on the investigated nucleus, are then converted into upper and lower limit distances that define the threshold limits for the exploitation of paramagnetic NMR data (see Figure 2).

All NMR experiments can be customized to extend these limits and therefore increase the number and the accuracy of paramagnetism-derived structural constraints: time consuming pulse sequence elements can be sacrificed to reduce the length of the sequence, and relaxation-weighted coherence transfer functions [17] are used to set the NMR parameters for mixing, acquisition and recovery delays. The γ^2 dependence of paramagnetic relaxation has led to many fruitful applications of heteronuclear direct detection [18,19] such as the use of CACO and CON experiments to decrease the "blind sphere" around the paramagnetic center or to obtain accurate measurement of RDC [20–23]. Two simple implementations that are particularly helpful to extend the detectability of "paramagnetic signals" are the inversion-recovery (IR) and the antiphase detection (AP). The IR building block, which is constituted by a non-selective 180° pulse on the spins of interest followed by a recovery period, acts as a longitudinal relaxation-based filter that allows the identification of weak, broad, and barely detectable signals also when they are overlapped by sharp, slow relaxing, signals

[1] for instance, the lower limit for PRE depends on the accuracy of the measurement and on the uncertainty of diamagnetic contributions to relaxation, while the upper limit depends on the fastest rate that can be measured with a given precision.

Figure 2



a. PRE R_2 values simulated for ^{1}H nuclei in a small protein (<10 kDa) at 500 MHz using different electronic correlation times. Assuming 50 s^{-1} and 0.5 s^{-1} as upper and lower limits for the detection of PREs, the regions affected by PREs have different dimensions. **b**. The comparison of a ^{15}N-HSQC-AP (black) with a routine HSQC experiment (red) identifies a number of signals, affected by paramagnetic relaxation, that are observable only in the HSQC-AP experiment. The selected rows show that shape of the antiphase doublet. The spectra have been collected, at 500 MHz, for the High Potential Iron–Sulfur Protein PioC, containing a $[Fe_4S_4]^{2+}$ cluster. Black labels are used to indicate residues that have been assigned only using the ^{15}N-HSQC-AP. **c**. In the R_2-weighted HSQC experiment, the coherence transfer delay is arrayed. The peak intensity depends on the efficiency of the scalar coupling under the effect of transverse relaxation. The position of the maximum peak intensity shifts to shorter times by increasing transverse relaxation. By fitting the experimental values, precise measurements of R_2 up to ca 400 s^{-1} are possible. Selected data are taken from the $[Fe_4S_4]^{2+}$ HiPIP protein PioC. **d**. The solution structure of the small paramagnetic protein PioC (which has been also used for panels **b** and **c**), has been solved by using a combination of standard and paramagnetism based NMR restraints and the effects of the various contributions has been analyzed and the number of PRE-based NMR restraints was found to be comparable to those obtained using routine NOEs. Regions where the number of PRE restraints exceeds that of long range NOE restraints are shown in red and orange; regions were the opposite occurs are shown in blue and light blue.

[24–26]. The AP detection scheme performs signal acquisition immediately after the last 90° mixing pulses of the sequence (^{13}C- or ^{15}N HSQC, HNCA, CACO …), the antiphase is converted into observable single quantum coherence during the evolution delay and the signals are acquired as antiphase doublets. By embedding chemical shift and scalar coupling evolution in the same delay, like a COSY approach, the refocusing spin-echo building blocks can be removed. This will revive signals that, in a standard experiment, vanish due to the effect of transverse relaxation during the refocusing step. A very relevant aspect of these building blocks is that they can also be used to measure R_1 and R_2 relaxation rates within ranges that are not accessible by standard experiments [24,27] and significantly increase the range of application of PREs and reinforce the role of paramagnetism-based NMR restraints for the solution structure of biomolecules (Figure 2).

Obtaining reliable paramagnetic restraints is of central importance for their use in the structural and dynamic characterization of biomolecular systems. For both

restraints, the lack of electron delocalization and Fermi-contact contributions to nuclear relaxation and NMR shift should be ensured. Their accuracy also depends on the structural invariance between the diamagnetic and the paramagnetic forms of the biomolecules. As already mentioned, in case of those systems that are made paramagnetic by the attachment of paramagnetic tags, it is very important that these tags are rigidly attached to the proteins and that the attachment sites do not experience local mobility.

Conformationally averaged paramagnetic NMR data

In the presence of mobility, PREs, PCSs, and RDCs are averaged over all sampled molecular conformations, and thus they can be used to recover information on the occurring structural rearrangements, as the reader will see in the following sections. In the analysis of the averaged PRE, PCS, and RDC data (Figure 1), attention should also be paid to the fact that these values depend on different motional averaging mechanisms [28]. Observed RDCs probe conformational rearrangements occurring on time scales of the order of, or faster than, milliseconds and that are faster than the chemical shift changes caused by the structural rearrangements. In the assumption that a protein domain moves rigidly with respect to the magnetic susceptibility tensor of the paramagnetic metal (located in a different domain), all experimental RDCs measured for the moving domain depend on a single mean tensor that results from the average of the magnetic susceptibility tensor with respect to the moving domain. Therefore, the RDCs can be analyzed as is done for rigid systems, with the difference that they depend on a tensor which is smaller the larger the mobility. In the extreme case of a domain mobility causing all orientations to be equally sampled, the mean tensor is zero, and so are all the RDCs. The detection of protein domains with RDCs in agreement with different mean tensors is a straightforward indication of the occurrence of interdomain mobility.

Also averaged PCSs result from the mean over all conformational rearrangements occurring faster than the difference in chemical shifts within the experienced conformations. However, at variance with respect to RDCs, even in the case of a domain moving rigidly with respect to the metal-bearing domain, the experimental PCSs do not depend on any single mean tensor (unless during the motion all nuclei maintain a fixed distance from the paramagnetic metal), and they should not be analyzed with the equation derived for the case of no mobility. When paramagnetic tags are attached to protein domains, only in the case of non-mobile tags, PCSs and RDCs can thus be fit jointly to provide a single tensor in agreement with both sets of data. The disagreement between PCS-derived tensor and RDC-derived tensor can be used to monitor tag mobility [29,30].

Observed PREs probe conformational rearrangements occurring on time scales faster than the chemical shift changes caused by the experienced conformations, and faster than the nuclear transverse relaxation times (in proteins of the order of milliseconds). At variance with PCSs and RDCs, experimental PREs cannot be determined from the weighted average of the values corresponding to the experienced conformations as if the protein were “frozen” in such conformations, because averaged PREs depend on the time of interconversion among the conformations, which can modulate the spin electron—nucleus dipole—dipole interaction. The influence of this time of interconversion on the PRE values also changes with the degree of spatial restriction of the conformational rearrangements, and can be modeled with an order parameter using the Lipari-Szabo approach [28,31].

Shedding light on structural rearrangements

Due to their long-range nature, paramagnetic data can easily show the occurrence of conformational rearrangements not detectable by X-ray crystallography. For instance, PCSs and RDCs measured for the paramagnetic tagged bacteriophage T4 lysozyme indicate that, in solution and in the absence of substrate, the protein structure is on average more open than suggested by the crystallographic closed conformation [32]. Paramagnetic data can also immediately show the occurrence of conformational rearrangements upon ligand binding. The large changes in the PCSs and RDCs values measured for the C-terminal domain of calmodulin, in the presence of a paramagnetic ion bound to its N-terminal domain, clearly point to the extensive interdomain rearrangements caused by ligand binding [33]. Similarly, ligand-driven conformational changes of the multi-domaindomain protein MurD were characterized from the analysis of the PCSs available upon paramagnetic tag binding of one domain: the protein passes from an open state in the apo form, to a semi-closed state upon ATP-Mg^{2+} binding, and to a fully closed state after hydrolysis of ATP [34].

Also PREs can reveal the occurrence of structural rearrangements: for instance, PREs were used in ^{19}F-NMR spectroscopy to assign the observed resonances and detect domain movements in a glutamate transporter homolog [35], and to probe the differential conformational change of the disordered part of an oncogenic transcription factor complex, the Myc-Max heterodimer, upon DNA binding [36].

In the presence of a paramagnetic cosolute, a protein nucleus may experience ensemble-averaged solvent PRE when the protein fluctuates between different conformational states [37]. Averaged solvent PREs were recently used to probe and quantitatively describe

cosolute–protein interactions at atomic resolution, in terms of the average of the interspin distance and an effective correlation time [38].

Unearthing minor states

Due to the inverse sixth-power dependence on the nucleus–metal distance, PREs are very large for nuclei at short distances from the paramagnetic metal, so that they can reveal the presence of these conformational states, although sampled only for a short time. Scarcely sampled conformations might thus be detected through the observation of large PREs that are not consistent with the available crystallographic structures [39,40].

Minor states can also be determined by coupling PCSs with Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion (PCS–CPMG). Relaxation dispersion experiments can allow observing the occurrence of conformational rearrangements of proteins made paramagnetic upon attachment of a paramagnetic tag. To this purpose, both the paramagnetic tag and the attachment site on the protein should be completely rigid and the paramagnetic center should not exchange between different coordination geometries [41]. A high-energy structure can be determined by extracting the PCS of each residue in the minor state from paired diamagnetic and paramagnetic $^{1}H^{N}$ CPMG relaxation dispersion experiments [42]. Of note, PCSs magnify CPMG relaxation dispersion profiles, thus enabling accurate measurement of both populations and NMR shifts. The approach was recently applied [42] to characterize the high-energy excursion of the multidomain enzyme adenylate kinase during catalysis. This metalloprotein was shown to access a partially open state, that corresponds to about 15° domain opening compared to the closed state.

Retrieving structural ensembles

The presence of molecular structural rearrangements poses the need of recovering conformational ensembles, defined as sets of structures with their corresponding statistical weights (not to be confused with the uncertainty ensembles—such as the NMR structure "families," which only reflect the limited information available on the system) [43]. The reconstruction of the conformation ensemble really sampled by the system cannot possibly be achieved, because the problem is severely underdetermined.[2] Different strategies were however introduced to recover at least some information on the structural variability of the system which are contained in the paramagnetic data [44,45].

Possible ensembles of conformations can be determined by selecting conformers from a precalculated pool of structures providing averaged data in agreement with the experimental data. This pool may comprise all sterically possible conformations (with properly optimized resolution), or it may be limited to the conformations calculated from a molecular dynamics trajectory (of sufficient length), or comprise only conformations previously selected on the basis of different experimental data. Since a unique probability distribution for protein conformations cannot be recovered from the averaged data, no reliability can be granted to any of the reconstructed ensembles nor to any conformation of the ensembles. Dramatically distinct ensembles can be obtained by different approaches [46], or even by independent runs from the same approach, and discerning their similarities and differences is essential [47].

The goal thus becomes that of pinpointing the conformations that are certainly the most representative to describe the conformational rearrangements of the protein. One strategy which can be implemented to this aim is that of using the smallest number of conformations that is sufficient to achieve a good agreement with the experimental data (maximum parsimony solution) [48]. This should provide at least an estimate of the conformational heterogeneity sampled by the system.

This approach can be implemented by considering a limited number of crystallographic structures, complemented by few structures selected within molecular dynamics calculations, to determine an ensemble fulfilling the experimental data [49]. Caution is however needed when this methodology is used because the presence of structural noise in the crystallographic structures may suggest a fictitious structural variability not really addressed by the data. Therefore, the approach requires that the experimental NMR data are preliminary analyzed to check whether they can be accommodated together with the crystallographic data to generate a refined structural model [33].

A different approach consists in recovering the ensemble with the broadest and flattest probability distribution in agreement with the averaged data (maximum entropy solution). These solutions result in ensembles with a very large number of conformers with very small probabilities, obtained by reweighting predefined pools calculated from statistical models or unbiased molecular dynamics simulations which often take advantage of enhanced sampling techniques.

Maximum parsimony ensembles are often used in the analysis of the conformational variability of two-domain proteins with flexible linkers. The protein Pin1 contains separate regulatory and catalytic domains that sample "extended" and "compact" states, and ligand binding changes this conformational equilibrium [50]. The

[2] The ensemble determination can be represented for most of the observables as finding the solutions of a linear system of equations (vide infra). By the Rouché-Capelli theorem, if the unknowns (i.e., the weights of the conformations if one assumes to be able to sample all the accessible conformations) outnumber the experimental observables, the number of solutions is infinite.

latter was characterized using time-averaged PREs, RDCs, interdomain NOEs and DEER data with a 70:30 population of the compact and extended states.

The dynamic interactions between a partially flexible dimeric protein, HIV-1 protease, and a paramagnetic variant of a metallacarborane-based ligand was recently analyzed using PCSs, molecular dynamics simulations and quantum chemistry calculations performed to estimate the magnetic susceptibility anisotropy tensor [51]. The qualitative features of the experimental PCS data could be reproduced by averaging over the conformations calculated in the molecular dynamics simulation. This approach has been suggested for the characterization of transient intermolecular interactions accompanied by conformational plasticity of the binding interfaces, fundamental to many biological processes.

Along the same lines, the structural rearrangements of the intrinsically disordered DNA-binding region of the MYC-associated transcription factor X was characterized by integrating PREs, molecular dynamics simulations and electron paramagnetic resonance (EPR) measurements, and showed the presence of highly flexible and spatially extended conformers together with hinged conformations, which modulates DNA-complex formation [52].

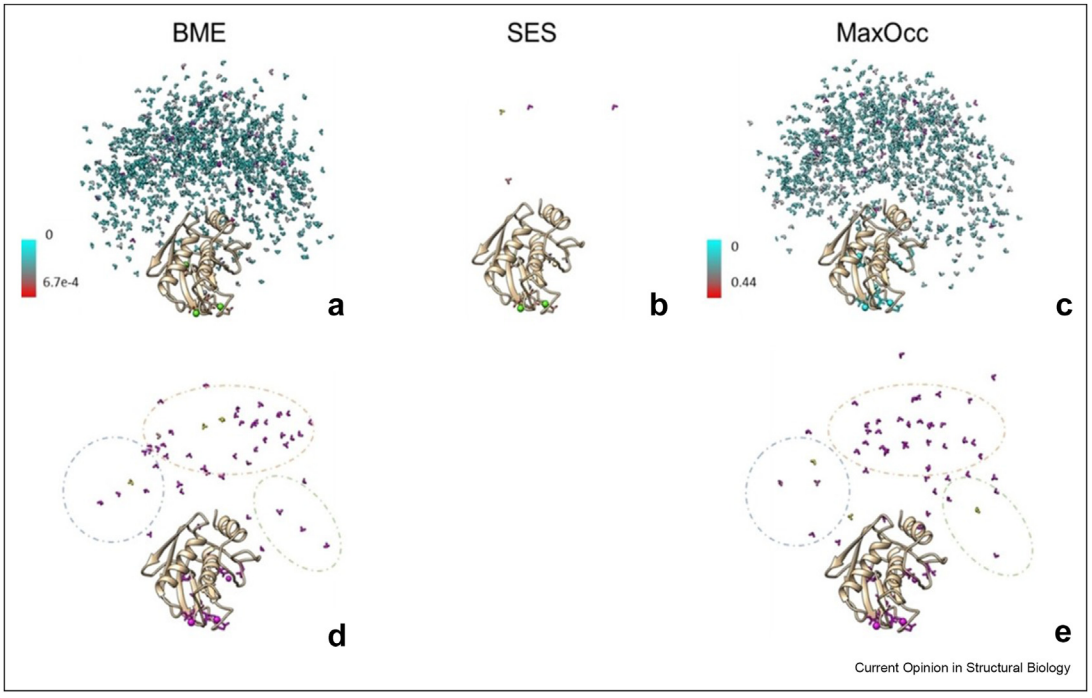
An integrated approach using PREs, NMR chemical shifts, FRET and SAXS data has been proposed to derive quantitative conformational ensembles describing intrinsically disordered proteins [53]. The analysis indicated complementarity between the different data (particularly FRET and PREs) and their importance to generate meaningful conformational ensembles.

Ranking conformers according to their maximum weight

With a different approach, the experimental average data can also be analyzed not to retrieve ensembles of structures, but rather to evaluate how each conformation is "compliant" with the experimental data. This approach provides information on the maximum weight, or Maximum Occurrence (MaxOcc), that a conformation can have [46]. The maximum occurrence value of each conformation is independent on the other conformations composing the ensemble, and the calculation requires no *a priori* assumptions, except that all possible conformers have been included in a precalculated pool of structures.

Using the MaxOcc approach it was thus possible, for instance, to determine the relative position of the two domains of the MMP-1 protein with the largest possible

Figure 3



3D representation of the conformational variability of MMP-1. The C-terminal domain in the different conformations is represented as a triad of axes positioned in the center of mass of the C-terminal domain. The figure shows the 500 conformations with highest probability in Bayesian maximum entropy (BME, **a**), the Sparsest Ensemble Selection ensemble comprising of 4 conformations with the lowest target function (SES, **b**), and the 500 conformations with higher MaxOcc values (**c**). For the sake of readability, we show also the 50 conformations with larger probability in BME (**d**) and with higher MaxOcc (**e**). Reproduced with permission from Ref [55] © 2020 Wiley-VCH GmbH.

probability in solution (Figure 3) [54]. This conformation is very different from that of the solid-state structure and suggests that the protein is poised to interact with collagen and can thus readily proceed along the steps of collagenolysis.

A comparison of the outcomes of MaxOcc, maximum parsimony and maximum entropy solutions indicates that the three approaches behave similarly in pointing out conformational preferences, and the comparison between the reconstructed areas is informative about the amount of information that is actually encoded in the data (Figure 3) [55]. Accordingly, the conformational space sampled by domain reorientation of linear diubiquitin, probed from PCSs and RDCs arising upon attachment of Tm^{3+}-tags in different positions of both ubiquitin units, was consistently characterized using maximum parsimony and MaxOcc approaches [56]. The analysis indicated that a significant population of compact conformers exists even in the absence of the target proteins, suggesting that conformational selection may contribute to protein binding.

The MaxOcc calculations, providing an upper bound to the statistical weight of each protein conformer, is expected to converge towards the true statistical weights by increasing the number of independent experimental datasets. The presence of restraints of different origin to be included together with the paramagnetic NMR restraints can be quite useful in this respect. SAXS and DEER data, for instance, can provide important complementarity information [11].

Paramagnetic data contain precious information also for the characterization of protein encounter complexes, which are formed as the protein partners approach each other in solution, before the formation of a specific protein–protein complex [57]. These encounter systems, very difficult to observe with traditional structural techniques, have been successful investigated using paramagnetic restraints, and PREs in particular. One example is the lowly populated encounter complex between cytochrome P450cam and putidaredoxin tagged with CLaNP-7 [58]. Using the MaxOcc approach, it was shown that putidaredoxin visits a large area of the surface of cytochrome P450cam and that several minor states are present that together represent a small percentage of the population in the presence of a strongly dominating major conformation of the protein complex. The distribution of these minor states correlates with the electrostatic potential map around cytochrome P450cam [59].

Conflict of interest statement

Nothing declared.

Data availability

No data was used for the research described in the article.

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