Supplementary Material

Towards A Unified Representation of Protein Structural Dynamics in Solution

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Reference 45 in full:

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Methods

Accelerated Molecular Dynamics:

The details of the accelerated molecular dynamics method have been discussed previously in the literature [1, 2] and is further described in the manuscript. The essential idea behind accelerated molecular dynamics is to define a reference, or 'boost energy', E_b, which is fixed above the minimum of the potential energy surface. It should be noted that if the boost energy is set too large for a given acceleration parameter, the modified potential energy surface becomes iso-energetic, resulting in a random walk through phase space. In the present work, we made considerable efforts to avoid this 'over-acceleration' regime: Comparison of the order parameters at different acceleration levels reveals that even at the most aggressive acceleration level employed in this work, the observed enhanced conformational space sampling is restricted to well defined regions of the protein, an observation that is not concurrent with a random walk. On increasing the boost energy further and entering the over-acceleration regime, we observed a global decrease in the order parameters across the entire protein. Furthermore, we performed additional AMD simulations using a slightly larger acceleration parameter, and observed similar conformational space sampling.

One of the favorable characteristics of AMD is that it yields a canonical average of an observable, so that thermodynamic and other equilibrium properties of the system can be accurately determined. The corrected canonical ensemble average of the system is obtained by re-weighting each point in the configuration space on the modified potential by the strength of the Boltzmann factor of the bias energy, \( \exp[\beta \Delta V(r)] \) at that particular point. Whilst AMD represents a robust free energy sampling method, obtaining an accurate estimate of the time-scale of the observed conformational space...
sampling is extremely difficult: The application of the bias potential has a strong effect on the transmission coefficient. The only way to avoid this problem would be to set the boost energy below the entire transition state region, thereby preserving the transition-state theory formalism. However, considering that proteins possess a highly rugged and structured potential energy landscape, setting the boost energy below the entire transition state region on the protein PES, would not allow acceleration over the larger energy barriers that separate the conformational sub-states.

Simulation Details:

The X-ray crystal structure of the protein ubiquitin (PDB code 1UBQ) was placed in a periodically repeating box with 6,500 water molecules. Initially a set of ten standard classical MD simulations were performed. In each case, the system was brought to thermodynamic equilibrium at 300K, 1 bar pressure using a Langevin thermostat with a collision frequency of 3-ps\(^{-1}\) and a Berendsen weak-coupling pressure-stat. For each of the ten simulations a different random seed generator was employed and, after equilibration, a 5-ns production MD simulation was performed under periodic boundary conditions with a time-step of 1-fs. Electrostatic interactions were treated using the Particle Mesh Ewald (PME) method [3] with a direct space sum limit of 10-A. The ff99SB force-field [4] was used for the solute residues and the TIP3P water force-field was employed for the solvent molecules. All simulations were performed using the AMBER8 code [5]. These initial ten 5-ns MD simulations provided the starting point for the biased potential accelerated molecular dynamics simulations discussed below, and were also used as a control set in order to assess the improvement in the calculation of the NMR observables on enhancing the conformational space sampling.

In total, twenty accelerated molecular dynamics (AMD) simulations were performed at eight different acceleration levels. The acceleration parameter, \(\alpha\), in each case was fixed at 60-kcal/mol and the acceleration level was controlled by varying the boost energy, \(E_b\). The acceleration was applied across all dihedral angle terms in the solute. The boost energy for the eight acceleration levels was set at 100, 150, 200, 250, 300, 350, 400 and 450-kcal/mol above the dihedral angle energy (estimated from the average dihedral angle energy from the unbiased 5-ns MD simulations). Each AMD simulation was performed for 8,000,000 steps (the equivalent of an 8-ns standard MD simulation) and the physical conditions, force-fields and all other simulation parameters employed were identical to those described above for the standard 5-ns MD control set. The atomic coordinates and necessary energetic data (such as the magnitude of the bias potential) were saved across each trajectory every 5 steps for analysis. All AMD simulations were performed using an in-house modified version of the AMBER 8 code.

For each AMD simulation, the conformational space for each trajectory was reweighted to obtain the correct canonical Boltzmann distribution. This means that at each acceleration level we have twenty free energy weighted trajectories, which are representative of twenty long time-scale 'brute-force' MD simulations. Although we accept the fact that the free energy weighting is only approximate for any single AMD trajectory, it does provide a reasonable representation of how much time the system spends in one region of conformational space before undergoing a stochastic transition to another conformational micro-state. However, we significantly improve our estimation of the free energy surface by performing trajectory averaging over the twenty free energy weighted AMD simulations obtained at each acceleration level: Obviously, the twenty AMD simulations at a given acceleration level do not sample exactly the same conformational space. Based on the seminal work of Frauenfelder [6], our understanding of long time-scale dynamics in small globular proteins, such as ubiquitin, is that the system sits in a well defined energy minimum and on slow time-scales undergoes stochastically mediated dynamic excursions to higher energy, low populated conformational states. The accelerated molecular dynamics approach increases the frequency of these dynamic excursions. After free energy
weighting of each trajectory, we find that the low energy regions of conformational space are well-conserved in each individual AMD trajectory, whilst the higher energy, low populated regions of conformational space are differentially sampled. As such, trajectory averaging not only provides a means of obtaining a meaningful ensemble average, but also improves the free energy weighting, particularly in the low energy, highly populated regions of the conformational space. As a direct result of this, no individual AMD trajectory provides \(R_{\text{cum}}\) values for any NMR observable as low as the trajectory averaged results.

After reweighting to the correct canonical Boltzmann distribution, a clustering analysis was performed on each AMD trajectory. The clustering protocol was based on QR factorization and principal components analysis and a series of short (3-ns) classical MD simulations were seeded from the resulting cluster. The initial 0.5-ns was discarded and a MM/PBSA analysis [7] on the resulting MD simulations was used to validate the AMD free energy weighting protocol. Using the approximate free energies, a set of large free energy weighted structural ensembles for each AMD trajectory was generated from the seeded MD simulations. These ensembles, which represent free energy weighted trajectories sampling the conformational space explored by the AMD trajectories at the relevant acceleration level, do not possess local structural distortions that potentially arise in the AMD trajectories through implementation of the bias potential.

### Calculation of Order Parameters:

For all molecular ensembles (standard classical MD and those generated from the seeded free energy weighted MD simulations generated from the AMD trajectories), order parameters, \(S^2\), were calculated within the framework of the Lipari-Szabo formalism [8]. The structures for each ensemble were superposed by mass-weighted backbone root-mean-square fitting to the average structure (residues 1-71), and the order parameters were calculated as [9]:

\[
S^2 = \frac{1}{2} \left[ \sum_{i,j=x,y,z} \left< \mu_i \mu_j \right> \right] - 1
\]

where \(\mu(i)\) are the Cartesian coordinates of the normalized inter-nuclear vector of interest (N-H) and \(i,j=x,y,z\). All order parameters were trajectory averaged.

### Singular Value Decomposition (SVD) and Calculation of RDCs and Scalar J-Couplings:

The principal difficulty concerned with the direct calculation of RDCs and scalar J-couplings arises in the determination of parameters defining the strength of the interaction. In the case of RDCs, five unknown parameters are required to explicitly define the alignment tensor. As our simulations are performed in explicit solvent, in the absence of any alignment medium, it is not possible to define explicitly from the simulation alone the preferential alignment of the molecule in a given alignment medium. The issue is further complicated by the fact that the structure, dynamics and preferential alignment of the molecule are mutually dependent: The alignment tensor depends on the shape and anisotropy of the molecule, which is specifically related to the structure. Dynamic motions on different time-scales result in small changes in the shape and anisotropy of the molecule, which, in turn result in small changes in the preferential alignment tensor for a given alignment medium. The approach taken in this work involves the use of a singular value decomposition (SVD) analysis to determine the optimal alignment tensor for each molecular ensemble.[10] SVD is an exquisite method for solving a set of simultaneous equations. Explicitly, the optimal alignment tensor and RDCs for each molecular ensemble were calculated in a reduced form as:
where $x, y, z$ are the Cartesian components of the normalized bond vector of interest (in the case of N-H RDCs, the N-H bond vector), $A_{ij}$ is a vector containing the five components necessary to completely define the 3x3 alignment tensor (bearing in mind that this tensor is symmetric and traceless) and $D_{\text{red}}$ is a vector containing the experimental RDCs for the particular alignment medium. The matrix on the left hand side of the equation, which describes the bond vector fluctuations, has dimensions $(N, 5)$, where $N$ is the number of RDCs in the given alignment medium. This matrix is formulated from the molecular ensemble where the brackets $<...>$ represent ensemble averages. Singular value decomposition of the matrix of bond vector fluctuations liberates the optimal alignment tensor components, from which the theoretical RDCs can be calculated. The principle behind such an analysis is that there should exist some optimal ensemble which represents the conformational space sampled by the system over the time-scales to which RDCs are sensitive (ie. up to 10-ms for N-H RDCs). For this optimal molecular ensemble, and its optimal SVD-calculated alignment tensor, the resulting theoretical RDCs will be in best agreement with the experimental observables. For molecular ensembles that sample too little or too much conformational space, the SVD analysis will attempt to find the best possible alignment tensor for that particular ensemble, but the resulting RDCs will not be optimal. As mentioned above, whilst the AMD approach represents a particularly efficient method for sampling conformational space and obtaining free energy weighted molecular ensembles, information concerning the specific time-scale of the conformational space sampling is not available. In the present work, we perform a series of AMD simulations at increasing acceleration levels to obtain a set of free energy weighted molecular ensembles that systematically sample an increasing amount of conformational space. By using the SVD analysis to obtain the optimal alignment tensor and hence the theoretical RDCs for each molecular ensemble, we can identify the most appropriate acceleration level (ie. the optimal conformational space sampling) to reproduce the experimental RDCs.

The analysis presented in the paper was performed over 23 experimental N-H RDC datasets. In total, 27 N-H RDC datasets were available in the literature. Of these 27 datasets, two were used to generate the fitted 1D3Z structure, which we use to gauge the accuracy of our simulations, and so these two datasets were not included in the analysis. A further two datasets were found to have a very low number of RDCs (less than 40), and these datasets were also found to be more noisy: Neither the 1D3Z structure, or any of our molecular ensembles could reproduce the RDCs in these two datasets to the accuracy obtained in the other 25 datasets, and so these two datasets were also rejected. The only N-H RDC not used in the analysis was that for residue 5. The N-H order parameter for residue 5 was found to be much higher than the associated experimental spin relaxation order parameter for this residue, even at the highest acceleration levels. This residue provided anomalously poor results across all the datasets compared to all other RDCs. Although we do not understand the reasons for this anomalous behavior, we assume that this is a force-field issue.

A similar approach was also applied to calculate the backbone scalar $J$-couplings: We calculated three backbone scalar $J$-couplings, $J^3(H^N, H\alpha)$, $J^3(H^N, C\beta)$ and $J^3(H^N, C\gamma)$. The magnitude of all these $J$-couplings is strongly related to the backbone $\phi$ angle, and can in general be described using the well-known Karplus equation[11]:
where A, B and C are the Karplus parameters, and θ is an offset angle, which typically has a value of 180° for $^3J(H^N, C^\gamma)$, -60° for $^3J(H^N, H\alpha)$ and 60° for $^3J(H^N, C\beta)$. In order to calculate these scalar J-couplings, we used the SVD analysis to obtain the optimal Karplus parameters for each molecular ensemble:

$$\begin{bmatrix}
<\cos^2(\phi_1+\theta)> & <\cos(\phi_1+\theta)> & 1 & J_1 \\
<\cos^2(\phi_2+\theta)> & <\cos(\phi_2+\theta)> & 1 & J_2 \\
... & ... & ... & ...
<\cos^2(\phi_N+\theta)> & <\cos(\phi_N+\theta)> & 1 & J_N
\end{bmatrix}$$

In each case, the analysis was initially performed using the typical θ offset angles defined above. The θ-offset values were then optimized by changing the θ-offset value in 1° steps and repeating the SVD analysis until the best reproduction of the experimental scalar J-couplings was achieved.

The R-factor:

The agreement between the theoretical and experimental RDCs and scalar J-couplings was monitored using the R-factor. Explicitly, for a given RDC or scalar J-coupling dataset, the R-factor is given by:

$$R_{factor} = \sqrt{\frac{\sum_{i=1}^{N}(X(i)_{theory} - X(i)_{exp})^2}{\sum_{i=1}^{N}(X(i)_{exp})^2}}$$

Where $X(i)_{theory}$ and $X(i)_{exp}$ are the theoretically determined and experimental observables respectively. As described in detail above, experimental RDCs and scalar J-couplings report both a time- and an ensemble average. In order to treat the statistical mechanical (ensemble) averaging, RDCs and scalar J-couplings were respectively calculated for every molecular ensemble generated at the same acceleration level, and the resulting values were then averaged. The R-factors for each RDC dataset or each scalar J-coupling were calculated using the trajectory averaged theoretical values. Finally these R-factors were summed to give a trajectory averaged cumulative R-factor ($R_{cum}$). In the case of the N-H RDCs, the cumulative R-factors were summed over all 23 RDC datasets. For the scalar J-coupling analysis, the summation was performed over the three scalar J-couplings. The R-factor is directly related to the Q-factor as:

$$R_{factor} = \frac{1}{\sqrt{2}} Q_{factor}$$

Accuracy of the Method:

The AMD/SVD approach discussed in this paper represents a fast and efficient means of accurately reproducing experimental NMR observables that report on structural/dynamic averages over long time-scales usually inaccessible to standard molecular dynamics simulations. Some points merit discussion: First, it should be stressed that the ability of the method to accurately reproduce RDCs and scalar J-couplings is strongly dependent on the accuracy of the force-field, which not only determines the structural quality of the molecular ensembles, but also defines the accuracy of the relative free energy weighting. The accuracy of the force-field has already been shown to have a significant effect on the ability of simulations to reproduce NMR observables [10], such as RDCs and spin relaxation order
parameters. Although we have used the latest generation ff99SB force-field, it is clear that this force-field is not exact, and force-field development is still an extremely active area of research. Many previously published papers concerning the structural/dynamic interpretation of RDCs have been based on rigorous fitting procedures, either to obtain a single copy representation of the time-ensemble-averaged structure (such as 1D3Z) or to generate a hypothetical optimal representative molecular ensemble using either ensemble- or time-averaged restraints (such as the EROS ensemble referred to in the paper). These rigorous fitting protocols which generally make use of a huge number of experimental observables (such as thousands of NoE distance restraints combined with information from multiple RDC datasets measured in multiple alignment media, as well as scalar J-couplings and chemical shift data) are not subject to the accuracy of the force-field. Indeed in many cases, such as the EROS ensemble, the force-field employed is usually minimalistic, only including bond, angle and simple VdW terms to define the connectivity and prevent the close approach of non-bonded atoms. In light of this, it is not surprising that a completely unrestrained molecular dynamic simulations cannot reproduce the raw experimental RDC data to the same level of accuracy as individual structures or ensembles generated through such rigorous fitting procedures. Indeed, the fact that the average RDC R-factor per alignment medium from our simulations is comparable to those obtained from the rigorously fitted 1D3Z structure or EROS ensemble is a tribute to the quality of the ff99SB force-field.

Another important factor that determines the accuracy of the result is obviously the accuracy of the experimental RDC datasets used to identify the optimal conformational space sampling. The 23 N-H RDC datasets that we have used in this study have been refined using a SECONDA analysis [12]. As a result, we consider these RDC datasets to be extremely accurate.

One of the most important questions to be discussed in the framework of this study is how accurately and realistically does the AMD method really sample conformational space compared to a brute-force long classical MD simulation? This question is difficult to answer as we simply don't have the computational power available to perform multiple brute force classical MD simulations over millisecond time-scales. However, in order to address this issue, we have performed a single MD simulation of ubiquitin over 150-ns. After performing the SVD analysis and calculating the RDCs for this trajectory, we obtained a RDC $R_{\text{cum}}$ value of 2.97, which is relatively similar to the $R_{\text{cum}}$ value that we obtained from the lowest level AMD simulations [$\left(E_b - V_{\text{dih}}\right) = 100$-kcal/mol]. In figure S1, we show a comparison of the effective order parameters obtained from the 150 ns MD simulation and the lowest level AMD simulations (which were averaged). This figure clearly shows that the order parameters are in good agreement, suggesting that the AMD simulation at this level is providing an accurate representation of the 150-ns brute force MD simulation. At this low level of acceleration, it appears therefore that we have achieved an effective 20-fold speed-up in the conformational space sampling.

**Statistical Noise and Free Energy Weighting:**

An important factor to be considered when performing AMD simulations is the issue of statistical noise, as has been referred to in a recent publication [13]. It is clear that as we accelerate more aggressively, we increase the perturbation on the simulation. This results in a less natural trajectory and also produces an enhanced amount of statistical noise. In extreme cases, particularly in the 'over-acceleration' limit, this statistical noise can adversely affect the accuracy of the free energy weighting procedure. We have implemented various techniques in the course of this study to try to address the issue of statistical noise, and moreover, to improve the free energy weighting analysis: First, for each AMD simulation, after performing the free energy weighting and clustering analysis, we have seeded numerous standard classical MD simulations from the AMD trajectory and performed a MM/PBSA analysis in order to validate the relative free energies obtained from the AMD procedure. In general,
whilst the absolute energetic values are obviously not the same, we found a reasonably good correlation between the AMD and MM/PBSA relative free energy analysis. Additionally, as discussed previously, we consider that performing multiple AMD simulations at each acceleration level and calculating trajectory averaged RDCs and scalar J-couplings also provides a significantly better representation of the free energy of the conformational space sampled at each acceleration level and reduces the effect of statistical noise. The question still remains however, whether the minimum in the $R_{\text{cum}}$ profile as a function of the acceleration level is genuinely real, or is just a manifestation of the enhanced perturbation at higher acceleration levels. In order to answer this question, we performed two very long AMD simulations at the acceleration levels $(E_b-V_{\text{dih}}) = 200$-kcal/mol and 250-kcal/mol. For the AMD simulation at $(E_b-V_{\text{dih}}) = 200$-kcal/mol, we found that over extended simulation times, the calculated RDCs and scalar J-couplings got slightly better, whereas for the AMD simulations performed at $(E_b-V_{\text{dih}}) = 250$-kcal/mol, the calculated RDCs and scalar J-couplings got slightly worse. This suggests that the observed minimum in the $R_{\text{cum}}$ profile is physically meaningful, though we do recognize that the rather dramatic increase in the $R_{\text{cum}}$ values at the highest acceleration levels may be partly due to the effects of statistical noise.

In general, it is clear that obtaining an accurate free energy surface over the explored conformational space is one of the principal aspects of this method that one would like to improve, not just in terms of dealing with the issue of statistical noise, but also in terms of the accuracy of the force-field. Although we certainly are not in a position to argue that the free energy surfaces that we have obtained are exactly correct, it is interesting to note that to our knowledge, this is the first and only study of RDCs to date that attempts to include a free energy weighting protocol in the analysis. All previous studies aimed at providing a structural/dynamic interpretation of RDC data either by implementing geometrical models, which do not include information concerning the free energy surface, or are based on simulations with experimental restraints. The presence of these restraints causes the simulation to react in an unpredictable manner and severely complicates the extraction of accurate free energy surfaces.

**Comparison between Unrestrained MD Simulation Results and those obtained from Rigorous Fitting Procedures:**

In the paper we compare the ability of the optimal AMD ensembles to reproduce experimental RDC and scalar J-coupling observables to previously published single-copy and restrained ensemble representations, most notably the single-copy representation of ubiquitin 1D3Z and the heavily empirically refined EROS ensemble. These comparisons have been made using the well-known R-factor. The 1D3Z structure was obtained by a rigorous simultaneous fitting procedure which included the use of over 2700 nOe-derived distance restraints, NC', NH, HC', H\textalpha-C\textalpha and C\textalpha-C' RDCs measured in two different alignment media as well as multiple scalar J-coupling datasets. Furthermore, it is also important to recognize that of the two alignment media used in the collection of the RDC datasets, one induced alignment by steric interaction, the other by electrostatic interaction. In light of this, whilst we use 23 NH RDC datasets that were explicitly not used in the construction of 1D3Z, some of these alignment media are strongly correlated to the two datasets used in the 1D3Z fitting procedure. The EROS ensemble was generated by employing over 2700 nOe-derived distance restraints, and then further refined against 47 NH RDC datasets (which included all 23 of the NH RDC datasets considered in this study), 6 HC' RDC datasets, 2 C\textalpha-C' RDC datasets, 2 C\textalpha-H\textalpha RDC datasets and 1 C\textalpha-C\beta dataset. It is therefore fair to say that both 1D3Z and the EROS ensemble are the products of extremely rigorous empirically derived fitting/refinement procedures. As we state in the paper, the average R-factors across the 23 NH RDC datasets used in this study are 0.066 (EROS), 0.093 (1D3Z) and 0.109 (AMD). In percentage terms therefore, the EROS ensemble is 4.1% better than the AMD
representation, and 1D3Z is 1.6% better than the AMD representation. The fact that the EROS ensemble gives the lowest R-factor is not surprising considering that unlike 1D3Z and the AMD ensembles, the EROS ensemble was actually refined against all the NH RDC datasets used in the present analysis. In the case of 1D3Z, even though none of the 23 NH RDC datasets were explicitly those used in the construction of this structure, the induced alignment in many of these datasets was in some cases similar for reasons discussed above, and this has a pronounced positive effect on the obtained R-factor for 1D3Z. In light of this, the fact that the trajectory averaged R-factor for the completely unrestrained optimal AMD ensembles is comparable to those values obtained from EROS and 1D3Z is a positive result, and a tribute to the ff99SB force-field.

The 116 member EROS ensemble was reprotonated using the 'protonate' algorithm in the AMBER suite. We then performed our SVD analysis on the AMBER-protonated ensemble for the 23 NH RDCs. The resulting average R-factor was found to be 0.101, in comparison to the original EROS ensemble value of 0.066. This reiterates the strong sensitivity of data reproduction on amino-proton positions, and indicates the difficulty commonly encountered when comparing ensembles to RDCs. It should be noted that most contemporary force-fields have a very small force-constant for the out-of-plane motion of the amino-proton, in line with the results of several studies [14]. As a result, during the refinement of the EROS ensemble using fairly large RDC restraints, the penalty energy of the RDC restraint readily outweighed the small energy cost of deviating the N-H bond vector orientation away from its standard geometry. The same (but slightly smaller) phenomenon is also observed for the 1D3Z structure, in which the position coordinates for the amino-protons were rigorously optimized during the fitting procedure.

Four experimental N-C' RDC datasets do exist which were neither employed in the 1D3Z fitting protocol, nor in the refinement of the EROS ensemble. However both 1D3Z and the EROS ensemble were generated using N-H, C'-H and Ca-C' RDCs in two alignment media, so that the N-C' bond vector orientations are already defined by these three RDCs. Nevertheless, we have performed our SVD analysis on these 4 N-C' RDC datasets. The average R-factors obtained were 0.158 (EROS), 0.163 (1D3Z) and 0.175 (AMD). Notably, the differences between these R-factors are smaller than those obtained for the 23 NH RDCs, and all three R-factors are within 2% of one-another. The 1UBQ structure has an R-factor of 0.186, and the average R-factor for the 5-ns standard classical MD simulations is 0.192. Therefore the optimal AMD ensembles perform better than both the X-ray crystal structure and the short 5-ns MD simulations.

Finally, we briefly discuss the results for the backbone scalar J-couplings. As described in the paper, the average R-factors for these three scalar J-couplings obtained from the optimal AMD ensembles (0.143) are better than both the 1UBQ structure (0.153) and the control set of 5-ns MD simulations (0.164). Remarkably, the AMD result is identical to the 1D3Z R-factor (0.143), despite the fact that these backbone scalar J-couplings were used in the fitting procedure for 1D3Z. The average R-factor for the three backbone scalar J-couplings obtained from the EROS ensemble was found to be 0.129, which is 1.4% better than the 1D3Z structure and the average optimal AMD-ensembles. However, closer inspection of the data reveals that the difference in this average R-factor for the EROS ensemble compared to the 1D3Z and AMD representations is predominantly due to the $^3J(H^N-C')$, and the associated EROS optimal Karplus curve for this scalar J-coupling looks rather unusual: Traditionally, the $\theta$-offset angle for the $^3J(H^N-C')$ Karplus curve is 180°. In line with this, the optimal $\theta$-offset angle is 179° and 177° for the AMD ensemble and the 1D3Z structure respectively. In the case of the EROS ensemble, the optimal $\theta$-offset value is found at 171°. One potential reason for this rather unusual $\theta$ angle could be that the structural integrity of the members of the EROS ensemble is rather poor: Most
notably, more than 40 of the peptide ω dihedral angles across the ensemble show deviations from the conventional geometry (180°) by more than 30°. It appears that the simultaneous implementation of multiple restraints in the refinement of the EROS ensemble has caused some distortion in the local peptide backbone geometry so that the validity of the improvement is unclear. In general, the average scalar J-coupling R-factors for EROS, 1D3Z and the AMD representations are quite similar.

**Principal Component Analysis and Comparison of Conformational Space Sampling:**

In Figure S2, free energy weighted structures of a representative RDC-optimal AMD ensemble have been projected onto their own backbone Cα (residues 1-71) lowest principal component eigenvectors (black dots) along with the average structure of this ensemble (green square), the 1D3Z structure (blue square), the average structure obtained from a standard 5-ns classical MD trajectory (magenta square) and the 166 member EROS ensemble (red circles). In principal component space, it is clearly visible that the average structure of the AMD ensemble is much more similar to the time- and ensemble-average structure 1D3Z than the standard 5-ns classical MD simulation, supported by the observation that the average structures obtained from the optimal molecular ensembles exhibit no violations to the nOe upper- and lower-bounds. Interestingly, the principal component projection plots (figure S2) show very large differences in the free energy of the system across the sampled conformational space. This large variation in the free energy is rather surprising on consideration that the backbone (residues 1-71) RMSD to the average structure for this ensemble is only 0.65 Å. To an approximation, it appears that there are two distinct conformational states that are heavily sampled. This observation is in agreement with a recent analysis by Clore and Schwieters,[15] who found that a 'double-copy' representation obtained by a fitting protocol provided a better representation of the available RDC data than a single-copy representation, whilst increasing the number of copies beyond this resulted in no further significant improvement.

With regards to the EROS ensemble, we find that before performing the free energy weighting analysis, the representative AMD ensemble sampled ostensibly all the conformational space of the EROS ensemble. However, after the free energy weighting protocol, it is clearly apparent that large amounts of this conformational space are energetically highly unstable. In conclusion therefore, we can say that the AMD/SVD approach described here has achieved the same conformational space sampling as the EROS ensemble, without the need to employ en masse ensemble-average restraints, and has further refined this conformational space sampling through a robust free energy weighting protocol, which is completely absent in the EROS approach, therefore providing a much more realistic unified structural dynamic representation of the system. Additionally, it is also worth mentioning that the order parameter scaling factor of 0.93 that was introduced into the EROS paper to account for librational motions that could not be adequately represented by the rather small 116 member ensemble is not necessary: On average, our molecular ensembles consist of some 20,000 members and yet we see no evidence of any additional librational motion that would require a re-scaling of the order parameters. Indeed, our optimal RDC order parameters are very similar to those of the EROS ensemble without this scaling factor. With regards to the order parameters, it is also worth noting that careful analysis of our AMD ensembles revealed that, on average, a small percentage (approximately 20%) of the free energy weighted ensemble, comprising structures that are distributed between high energy micro-states contribute to about 50% of the observed decrease in the order parameters. So, whilst this relatively small subset of the free energy-weighted ensemble provides a modest contribution to the observed improvement in the RDCs, its contribution to the order parameters is much more substantial.
Figure S1. Comparison of order parameters derived from a single AMD trajectory, using the equivalent number of steps to 8ns of classical MD, with acceleration parameters of $(E_{\text{boost}} - V_{\text{dih}})=100 \text{ kcal.mol}^{-1}$, $\alpha=60 \text{ kcal.mol}^{-1}$) (red) and a single 150ns classical MD trajectory (black). As both the N-H$^N$ RDC R-factors and order parameters are comparable, this demonstrates that the AMD approach samples meaningful conformational space analogous to standard long MDs.
Figure S2. Principal component projection plots. The lowest backbone C\(^\alpha\) principal component eigenvectors for a representative RDC-optimal free energy weighted AMD ensemble were calculated and the members of this ensemble were projected onto the collective coordinates (black dots) along with the average structure (green square), the average structure obtained from a standard 5-ns MD simulations (magenta square), the 1D3Z structure (blue square) and the EROS ensemble (red circles). Although these PC projection plots accentuate the observed collective motions of the system, it is important to note that the backbone RMSD to the average structure for this AMD ensemble is only 0.65-A.

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