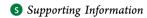


# Probing the Structure and Dynamics of Proteins by Combining Molecular Dynamics Simulations and Experimental NMR Data

Jane R. Allison,<sup>†,#</sup> Samuel Hertig,<sup>‡</sup> John H. Missimer,<sup>§</sup> Lorna J. Smith,<sup>∥</sup> Michel O. Steinmetz,<sup>§</sup> and Jožica Dolenc\*,<sup>†,⊥</sup>

<sup>&</sup>lt;sup>1</sup>Faculty of Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia



ABSTRACT: NMR experiments provide detailed structural information about biological macromolecules in solution. However, the amount of information obtained is usually much less than the number of degrees of freedom of the macromolecule. Moreover, the relationships between experimental observables and structural information, such as interatomic distances or dihedral angle values, may be multiple-valued and may rely on empirical parameters and approximations. The extraction of structural information from experimental data is further complicated by the time- and ensemble-averaged nature of NMR observables. Combining NMR data with molecular dynamics simulations can elucidate and alleviate some of these problems, as well as allow inconsistencies in the NMR data to be identified. Here, we use a number of examples from our work to highlight the power of molecular dynamics simulations in providing a structural interpretation of solution NMR data.

## INTRODUCTION

NMR is one of a host of experimental methods capable of providing structural information about biological macromolecules such as proteins, and one of the few that provides detailed atomic-level information. An additional advantage of NMR is that it reflects dynamics as well as structure. The different quantities measurable in an NMR experiment, such as nuclear Overhauser enhancements (NOEs), <sup>3</sup>*J*-coupling constants, and residual dipolar couplings (RDCs), report on different structural features of molecules and are responsive to dynamics on different time scales. While NMR data are informative in their own right, their ability to shed light on molecular structure and dynamics is greatly enhanced when they are augmented with classical molecular dynamics (MD) simulations.<sup>3</sup>

In order to incorporate NMR data into MD simulations, the force field, which specifies the covalent and noncovalent interaction functions, is generally supplemented by a special potential energy function that depends on how well the simulated structures agree with the experimental data. The simplest forms of this special function, which may be a restraining or a biasing potential, are based on harmonic functions, although more complex forms have been explored.<sup>6–12</sup> Either the primary data, i.e., the measured observable, or the secondary data, i.e., the related structural property, may be restrained. A relationship  $Q(\mathbf{r})$ linking the measured observable Q to the structural property of the molecule that it describes, specified by the Cartesian coordinates of the molecule r, is therefore required. For NOEs, typically the secondary data, in the form of interatomic distances, are restrained; for RDCs, the primary data; and for <sup>3</sup>J values, either the couplings themselves (primary) or the corresponding dihedral angle values (secondary) are restrained.

One factor that is particularly important when interpreting NMR data as well as when incorporating them into MD simulations is the fact that NMR observables are averages over both the number of molecules present and the time taken to carry out the experimental measurement. Failure to account for this averaging correctly, by forcing a single structure to satisfy all experimental data simultaneously, can result in structures that are incorrect and, at worst, physically impossible. This problem can be avoided by restraining or biasing the ensemble- or timeaverage of the calculated value of the observable to fit the experimental data and by ensuring that the relative weights of the force field and special interaction potentials are appropriate. 13,14 While in restrained time-averaged MD, the simulated molecule experiences a penalty potential if the time average of an NMR observable calculated from a MD trajectory differs from the experimental value, 15-18 in restrained ensemble-averaged MD, multiple configurations are simulated in parallel and a penalty potential is applied if the ensemble-averaged NMR observable does not match the experimental value at each step of the simulation.  $^{19-24}$ 

The conversion between primary and secondary data is fraught with a number of difficulties. The relationship between an observable quantity  $Q(\mathbf{r}^N)$  and the structure of a molecule represented by the Cartesian coordinates  $\mathbf{r}^N \equiv (\mathbf{r}_1, \mathbf{r}_2, ..., \mathbf{r}_N)$  of its N atoms may be approximate in nature and may rely on empirically determined parameters. It may be nonlinear, in

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<sup>&</sup>lt;sup>†</sup>Laboratory of Physical Chemistry, Swiss Federal Institute of Technology ETH, 8093 Zürich, Switzerland

<sup>&</sup>lt;sup>‡</sup>Department of Health Sciences and Technology, Swiss Federal Institute of Technology ETH, 8093 Zürich, Switzerland

<sup>§</sup>Biomolecular Research, Paul Scherrer Institut, 5232 Villigen, Switzerland

Department of Chemistry, University of Oxford, Oxford, United Kingdom

which case averaging over structures,  $\langle \mathbf{r}^N \rangle$ , will yield a different result from averaging over Q values:  $\langle Q(\mathbf{r}^N) \rangle \neq Q(\langle \mathbf{r}^N \rangle)$ . Finally,  $Q(\mathbf{r}^N)$  may be multiple-valued, meaning that there is not a unique one-to-one relationship between the value of the structural property and the value of the observable. This can further complicate the effects of conformational averaging. A key example of all of these problems is the Karplus relation used to convert between NMR <sup>3</sup>I couplings and dihedral angle values. As well as being nonlinear and multiple-valued, the many different parameter sets available for this relation can quite markedly change the nature of the relation. 25,26 Moreover, the empirical parametrization methods generally used mean that the parameters often already incorporate some degree of conformational averaging, making their application for the analysis of MD simulations problematic. Various means of accounting for this have been proposed, 27-31 although no single method has been widely adopted.

A further issue specific to the use of NMR data in structure determination or refinement is that the number of observables  $N^{\mathrm{obs}}$  at a particular thermodynamic state point is in general much smaller than the number of degrees of freedom of the molecule  $N^{df}$ , even when a number of different observables are measured. This scenario, termed "under-fitting", may be tested for by cross-validating the ensemble of structures produced against independent "free" data not used as restraints. 32-34 Satisfaction of the restraints generally improves with more degrees of freedom, whereas reproduction of the free data becomes worse. The removal of data to form the free set of course further reduces  $N^{\text{obs}}$  relative to  $N^{\text{df}}$ . Moreover, some data are correlated, reducing the number of independent observables. Cross-validation can also be usefully applied to identify overfitting of the data, in which the restraints are too stringently enforced, and incorrect assignment of the data.

Ideally, many different types of data reporting on different aspects of the structure should be used to fully characterize the structural ensemble and its dynamics. In the case of multiple-valued functions  $Q(\mathbf{r})$ , measurement of different observables Q reporting on the same structural feature can help to overcome the degeneracy of the solutions of  $\mathbf{r}(Q)$ . Using multiple types of data can also help to identify inconsistencies in the data itself. When combining data, however, it is important to consider the thermodynamic state point at which each data set was measured, as a molecule may not have the same structural or dynamic features at different thermodynamic state points. The time-scale of the measurement should also be taken into account when the data are to be interpreted in terms of dynamics.

Here, we explore the effects of the aforementioned issues on the interpretation of NMR data in terms of molecular structure. Using four model systems from our own work, we illustrate how MD simulations can improve the interpretation of NMR data by properly accounting for the average nature of measured observables in the structure refinement protocol, and by identifying misassignments and inconsistencies in the NMR data.

### **■ THEORY**

In order to bias the sampling in a MD simulation toward a particular measured value, a penalty function  $V^{\text{restr}}$  can be added to the physical force field term  $V^{\text{phys}}$  for the potential energy:

$$V^{\text{pot}}(\mathbf{r}^{N}(t)) = V^{\text{phys}}(\mathbf{r}^{N}(t)) + V^{\text{restr}}(\mathbf{r}^{N}(t))$$
(1)

We begin by briefly reviewing the different restraining functions that have been used in this work.

In NMR structure refinement based on experimental data, a harmonic restraining potential energy function is usually used:

$$V^{\text{restr}}(\mathbf{r}^{N}(t)) = \frac{1}{2} \sum_{k=1}^{N^{\text{restr}}} K_{k}^{\text{restr}} (f_{k}(\mathbf{r}^{N}(t)) - f_{k}^{0})^{2}$$
(2)

in which  $N^{\rm restr}$  is the number of restraints,  $K_k^{\rm restr}$  is the force constant for each restraint,  $f_k({\bf r}^N(t))$  is the value of the observable calculated from the coordinates of the molecule in the simulation at the current point in time, and  $f_k^0$  is the value of the observable measured experimentally. The penalty function  $V^{\rm restr}$  raises the energy of the system as the deviation of the calculated value of the observable  $f_k({\bf r}^N(t))$  from the experimentally measured value  $f_k^0$  increases. The harmonic potential may have a flat bottom, if some degree of deviation from the experimentally measured value is allowed without penalty, or it may be half-harmonic, such as in the case of NOE distances:

$$V^{\text{NOE}}(\mathbf{r}^{N}(t)) = \begin{cases} \frac{1}{2} \sum_{k=1}^{N^{\text{NOE}}} K_{k}^{\text{NOE}} (r_{nn}, -r_{k}^{0})^{2} & \text{if } r_{nn}, > r_{k}^{0} \\ 0 & \text{otherwise} \end{cases}$$
(3)

where  $f_k(\mathbf{r}^N(t))$  is given by  $r_{nn'}$ , the distance between the kth pair of atoms n and n' that are subject to the NOE upper distance bound  $r_k^0$ . This potential can be made linear with respect to violations above a defined distance threshold.

A more tolerant approach to impose restraints is to treat the NMR data as quantities satisfied only on average over the course of a restrained MD simulation. This can be achieved by using the weighted temporal average during the simulation: 15,16,35

$$\overline{f_k(\mathbf{r}^N(t))} = \frac{1}{\tau(1 - \exp(-t/\tau))} \times \int_0^t \exp\left(\frac{t' - t}{\tau}\right) f_k(\mathbf{r}^N(t')) dt' \tag{4}$$

instead of  $f_k(\mathbf{r}^N(t))$  in eq 2. The characteristic time for the exponential decay,  $\tau$ , should ideally be longer than the period of the natural motions of the system, <sup>36,37</sup> although in practice, this is difficult to know prior to carrying out the simulation.

The inherent problem of standard time-averaging approaches is that the average value lags behind the instantaneous one. In fact, when the restrained observable has a long memory-relaxation time, the slowly changing average may drive the system away from conformations that are in agreement with the experimental data. This problem is particularly severe in the case of <sup>3</sup>*J* couplings, where standard time-averaging can cause artificially induced structural fluctuations. In order to avoid this, a biquadratic potential that takes into account both the time-averaged and instantaneous values of the calculated <sup>3</sup>*J* value should be used: <sup>11,18</sup>

$$V^{J}(\mathbf{r}^{N}(t)) = \frac{1}{2} \sum_{k=1}^{N^{J}} K_{k}^{J} (^{3}J(\phi_{k}(\mathbf{r}^{N}(t))) - ^{3}J_{k}^{0})^{2} \times (^{3}J(\phi_{k}(\mathbf{r}^{N}(t))) - ^{3}J_{k}^{0})^{2}$$
(5)

where  ${}^{3}J(\phi_{k}(\mathbf{r}^{N}(t)))$  replaces  $f_{k}(\mathbf{r}^{N}(t))$ ,  $\phi_{k}$  is the torsional angle, and eqs 2 and 4 are combined.

No matter how sophisticated the restraining potential function, high-energy barriers between different conformations may prevent sampling of the entire range of values of the structural property that contributes to the measured experimental observable. Improved sampling of conformational space during restrained simulations may be obtained by using the local elevation biasing method. In the case of  ${}^3J$  values, the restraining potential energy function  $V_k^I$  for the kth  ${}^3J$  value related to the torsional angle  $\phi_k$  is a sum of  $N^{\text{LE}}$  local elevation terms:

$$V_k^J(\phi_k(\mathbf{r}^N(t))) = \sum_{i=1}^{N^{\text{LE}}} V_{ki}^{\text{LE}}(\phi_k(\mathbf{r}^N(t)))$$
(6)

in which the penalty terms are Gaussian functions centered at  $\phi_{b}^{0}$ :

$$V_{ki}^{\text{LE}}(\phi_k(\mathbf{r}^N(t))) = K_k^J \omega_{\phi_{ki}}(t) \times \exp(-(\phi_k(t) - \phi_{ki}^{\ 0})^2 / 2(\Delta \phi^0)^2)$$
(7)

with  $\Delta\phi^0=360^\circ/N^{\rm LE}$ . The ideal number of local elevation bins  $N^{\rm LE}$  to use is a balance between computational efficiency and the level of detail of the resulting local elevation potential energy landscape.  $K_k^I$  is the overall  $^3J$ -value penalty function force constant and  $\omega_{\phi_k}(t)$  is the weight function of the ith local elevation Gaussian penalty function. The latter is calculated using a biquadratic product of two, usually flat-bottomed (fb), terms in order to determine if the instantaneous or time-averaged  $^3J$  value deviates from the experimental one:

$$\omega_{\phi_{kl}}(t) = t^{-1} \int_{0}^{t} \delta_{\phi_{k}(\mathbf{r}^{N}(t'))\phi_{kl}^{0}} V^{\text{fb}}(^{3}J(\phi_{k}(\mathbf{r}^{N}(t'))))$$

$$\times V^{\text{fb}}(^{3}J(\phi_{k}(\mathbf{r}^{N}(t')))) dt'$$
(8)

The weight function is nonzero if the instantaneous  $\phi_k(\mathbf{r}^N(t'))$  value is in the bin of  $\phi_{k:}^0$ :

$$\delta_{\phi_k(\mathbf{r}^N(t'))\phi_{ki}^0} = \begin{cases} 1 & \text{if } \phi_{ki}^0 - \Delta \phi^0/2 < \phi_k(\mathbf{r}^N(t')) \\ & \text{and } \phi_k(\mathbf{r}^N(t')) < \phi_{ki}^0 + \Delta \phi^0/2 \\ 0 & \text{otherwise} \end{cases}$$
(9)

and both the instantaneous  ${}^3J(\phi_k(\mathbf{r}^N(t)))$  and the time-averaged  ${}^3J(\phi_k(\mathbf{r}^N(t)))$  deviate more than  $\Delta J^0$  from the experimental value  ${}^3J_k^0$ :

$$\begin{split} V^{\text{fb}}(^{3}\!J(\phi_{k}(\mathbf{r}^{N}(t)))) \\ &= \begin{cases} (^{3}\!J(\phi_{k}(\mathbf{r}^{N}(t))) - ^{3}\!J_{k}^{\ 0} - \Delta^{3}\!J^{0})^{2} \\ &\text{if } ^{3}\!J(\phi_{k}(\mathbf{r}^{N}(t))) > ^{3}\!J_{k}^{\ 0} + \Delta^{3}\!J^{0} \\ (^{3}\!J(\phi_{k}(\mathbf{r}^{N}(t))) - ^{3}\!J_{k}^{\ 0} + \Delta^{3}\!J^{0})^{2} \\ &\text{if } ^{3}\!J(\phi_{k}(\mathbf{r}^{N}(t))) < ^{3}\!J_{k}^{\ 0} - \Delta^{3}\!J^{0} \\ 0 & \text{otherwise} \end{cases} \end{split}$$

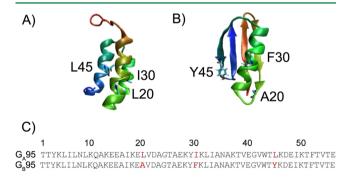
$$(10)$$

In  $V^{\text{fb}}(\overline{{}^3J(\phi_k(\mathbf{r}^N(t)))})$ ,  ${}^3J(\phi_k(\mathbf{r}^N(t)))$  is replaced by  $\overline{{}^3J(\phi_k(\mathbf{r}^N(t)))}$ , which is the exponentially damped temporal average over the course of a MD simulation, calculated using eq 4.

### METHODS

Model Systems. FKBP. The FK506 binding protein (FKBP) is a cis/trans peptidylprolyl isomerase that is an immunophilin when bound to immunosuppressive drugs such as FK506 and ascomycin. Up to four different types of stereospecifically assigned side-chain <sup>3</sup>*J* couplings, namely  $^{3}J_{\text{H}\alpha\text{H}\beta}$ ,  $^{3}J_{\text{H}\alpha\text{H}\beta}$ ,  $^{3}J_{\text{NH}\beta}$ , and  $^{3}J_{\text{NH}\beta}$ , have been acquired for 69 of its 107 residues, giving 197 in total, i.e., more than one  ${}^{3}J$  coupling per residue. This raises the possibility that the degeneracy inherent in the inference of structural properties from <sup>3</sup>I values might be resolved. Although each <sup>3</sup>I value can correspond to up to four dihedral angle values when the standard Karplus relation is used to convert between <sup>3</sup>I value and dihedral angle value, it should be possible to select an appropriate set of dihedral angle values in keeping with all experimental data if all four types of <sup>3</sup>J couplings can be measured for the same  $\chi_1$  angle. This solution could take the form of a single range of dihedral angle values, which may be broad or narrow, depending on the mobility of the side chain in question, or may comprise several different ranges of dihedral angle values. It is also necessary to account for the uncertainty introduced by the parametrization of the Karplus relation; thus <sup>3</sup>*I* couplings are commonly interpreted with an uncertainty of  $\pm 1$  Hz. Possible solutions were sought using three different levels of investigation: First, the ranges of dihedral angle values corresponding to the measured  $^3J$  couplings given an uncertainty of  $\pm 1$  Hz were back-calculated. Second, MD simulations of a model dihedral angle (MDA), comprising a united-atom butane molecule with all dihedral angle and 1-4 noncovalent interactions removed, were carried out using three different restraining or biasing methods. Last, MD simulations of the entire protein using the same three simulation methods were performed.<sup>25</sup>

 $G_A95$  and  $G_B95$ .  $G_A95$  and  $G_B95$  are two proteins whose sequences are 95% similar, differing by just three residues, but fold into topologically distinct structures <sup>40</sup> (Figure 1). They are



**Figure 1.** NMR model structures of (A)  $G_A95$  and (B)  $G_B95$  and (C) sequences of both proteins. Coloring is from red to blue from the N- to C-terminus for the protein backbone and according to atom type for individual residues. The location, number, and type of the residues that differ between the two proteins and the change in residue type upon switching structure (A  $\Leftrightarrow$  B) or upon creation of the homology models (A)  $\beta/\alpha$  and (B)  $\alpha/\beta$  are labeled in A and B and highlighted in red in C.

part of a series of pairs of proteins derived from the binding domains of *Streptococcus* protein G that were systematically modified to have increasing sequence identity (SI) while retaining different structures and binding functionalities.

G<sub>A</sub>95 was derived from the 45-residue G<sub>A</sub> domain, which binds human serum albumin, and G<sub>B</sub>95 was derived from the 56residue G<sub>R</sub> domain, which binds the constant Fc region of IgG. The naturally occurring versions of these two domains have only 16% SI and fold into  $3\alpha$  and  $4\alpha+\beta$  structures, respectively. During the switch between the two structures, 85% of the residues change their secondary structure, with just eight residues assuming  $\alpha$ -helical structure in both folds.  $G_A95$  and  $G_B95$ were the focus of the CASP8 structure prediction competition. 43 Only four of the competing groups correctly predicted the two different folds, with most Web servers predicting a  $4\alpha + \beta$  fold for both sequences. NMR model structures have also been determined for these two proteins. 40 In addition, "crossover" models, in which each sequence was homologymodeled onto the structure associated with the other sequence, have been created and studied computationally. 44 Calculation of the NOE distances used in the structure determination from the NMR model structures and from the crossover models allowed the information content of the NOE data to be assessed.

GCN4p16-31. The leucine zipper of the yeast transcriptional activator GCN4 is a 33-residue peptide that has attracted particular attention because of its two-stranded, parallel coiledcoil motif in which two  $\alpha$ -helices are wound around each other to form a superhelix. The folding of the leucine zipper is proposed to proceed via a short autonomous helical folding unit also called a trigger sequence. Biophysical analyses of GCN4 suggest that its C-terminal half has a propensity to form a stable helix. In order to obtain detailed insight into the interactions that initiate GCN4 leucine zipper formation, the NMR model structure of the C-terminal half, denoted GCN4p16-31, has been derived from NOE intensities and <sup>3</sup>J<sub>Ho.H.</sub>-coupling constants using a standard single-structure refinement protocol based on 172 NOE distance restraints and 14 hydrogen-bond and 11  $\psi$  torsional-angle restraints assumed from the measured  ${}^{3}J_{H_{N}H_{\alpha}}$ -coupling constants and secondary  $C\alpha$ and  $H\alpha$  chemical shifts. 45 The resulting set of 20 NMR model structures exhibits a regular  $\alpha$ -helix extending from residues 18 to 28. However, the calculated set of model structures does not reproduce all 15 measured  ${}^{3}J_{H_{N}H_{\alpha}}$ -coupling constants within an uncertainty of  $\pm 1$  Hz, suggesting that the  $\alpha$ -helical propensity should not in fact be uniform along the sequence. 46 With the aim of exploring averaging effects implicit in NMR spectroscopic data and to resolve structures compatible with the entire set of NMR data, restrained molecular dynamics simulations of the GCN4 trigger sequence in solution using time-averaged distance restraints based on the measured NOEs in combination with local elevation biasing of <sup>3</sup>J-coupling constants have been performed.46

 $^{1}$ F1 $^{12}$ F1-B3 Complex. Fibronectin is a large extracellular glycoprotein that is involved in a number of important physiological processes such as cellular adhesion, differentiation, and wound healing. It is composed of three types of modules, F1, F2, and F3, which are separated by short sequences of amino acids.  $^{47}$  The N-terminal region of fibronectin has been shown to contain binding sites for pathogenic bacteria which bind to fibronectin through fibronectin-binding proteins anchored to the bacterial cell wall. This region of fibronectin is composed of a string of five F1 modules, each of which consists of a double-stranded and a triple-stranded antiparallel β-sheet. Understanding the interactions between

pathogenic bacteria and fibronectin is of vital importance for the development of novel therapeutic strategies.

A solution NMR structure of the human <sup>1</sup>F1<sup>2</sup>F1 complex (where 1 and 2 denote the number of the module as counted from the N-terminus) bound to a streptococcal peptide B3 has been determined and shows that the bacterial peptide binds to  $^{1}\text{F}1^{2}\text{F}1$  by forming an additional antiparallel  $\beta$ -strand with the two sequential modules. This novel mechanism of proteinprotein recognition is also named a tandem  $\beta$ -zipper. 48 Despite the fact that 14 out of 15 model structures of the <sup>1</sup>F1<sup>2</sup>F1-B3 complex deposited in the PDB data bank show an elongated conformation of the complex, the relative orientation of the modules <sup>1</sup>F1 and <sup>2</sup>F1 is, due to the lack of intermodule NOEs, rather underdetermined, allowing a certain degree of variability. Yet the characterization of the intermodule orientations is necessary to understand the structure and function of fibronectin. This prompted us to perform a series of unrestrained and restrained MD simulations of the <sup>1</sup>F1<sup>2</sup>F1-B3 complex starting from the two NMR model structures with the most different intermodule orientations.

Model Generation and MD Simulations. FKBP. The initial coordinates of FKBP bound to ascomycin were provided by the X-ray structure of FKBP bound to FK506.<sup>49</sup> Coordinates for ascomycin were derived by simply removing the additional allyl group of FK506. A new GROMOS building block was made for ascomycin by analogy with the building blocks of standard molecules with similar chemical structure. A GROMOS building block was also created for the model dihedral angle (MDA) based on united atom butane and using standard force field parameters. To allow complete, free rotation through 360°, the 1-4 noncovalent interaction was removed from the GROMOS molecular topology file, and the torsion angle forces were set to zero in the MD input file. For the simulations in explicit water, the FKBP/asc complex was placed in a pre-equilibrated box of approximately 9500 SPC<sup>50</sup> water molecules with a minimum solute-wall distance of 1.2 nm, and seven Na<sup>+</sup> counterions were added to neutralize the −7e charge of FKBP. Starting structures were energy minimized before and, for the explicit water simulations, after solvation.

The  ${}^{3}J$ -value memory relaxation time,  $\tau_{I}$ , was 5.0 ps for all simulations. 35,37 The number of Gaussian functions comprising the local elevation potential energy term was  $N^{LE} = 36$ , and the width of the flat bottomed well was  $\Delta^3 I^0 = 1$  Hz to account for the uncertainty that stems from the parametrization of the Karplus relation. The force constants,  $K_k^I$ , were calibrated independently for each molecule and each simulation method, as explained in ref 25. The resulting  $K_k^J$  values were  $K_k^{J_{INSR}} =$ 0.5 kJ mol<sup>-1</sup> Hz<sup>-2</sup> for instantaneous restraining,  $K_k^{\gamma_{\text{TAVR}}} =$ 0.05 kJ mol<sup>-1</sup> Hz<sup>-4</sup> for biquadratic time-averaged restraining, and  $K_k^{J_{\text{TLEB}}} = 5 \times 10^{-5} \text{ kJ mol}^{-1} \text{ Hz}^{-4}$  for biquadratic timeaveraged local elevation biasing of the MDA and  $K_k^{I_{\text{INSR}}} =$ 5 kJ mol<sup>-1</sup> Hz<sup>-2</sup> for instantaneous restraining,  $K_k^{J_{\text{TAVR}}} =$ 0.1 kJ mol<sup>-1</sup> Hz<sup>-4</sup> for biquadratic time-averaged restraining, and  $K_k^{J_{\text{TLEB}}} = 1 \times 10^{-3} \text{ kJ mol}^{-1} \text{ Hz}^{-4}$  for biquadratic timeaveraged local elevation biasing of FKBP/asc. When timeaveraging was used, it was necessary to include an additional weight factor so that the restraining function associated with the  ${}^{3}J_{NH\beta}$  couplings was of an equivalent magnitude to that of the  ${}^3J_{{\rm H}\alpha{\rm H}\beta}$  couplings. Multiplying the contribution of the  ${}^3J_{{\rm NH}\beta}$  couplings by 10 relative to the  ${}^3J_{{\rm H}\alpha{\rm H}\beta}$  couplings proved to be

Unrestrained (10 ns) and restrained (1 ns) simulations of the MDA were run in vacuum using the GROMOS 45B3 force

field, which is derived from the 45A3 force field<sup>51</sup> by neutralizing the  $\pm 1e$  charges of charged side chains (Arg, Lys, Asp, Glu) and the charged termini of the polypeptide backbone and reducing their repulsive van der Waals parameters.<sup>52</sup> Unrestrained, restrained, and local elevation biased simulations of FKBP bound to ascomycin (FKBP/asc) were run in a vacuum for 22 ns using the 45B3 force field and in explicit water for 20 ns using the 45A4 force field.<sup>51</sup> All simulations were carried out at 300 K and 1 atm.

 $G_A95$  and  $G_B95$ . Initial coordinates of  $\alpha/\alpha$  and  $\beta/\beta$  were taken from the NMR model structures deposited in the PDB with entry codes 2KDL and 2KDM, respectively. Homology models of alternate sequence/structure combinations were created using the I-TASSER server. For instance, to create  $\alpha/\beta$ , the sequence of 2KDL was submitted along with the structure of 2KDM. Hydrogens were added to the homology models according to standard geometric criteria. The protonation state of the ionizable residues was chosen according to the pH (7.2) of the NMR experiments. Each sequence/structure combination was energy-minimized in the 54A7<sup>54</sup> GROMOS force field prior to further analysis.

GCN4p16-31. The initial coordinates of GCN4p16-31 were taken from the first model structure of the set of NMR model structures (PDB entry 2OVN). The GCN4p16-31 peptide comprises the sequence Ac-16Asn-17Tyr-18His-19Leu-20Glu-21Asn-22Glu-23Val-24Ala-25Arg-26Leu-27Lys-28Lys-29Leu-30Val-31Gly-NH2. The His residue was protonated at NE2, and the Arg and Lys side chains were protonated with charge +1e. GCN4p16-31 was solvated in a rectangular box of approximately 3000 pre-equilibrated SPC<sup>50</sup> water molecules.

The time-averaged NOE distance restraints were imposed with a force constant of  $K^{\rm NOE}=6000~{\rm kJ~mol^{-1}~nm^{-2}}$  and with a memory relaxation time of  $\tau_{\rm NOE}=20~{\rm ps}$ . The local elevation  $^3J_{\rm coupling}$  biasing used a memory relaxation time  $\tau_J$  of 5 ps. The number of local elevation Gaussian functions per dihedral angle was set to  $N^{\rm LE}=36$ , and the restraints were imposed with a force constant  $K^J=0.005~{\rm kJ~mol^{-1}~Hz^{-4}}$ . Additionally, due to the uncertainty in the  $^3J_{\rm H_NH_a}$ -coupling constants calculated from the corresponding angles via the Karplus relation, a flat-bottomed restraining energy term with a width of 2 Hz ( $\Delta^3J^0=1~{\rm Hz}$ ) was used. The simulations were carried out at 278 K and 1 atm for 10 ns using the GROMOS 53A6 force field.  $^{55,56}$ 

<sup>1</sup>F1<sup>2</sup>F1-B3 Complex. The simulations of the <sup>1</sup>F1<sup>2</sup>F1-B3 complex were started from the first and the last of the 15 NMR model structures deposited in the protein database (PDB entry 109A). The protonation states of protonatable groups were selected to correspond to a pH of 7. Each complex was solvated in a rectangular box containing 51 sodium ions, 48 chlorine ions, and 35 336 SPC<sup>50</sup> water molecules in the case of model 1 or 19 622 SPC<sup>50</sup> water molecules in the case of model 15. The dimensions were chosen such that the minimum distance from the <sup>1</sup>F1<sup>2</sup>F1-B3 complex to the box wall was 1.4 nm in the starting configuration.

Four MD simulations were performed: two 20 ns unrestrained MD simulations starting from NMR model 1 and NMR model 15 and two 10 ns restrained MD simulations starting from NMR model 1 and NMR model 15. NOE distance restraints were imposed as time-averaged restraints using a force constant of  $K^{\rm NOE} = 6000 \ \rm kJ \ mol^{-1} \ nm^{-2}$  and a memory

relaxation time of  $\tau_{\rm NOE}$  = 20 ps. The simulations were carried out at 310 K and 1 atm using the GROMOS 54A7 force field.<sup>54</sup>

General Simulation Protocol. All MD simulations were carried out using the GROMOS biomolecular simulation software \$2,57-59 under periodic boundary conditions. Constant temperature and pressure were maintained using the weak coupling algorithm, 60 with relaxation times of  $\tau_T = 0.1$  ps and  $\tau_v =$ 0.5 ps and an estimated isothermal compressibility of  $4.575 \times 10^{-4} \, (kJ \, mol^{-1} \, nm^{-3})^{-1.52} \,$  The SHAKE algorithm  $^{61}$  was used with a geometric tolerance of 10<sup>-4</sup> to constrain bond lengths and the geometry of the water molecules, allowing for an integration time step of 2 fs. The center of mass motion was removed every 1000 time steps. Noncovalent interactions were calculated using a triple-range cutoff scheme with cutoffs of 0.8 and 1.4 nm. To account for the influence of the dielectric medium outside the cutoff sphere, a reaction-field force was added based on a relative dielectric permittivity  $\varepsilon$  of 1 for the vacuum simulations, 61<sup>62</sup> for the explicit water simulations of FKBP and <sup>1</sup>F1<sup>2</sup>F1-B3 or 66.6<sup>63</sup> for the explicit water simulations of GCN4p16-31.

**Analysis.** All analyses described here were carried out using the GROMOS++ suite of analysis programs.<sup>64</sup>

Comparisons to experimental NOE data were made by comparing the interproton distances derived from the NOE cross-peak intensities to the average interproton distances calculated from the simulated trajectories. The results are presented as NOE upper distance bound deviations, i.e., as a difference between the distances averaged over the simulation and the corresponding NMR-derived upper distance bounds. Because the GROMOS force fields make use of united atoms, positions of aliphatic hydrogen atoms of interest were constructed on the basis of standard geometries. <sup>52</sup> If a NOE upper bound involved nonstereospecifically assigned protons, a pseudo-atom was constructed. <sup>52</sup>

<sup>3</sup>*J*-coupling constants were calculated from the coordinates of simulated structures using the Karplus relation: <sup>65</sup>

$${}^{3}J(\phi(\mathbf{r}(t))) = a\cos^{2}\phi(\mathbf{r}(t)) + b\cos\phi(\mathbf{r}(t)) + c$$
 (11)

where  $\phi$  is the torsional angle defined by the four covalently bound atoms that determine a particular  $^3J$ -coupling constant;  $\mathbf{r}(t)$  denotes a molecular conformation as a function of time; and a, b, and c are empirical coefficients. The parameters of DeMarco<sup>66,67</sup> were used for the side-chain  $^3J$  couplings measured for FKBP, and the parameters of Pardi<sup>68</sup> were used for the backbone  $^3J_{\mathrm{H_NH_{eff}}}$  couplings measured for GCN4p16-31.

The ensemble averaged chemical shifts were calculated by using the program SHIFTX2. Secondary structure assignments were carried out using the Kabsch and Sander rules. Hydrogen bonds were analyzed according to a geometrical criterion, where a hydrogen bond is defined by a minimum donor—hydrogen—acceptor angle of 135° and a maximum hydrogen—acceptor distance of 0.25 nm. For visual analysis, the visual molecular dynamics (VMD)<sup>71</sup> program was used.

The simulations of the GCN4p16-31 were subjected to a conformational clustering analysis which was performed on every second frame of the simulated trajectories, that is, at 1 ps intervals. The clustering algorithm, which uses the atom-positional RMSD as a similarity criterion, has been described in previous studies of peptide dynamics.<sup>72</sup> We performed a translational superposition of the centers of mass and a rotational least-squares fit for every pair of configurations using all heavy backbone atoms of the peptides and calculated the corresponding

atom-positional RMSD for the same set of atoms. The cluster membership criterion was a backbone atom-positional RMSD of less than 0.1 nm.

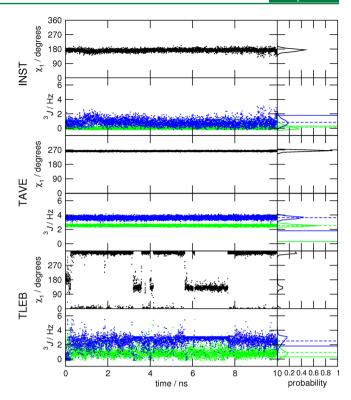
#### RESULTS AND DISCUSSION

Interpretation of Experimental Data Using Simulation. Although all of the experimental data discussed here can be related to some structural property of the molecule under study, attempting to build an image of the molecule without recourse to additional information is a herculean task. Combining NMR data with MD simulations provides a much simpler route toward extracting information about the structure and dynamics of the molecule from the experimental data. The way in which this is done, however, requires careful consideration.

For instance, when restraining or biasing MD simulations to fit experimental data, the choice of simulation methodology should reflect the degree of conformational averaging encompassed by the measured value. For NMR data, which are generally measured for molecules in solution at temperatures at which some degree of mobility is expected, this means that instantaneous restraining, in which a single copy of the molecule is expected to fulfill all of the experimental data at each point in time, is unlikely to be suitable. The choice of simulation methodology can also greatly affect the ensemble of structures obtained, an issue that is seldom mentioned.

An example that highlights the inappropriateness of using instantaneous or even simple time-averaged restraining when conformational motion occurs is the behavior of residue 44 of FKBP when the whole protein is simulated with <sup>3</sup>*I*-coupling restraints (Figure 2). Using instantaneous restraining with a single harmonic potential function, the  $\chi_1$  dihedral angle of this residue has a value of approximately 180°. While the corresponding  ${}^{3}J_{NH\beta_{1}}$  value of 0.15 Hz is close to the experimental value of 0.3 Hz, the  ${}^{3}J_{NH\beta_{2}}$  value of 0.84 is almost 1 Hz lower than the experimental value of 1.8 Hz. When time-averaging with a biquadratic potential function is used, the dihedral angle samples a very narrow range of angle values around 270°, which gives rise to  ${}^{3}J_{NH\beta_{3}}$  and  ${}^{3}J_{NH\beta_{3}}$  values of 2.54 and 3.62 Hz, respectively, both of which are significantly larger than the experimental values. Only when biquadratic time-averaged local elevation biasing is used are the  ${}^{3}J_{NH\beta_{2}}$  and  ${}^{3}J_{NH\beta_{3}}$  values (0.91) and 2.51 Hz, respectively) both well within the accepted uncertainty of ±1 Hz from the experimental values. In this simulation, the dihedral angle undergoes several transitions between values around 135° and 360°. With instantaneous restraining, only one of these two dihedral angle values is sampled, and with normal time averaging, the dihedral angle becomes stuck in a conformation that does not agree well with either of the experimental <sup>3</sup>*J* couplings.

Further illustration of the importance of choosing an appropriate simulation methodology is provided by residue 96 of FKBP (Figure 3). This residue is a threonine, which has only one H $\beta$  proton attached to the C $\beta$  atom, so that only two  ${}^3J_{{\rm H}\alpha{\rm H}\beta}$  couplings can be measured. In this case, use of instantaneous restraining gives rise to dihedral angle values around 270°. The corresponding  ${}^3J_{{\rm H}\alpha{\rm H}\beta}$  and  ${}^3J_{{\rm N}{\rm H}\beta}$  values of 11.19 and 1.78 Hz, respectively, are in fact not too different from the experimental values of 10.5 and 1.2 Hz. When time-averaged biquadratic restraining is used, a slightly broader range of dihedral angle values, centered on 120°, is sampled. The  ${}^3J_{{\rm N}{\rm H}\beta}$  value of 1.28 Hz now agrees very well with the experimental



**Figure 2.** Time-series and distributions of the  $\chi_1$  dihedral angle value and  ${}^3J_{\mathrm{NH}\beta_2}$  (green) and  ${}^3J_{\mathrm{NH}\beta_3}$  couplings (blue) for residue 44 during simulations of FKBP bound to ascomycin with  ${}^3J$ -value restraints enforced using a single-harmonic instantaneous restraining function (INST) or a biquadratic time-averaged restraining function (TAVE) in a vacuum (45B3 force field) or using biquadratic time-averaged local elevation biasing (TLEB) in explicit water (45A4 force field). The bin width for the histograms is  $10^\circ$  for the  $\chi_1$  angle values and 0.2 Hz for the  ${}^3J$  values. In the right-hand panels, the experimental value of each coupling (solid lines) and the average calculated from the simulation (dashed lines) are also shown.

value, but the  $^3J_{\text{H}\alpha\text{H}\beta}$  value of 9.27 Hz is not in good agreement with the experimental value. As for residue 44, using biquadratic time-averaged local elevation biasing results in the dihedral angle switching between two sets of values, around 270° and 120° i.e., the ranges of angle values sampled individually by the instantaneous and time-averaged simulations are now both sampled in the same simulation. The corresponding  $^3J_{\text{H}\alpha\text{H}\beta}$  and  $^3J_{\text{NH}\beta}$  values of 10.77 and 1.75 Hz are both in very good agreement with the experimental data. What is particularly noticeable for this residue is that despite the quite different distributions of dihedral angle values, the  $^3J$  couplings calculated from the instantaneously restrained and biquadratic time-averaged local elevation-biased simulations are rather similar, making it difficult to ascertain which distribution is the most correct

These residues are just two examples of cases where even time-averaging with a biquadratic restraint potential does not result in agreement with the experimental data because of insufficient sampling due to high energy barriers between conformations. In general, if a restrained simulation gives rise to a narrow range of the structural property associated with the restrained experimental observable, yet the back-calculated value of this observable does not agree with the experimental value, this can be interpreted as an indication that an enhanced sampling method such as local elevation is required.

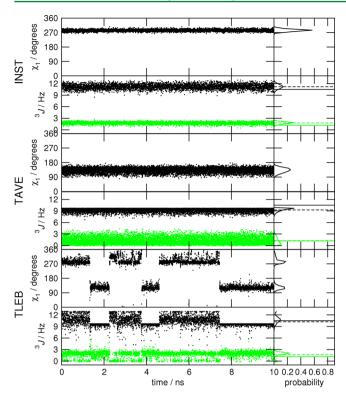
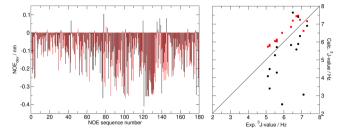


Figure 3. Time-series and distributions of the  $\chi_1$  dihedral angle value and  ${}^3J_{{\rm H}a{\rm H}\beta}$  (black) and  ${}^3J_{{\rm N}{\rm H}\beta}$  couplings (green) for residue 96 during simulations of FKBP bound to ascomycin with  ${}^3J$  value restraints enforced using a single-harmonic instantaneous restraining function (INST) or a biquadratic time-averaged restraining function (TAVE) in a vacuum (45B3 force field) or using biquadratic time-averaged local elevation biasing (TLEB) in explicit water (45A4 force field). The bin width for the histograms is  $10^\circ$  for the  $\chi_1$  angle values and 0.2 Hz for the  ${}^3J$  values. In the right-hand panels, the experimental value of each coupling (solid lines) and the average calculated from the simulation (dashed lines) are also shown.

A second example that illustrates the effect of the structure refinement protocol on the calculated conformational ensemble concerns the 16-residue peptide GCN4p16-31 for which a single-structure refinement protocol using the X-PLOR software<sup>73</sup> and a standard simulated-annealing approach<sup>74</sup> in vacuo failed to produce a set of model structures in agreement with all of the measured experimental data. While the set of 20 NMR model structures exhibits only a few small NOE upper-bound violations, a comparison of the  ${}^{3}J_{H_{N}H_{q}}$ -coupling constants backcalculated from the set of 20 NMR model structures with the corresponding experimental values shows that the calculated  ${}^{3}J_{\mathrm{H_{N}H_{a}}}$ -coupling constants for residues 18His, 19Leu, and 23Val deviate from the measured ones by more than 1.5 Hz, i.e., by 4.0, 3.4, and 1.8 Hz, respectively (Figure 4). The poor agreement of these <sup>3</sup>I values with the experimental ones is most likely due to the assumption of standard  $\alpha$ -helical hydrogen bonds and the imposition of  $\phi$ -torsional angle restraints in the single-structure refinement that produced the NMR model structures.46

With the aim of improving the agreement of the calculated conformational ensemble with the measured experimental data, we performed MD simulations in which time-averaged NOE restraints in conjunction with local elevation biasing of the  ${}^3J$  values were used. This combination of methods allowed us to account for the average nature of NOE signals and  ${}^3J$  couplings

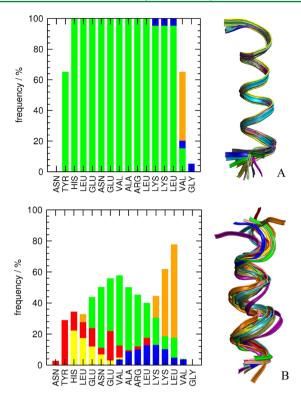


**Figure 4.** Deviation from the experimentally derived NOE upper distance bounds (NOE<sub>dev</sub>) as a function of the NOE sequence number (left-hand panel) and comparison of the experimental and calculated  ${}^3J_{\rm H_NH_a}$ -coupling constants (right-hand panel) for the set of NMR model structures (black) and restrained simulations (red) of GCN4p16-31.

appropriately as well as accommodate the nonlinear nature of the Karplus relation that links  $^3J$  couplings to dihedral angle values. As evident from Figure 4, the NOE violations and in particular the deviations of the calculated  $^3J$  couplings from the experimental values for the simulated ensemble are significantly lower than those obtained from the deposited set of NMR model structures.  $^{46}$  Moreover, the simulated ensemble shows much more conformational variability than the set of NMR model structures, manifest as a proliferation of interactions between backbone and side chains and between side chains not found in the NMR model structures.  $^{75}$ 

The differences in the conformational space explored using a standard simulated-annealing protocol in vacuo and using a time-averaged restrained MD simulation in explicit solvent are reflected in the secondary structure analysis presented in Figure 5. When the standard refinement protocol is used, 14 of the 16 residues of GCN4p16-31 exhibit a clear  $\alpha$ -helical structure. On the other hand, applying time-averaged NOE restraints together with local elevation biasing of the <sup>3</sup>*I*-coupling constants yields a decrease of the total helical population to less than 60% for all residues, and the nonhelical secondary structure, denoted as "bend", becomes dominant for four residues near the termini. This is in line with the  $\alpha$ -helical population as determined from the  $^{13}C\alpha$  chemical shift data.  $^{45}$  Crossvalidation of the NMR bundle and the simulated ensemble wagainst the chemical shift-derived  $\alpha$ -helical population (Figure S1, Supporting Information) shows that the  $\alpha$ -helical fraction calculated from the simulated ensemble agrees better with that calculated from the experimental chemical shifts than the  $\alpha$ -helical fraction calculated from the set of NMR model structures. The conformational space of the backbone of GCN4p16-31 sampled in our simulations is notably larger than that of the set of structures obtained by conventional single-structure NMR refinement. This demonstrates that the experimentally derived NOE distance bounds and <sup>3</sup>J-coupling constants require substantial flexibility in the backbone which cannot be captured if secondary, nonobserved structural data instead of primary, observed structural data are used in NMR structure determination.

**Detection of Inconsistencies in Experimental Data.** Use of simulation to aid the interpretation of experimental data can help to isolate errors and inconsistencies in the data that might not otherwise be obvious. Two examples from the study of FKBP are residues 8 and 43, for which all four types of  $^3J$  couplings were measured. The ranges of dihedral angles corresponding to each coupling, given an uncertainty of  $\pm 1$  Hz, are shown in the left-hand panels of Figures 6A and 7A. It is clear that, in both cases, there is no single region in which the ranges



**Figure 5.** Occupation frequencies for different secondary structure types of each residue of GCN4p16-31 and superposition of conformations for the entire set of NMR structures (A) and for the restrained simulation (B). In the case of the restrained simulation, the central members of the first 10 conformational clusters are shown. The secondary structure assignment in A is calculated according to Kabsch and Sander and is colored according to  $3_{10}$ -helix (yellow),  $\alpha$ -helix (green),  $\pi$ -helix (blue), bend (orange), and turn (red). The structures in B are superimposed using the heavy atoms of the backbone onto the first model or central member structure.

of dihedral angle values in keeping with all four couplings overlap. Given the nonlinearity of the Karplus relation, this may indicate that the measured <sup>3</sup>*J* couplings are averages over many different conformations. It is difficult to investigate this possibility by simply calculating the ranges of dihedral angle values corresponding to the measured <sup>3</sup>*J* couplings, but MD simulations allow the effects of conformational averaging to be explored. Rather than attempt to study the entire protein simultaneously, a model dihedral angle (MDA) was restrained or biased with the experimental data for one side chain at a time, thus avoiding any complications that might arise from interaction of the side chain under study with the remainder of the protein.

Using single-harmonic instantaneous or biquadratic time-averaged restraining resulted in the MDA sampling dihedral angle values around 250° for the residue 8 data set (Figure 6A) and 240° for the residue 43 data set (Figure 7A). When biquadratic time-averaged local elevation biasing was used, bimodal distributions were obtained, with the dihedral angle switching almost continuously between a broad range of angles from 45–100°, and a narrower range of angles centered on 250° (Figure 6B and Figure 7B, left-hand panels, top row). In all cases, other than the  $^3J_{\text{H}\alpha\text{H}\beta_2}$  coupling in the instantaneous and time-averaged restrained simulations, the  $^3J$  couplings calculated from the simulated structures are more than 1 Hz from the experimental values (Table 1). Thus, in comparison to

the cases of residues 44 and 96 discussed above, switching between multiple different conformations when biquadratic time-averaged local elevation biasing is used is not sufficient to fulfill the experimental data. Indeed, the local elevation biasing potentials increase throughout the simulations, and the final local elevation potentials for the four couplings conflict with one another (Figures 6B and 7B, central and right-hand panels, top row). This indicates that regardless of which dihedral angle value is sampled, at least one of the back-calculated <sup>3</sup>*J* values does not fit the corresponding experimental <sup>3</sup>*J* value, resulting in continuous building of the local elevation biasing potential and suggesting that one or more of the <sup>3</sup>*J* couplings is inconsistent with the remainder of the data.

Scrutinizing the predicted ranges of dihedral angle values (Figures 6A and 7A, left-hand panels) indicates that removal of the  ${}^{3}J_{NH\beta}$ , coupling from the residue 8 data set results in a region around 60° in which the predicted ranges of dihedral angle values agree (indicated by a cyan oval on Figure 6A). This makes particular sense given that both of the  ${}^{3}J_{NH}$ -coupling values are 1.8 Hz, suggesting that a misassignment or typographical error may have occurred at some point. In addition, it is clear from Figure 6A that the ranges of dihedral angle values compatible with  ${}^3J_{{\rm NH}\beta_2}$  and  ${}^3J_{{\rm NH}\beta_3}$  couplings of 1.8 Hz do not overlap, although the predicted ranges of dihedral angle values do not account for conformational averaging. For the residue 43 data set, removal of the  ${}^{3}J_{H\alpha H\beta_{3}}$  coupling gives rise to a possible solution around 170° (cyan oval, Figure 7A), and removal of the  ${}^{3}J_{NH\beta_{3}}$  coupling leads to a solution around 70° (magenta oval, Figure 7A). The experimental range for  ${}^{3}J_{\text{NH}\beta}$  couplings is much smaller than for  ${}^{3}J_{\text{H}\alpha\text{H}\beta}$  couplings, meaning that even small errors in a  ${}^3J_{\rm NH\beta}$  coupling can lead to quite significant errors in the corresponding dihedral angle values and suggesting that, for residue 43, it may make more sense to remove the  ${}^3J_{\mathrm{NH}\beta_2}$  coupling.

To investigate whether these predictions remain relevant once conformational averaging is included, simulations were carried out in which each of the <sup>3</sup>J couplings were removed from each data set in turn. For both residues, only removal of the  ${}^{3}J_{NH\beta}$ , coupling results in the remainder of the experimental data being satisfied. MD simulations of the MDA using biquadratic time-averaged local elevation biasing with edited data sets, from which the  ${}^{3}J_{NH\beta_{2}}$  couplings were removed, results in sampling of single, broad ranges of dihedral angle values around 60° for residue 8 (Figure 6A, right-hand panel) and from 40 to 100° for residue 43 (Figure 7A, right-hand panel). The <sup>3</sup>*J* values are all within 1 Hz of the experimental values (Table 1). Initially, during the local elevation build-up phase in which all dihedral angle values are sampled, the local elevation biasing potentials increase, but once the local elevation biasing potentials defined by the experimental data are established, the potentials experienced by the MDA decrease and stay small for the remainder of the simulation (Figures 6B and 7B, central panels, bottom row), as it now samples angle values that lie in minima of the biasing potentials.

These two examples illustrate clearly how use of MD simulation, in particular of small model systems, can aid the identification and elimination of inconsistencies in experimental data

Underdetermination of NMR Model Structures. In general, for solution structures of proteins derived from NMR data, the number of items of data  $N^{\rm data}$  is much less than the

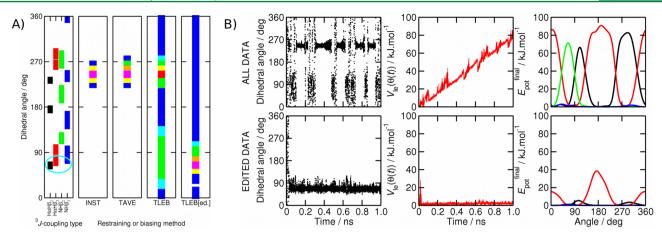


Figure 6. (A) Predicted ranges and populations of dihedral angle values according to  ${}^3J$ -values measured for residue 8. Left panel: ranges of dihedral angle values calculated from the experimentally measured  ${}^3J$  values  $\pm$  1 Hz, colored according to  ${}^3J$ -value type,  ${}^3J_{\text{H}\alpha\text{H}\beta_2}$  (black),  ${}^3J_{\text{H}\alpha\text{H}\beta_3}$  (red),  ${}^3J_{\text{NH}\beta_2}$  (green), and  ${}^3J_{\text{NH}\beta_3}$  (blue). Remaining panels: populations of dihedral angle values sampled during simulations of the MDA with  ${}^3J$ -value restraints enforced using a single-harmonic instantaneous restraining function (INST) or a biquadratic time-averaged restraining function (TAVE) or using biquadratic time-averaged local elevation biasing (TLEB) in vacuum (45B3 force field). "Edited" refers to the data set obtained by eliminating inconsistent data. The populations are colored according to 0–1% (blue), 1–2% (cyan), 2–10% (green), 10–20% (yellow), 20–30% (orange), 30–40% (red), 40–100% (magenta). (B) Behavior of the MDA and the associated biasing potential energy function during MD simulations biased with  ${}^3J$  values measured for residue 8 enforced using biquadratic time-averaged local elevation biasing (TLEB) in vacuum (45B3 force field). In the top row are the results when all experimental data are used, and in the bottom row, the results when the edited data set obtained by eliminating inconsistent data are used. Left-hand panels, time-series of the dihedral angle value; central panels, time-series of the local elevation biasing potential function; right-hand panels, final local elevation biasing potentials, colored according to the type of  ${}^3J$  value each belongs to:  ${}^3J_{\text{H}\alpha\text{H}\beta_2}$  (black),  ${}^3J_{\text{H}\alpha\text{H}\beta_3}$  (red),  ${}^3J_{\text{NH}\beta_2}$  (green), and  ${}^3J_{\text{NH}\beta_2}$  (black),

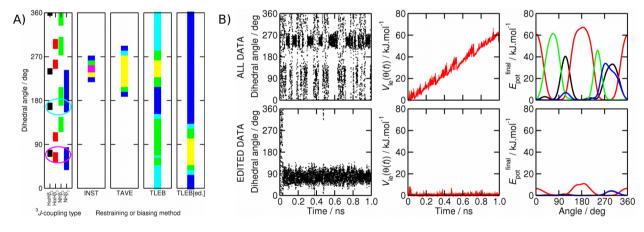


Figure 7. (A) Predicted ranges and populations of  $χ_1$  dihedral angle values for residue 43. Left panel: ranges of  $χ_1$  dihedral angle values calculated from the experimentally measured  ${}^3J$  values  $\pm 1$  Hz, colored according to  ${}^3J$ -value type:  ${}^3J_{\text{H}aH\beta_2}$  (black),  ${}^3J_{\text{H}aH\beta_3}$  (red),  ${}^3J_{\text{NH}\beta_2}$  (green), and  ${}^3J_{\text{NH}\beta_3}$  (blue). Remaining panels: populations of  $χ_1$  dihedral angle values sampled during simulations of FKBP bound to ascomycin with  ${}^3J$ -value restraints enforced using a single-harmonic instantaneous restraining function (INST) or a biquadratic time-averaged restraining function (TAVE) in vacuum (45B3 force field) or using biquadratic time-averaged local elevation biasing (TLEB) in explicit water (45A4 force field). "Edited" refers to the reduced data set obtained by eliminating inconsistent data. The populations are colored according to 0–1% (blue), 1–2% (cyan), 2–10% (green), 10–20% (yellow), 20–30% (orange), 30–40% (red), 40–100% (magenta). (B) Behavior of the MDA and the associated biasing potential energy function during MD simulations biased with  ${}^3J$  values measured for residue 43 enforced using biquadratic time-averaged local elevation biasing (TLEB) in vacuum (45B3 force field). In the top row are the results when all experimental data are used, and in the bottom row, the results when the edited data set obtained by eliminating inconsistent data are used. Left-hand panels, time-series of the dihedral angle value; central panels, time-series of the local elevation biasing potential function; right-hand panels, final local elevation biasing potentials, colored according to the type of  ${}^3J$  value each belongs to:  ${}^3J_{\text{HaH}\beta_3}$  (black),  ${}^3J_{\text{HaH}\beta_3}$  (red),  ${}^3J_{\text{NH}\beta_3}$  (green), and  ${}^3J_{\text{NH}\beta_3}$  (blue).

number of degrees of freedom of the protein structure  $N^{\mathrm{df}}$ . If a single structure is to be determined,  $N^{\mathrm{df}}=3N$ , where N is the number of atoms in the protein. Use of even the most basic molecular mechanics force fields, however, severely restricts the motion of the bond lengths, bond angles, and some of the torsional angles, such that  $N^{\mathrm{df}} \approx N$ . Even so, most NMR model

structures are significantly underdetermined: ideally, the ratio of the number of independent items of data  $N^{\rm idata}$  and  $N^{\rm df}$ ,  $N^{\rm idata}/N^{\rm df}$ , should be much greater than 1, but in practice, it seldom is, due to correlation between elements of the data, such as when a single atom is involved in multiple NOE distance restraints. Additionally, a large proportion of NOE distances, which often

Table 1.  $^3J$  Values Measured Experimentally (EXP) and Calculated As Averages over the Trajectory Obtained from Simulations of the MDA Using Single-Harmonic Instantaneous (INST) or Biquadratic Time-Averaged (TAVE) Restraining or Biquadratic Time-Averaged Local Elevation Biasing (TLEB) with the Complete Data Set (All Four Types of  $^3J$  Coupling) or Biquadratic Time-Averaged Local Elevation Biasing with the Edited Data Set (NH $_{\beta_2}$ -Coupling Removed, TLEB[ed.]) for Residues 8 and 43 of FKBP

residue	type	EXP	INST	TAVE	TLEB	TLEB[ed.]
8	$H_{\alpha}H_{\beta_2}$	4.0	5.86	6.04	6.30	4.70
	$H_{\alpha}H_{\beta_3}$	2.0	4.21	4.11	4.35	2.77
	$\mathrm{NH}_{eta_2}$	1.8	3.05	3.01	3.07	
	$\mathrm{NH}_{eta_3}$	1.8	2.04	2.13	2.07	0.97
43	$H_{\alpha}H_{\beta_2}$	5.0	5.68	5.39	6.63	5.80
	$H_{\alpha}H_{\beta_3}$	3.1	4.37	5.35	4.97	2.90
	$\mathrm{NH}_{eta_2}$	1.0	3.06	2.75	2.65	
	$\mathrm{NH}_{eta_3}$	0.8	1.95	1.78	2.21	1.45

comprise the largest proportion of the NMR data used in protein structure determination, pertain to pairs of atoms that are close together in sequence, greatly reducing the amount of information that can be obtained about the global fold of the protein. This was nicely illustrated by the work of Nabuurs et al. in their introduction of the QUEEN method for evaluating the information content of NMR data. Another complication not accounted for by the QUEEN method is the conformational averaging inherent in solution NMR data. Accounting for this by carrying out ensemble- or time-averaging, although more correct, further increases the number of degrees of freedom.

 $G_A95$  and  $G_B95$ , two proteins that differ in sequence by only three residues and yet fold into topologically distinct  $3\alpha$  (" $\alpha$ ") and  $4\alpha+\beta$  (" $\beta$ ") structures (Figure 1), provide an opportunity to quantify the information content of NMR data due to the availability of crossover homology models, in which a given sequence is fitted onto the structure corresponding to the other sequence. <sup>44</sup> For example,  $\alpha/\beta$  is a homology model created by fitting the  $\alpha$  sequence onto the  $\beta$  structure. The number of violations, defined as a positive deviation from the upper bound of the distance restraint of more than 0.1 nm when the NOE distances are back-calculated from the energy-minimized NMR model structures and from the homology model structures, allows the number of NOE distance restraints that actually define the three-dimensional structure of the protein to be identified.

The full sets of NOE distance restraints used to determine the NMR model structures of  $G_A95$  and  $G_B95$ ,  $\alpha F$  and  $\beta F$ , were divided into two subsets:  $\alpha B$  and  $\beta B$ , containing NOEs between atoms in the protein backbone, and  $\alpha S$  and  $\beta S$ , containing NOEs where one or both of the atoms are in the amino acid side chains. The NOEs in the  $\alpha B$  and  $\beta B$  subsets can be calculated from both the NMR model structures and from the homology-modeled crossover structures, whereas data sets  $\alpha F$  and  $\beta F$  and subsets  $\alpha S$  and  $\beta S$  can only be calculated from structures where the sequence matches the sequence of the protein for which they were measured; e.g.,  $\alpha F$  can only be calculated from  $\alpha/\alpha$  and  $\alpha/\beta$  (see Table 2). These data sets were further subdivided according to the sequence separation

(in numbers of residues) between the pairs of atoms involved in each NOE distance restraint.

Only a small fraction, 7%, of the  $\alpha$ F data set comprises longrange NOE distance restraints, defined as those between pairs of atoms separated by more than three residues. NOEs between residues that are closer together in sequence are unlikely to be violated due to the connectivity of the polypeptide chain. Indeed, when the  $\alpha F$  data set is back-calculated from the  $\alpha/\beta$ homology model structure, it is mostly the long-range NOEs, along with those between atoms separated by three residues, that are violated. The large number of violations for pairs of atoms separated by three residues makes sense, as these are the key NOE data that define helices. Overall, 10.4% of the  $\alpha F$ NOE distance restraints are violated by the  $\alpha/\beta$  homology model structure. The 216 NOE distance restraints in  $\alpha B$  can be back-calculated from any sequence/structure combination.  $\alpha/\alpha$ and  $\beta/\alpha$ , which both have the  $\alpha$  structure, satisfy all 216  $\alpha B$ NOEs, whereas  $\alpha/\beta$  and  $\beta/\beta$ , which exhibit the  $\beta$  structure, violate 11.6% and 12.5% of the  $\alpha B$  NOEs, respectively, with two-thirds of these violations being for pairs of atoms three residues apart.  $\alpha/\alpha$  also satisfies all but one of the  $\alpha$ S data, whereas 9.9% are violated by  $\alpha/\beta$ . Overall, these results show that for both the  $\alpha F$  data set, which contains both backbone and side-chain NOEs, and the subset containing only backbone NOEs, approximately 90% of the NOEs are not informative about the global topology.

The  $\beta F$  data set contains more NOE distance restraints, of which a greater proportion are long-range, than the  $\alpha F$  data set. Accordingly, the number of violations and the proportion of the entire data set that is violated by the  $\alpha$  structures are larger than when the  $\alpha$  data sets are calculated from the  $\beta$  structures. For the  $\beta F$ ,  $\beta B$ , and  $\beta S$  data sets, 16–18% of the data are violated when the NOE distances are back-calculated from  $\beta/\alpha$  or  $\alpha/\alpha$ . Almost all of these violations are for long-range NOE distance restraints, indicating that for the  $\beta$  structure, even more than for the  $\alpha$  structure, the long-range NOEs provide the most information about the overall structure.

The number of theoretically possible NOE distances, i.e., the number obtainable in an ideal case where the interactions between all pairs of protons sufficiently close to one another give rise to a measurable and distinguishable NOE intensity, was calculated for the energy-minimized NMR model structures  $\alpha/\alpha$  and  $\beta/\beta$  and crossover homology model structures  $\alpha/\beta$  and  $\beta/\alpha$  by calculating the distances between all pairs of protons and retaining those less than 0.55 nm, as described by Zagrovic and van Gunsteren (Table 3). The first point to note is that not only are there, as expected, many more NOE distances than are measured experimentally, the number of long-range (sequence separation of more than three residues) distances, which are crucial for determining the overall topology, is significantly larger. While not all of these NOEs are expected to be observed experimentally, for a variety of reasons including spectral overlap and spin diffusion, this data set is informative as it offers an opportunity to determine how much improvement could be expected from measuring more data.

The amount of data informative about the overall structure can be quantified by considering the overlap between the data sets calculated for a given sequence in each of the two structures. For both structures, slightly more than half of the possible NOEs would be detected for both structures, but only a small percentage of these are long-range (5.6% for the  $\alpha$  structure and 3.9% for the  $\beta$  structure). In contrast, of the false positives (NOEs calculated from the crossovers that are not

Table 2. Numbers and Violations of Experimentally Measured NOE Distances<sup>a</sup>

		sequence separation (num. res.)							long-range		total	
data set	seq/struct	0	1	2	3	4	5	>5		%		%
αF		553	158	20	41	12	4	42	58	7.0%	830	
	lpha/lpha	1	0	0	0	0	0	0	0	0.0%	1	0.1%
	lpha/eta	1	4	7	29	8	2	35	45	5.4%	86	10.4%
$\alpha \mathrm{B}$		95	81	16	17	2	0	5	7	3.2%	216	
	lpha/lpha	0	0	0	0	0	0	0	0	0.0%	0	0.0%
	lpha/eta	0	0	4	14	2	0	5	7	3.2%	25	11.6%
	eta/lpha	0	0	0	0	0	0	0	0	0.0%	0	0.0%
	eta/eta	0	0	7	14	2	0	4	6	2.7%	27	12.5%
$\alpha$ S		458	77	4	24	10	4	37	51	8.3%	614	
	lpha/lpha	1	0	0	0	0	0	0	0	0.0%	1	0.2%
	$\alpha/eta$	1	4	3	15	6	2	30	38	6.2%	61	9.9%
etaF		636	196	12	32	9	4	152	165	15.9%	1041	
	eta/eta	0	0	0	0	0	0	0	0	0.0%	0	0.0%
	eta/lpha	2	4	8	10	4	4	144	152	14.6%	176	16.9%
$\beta$ B		89	100	3	7	2	3	35	40	16.7%	239	
	eta/eta	0	0	0	0	0	0	0	0	0.0%	0	0.0%
	eta/lpha	0	0	1	2	1	3	35	39	16.3%	42	17.6%
	lpha/eta	0	0	0	0	0	0	1	1	0.4%	1	0.4%
	lpha/lpha	0	0	1	2	1	3	35	39	16.3%	42	17.6%
$\beta$ S		547	96	9	25	7	1	117	125	15.6%	802	
	eta/eta	0	0	0	0	0	0	0	0	0.0%	0	0.0%
	eta/lpha	2	4	7	8	3	1	109	113	14.1%	134	16.7%

<sup>a</sup>The numbers of NOEs between pairs of residues at a given sequence separation are listed for the full (F), backbone-only (B), and remainder (S) NOE data sets measured for  $G_A95$  and  $G_B95$  along with the percentage that are long-range (sequence separation of more than three residues). For each subset of the data, the number and percentage that are violated (defined as a positive deviation from the experimental value of more than 0.1 nm) when the NOE distances are calculated from the specified NMR or homology model structures energy-minimized in the GROMOS 54A7 force field are given.

Table 3. Comparison of Number of Theoretically Possible NOE Distances<sup>a</sup>

	sequence separation (num. res.)							long-		
data set	0	1	2	3	4	5	>5		%	total
$\alpha/\alpha$	1039	971	308	509	252	41	744	1037	26.8%	3864
lpha/eta	1034	882	367	241	156	93	1205	1454	36.6%	3978
$\alpha/\beta \in \alpha/\alpha$	1027	674	106	124	28	14	72	114	5.6%	2045
$\alpha/\beta \notin \alpha/\alpha$	7	208	261	117	128	79	1133	1340	69.3%	1933
$\beta/\beta$	1036	912	285	196	166	81	1266	1513	38.4%	3942
eta/lpha	1039	888	308	527	270	29	644	943	25.5%	3705
$\beta/\alpha \in \beta/\beta$	1025	680	100	111	37	3	38	78	3.9%	1994
$\beta/\alpha \notin \beta/\beta$	14	208	208	416	233	26	606	865	50.6%	1711

"The set of theoretically possible NOE distances for the energy-minimized NMR model structures of  $G_A95$  and  $G_B95$  ( $\alpha/\alpha$  and  $\beta/\beta$ ) and crossover homology model structures ( $\alpha/\beta$  and  $\beta/\alpha$ ) are defined as the distances between pairs of protons that are less than 0.55 nm. Long-range distances are defined as those between protons with a sequence separation of more than three residues. The overlap between the data sets is given by  $\alpha/\beta \in \alpha/\alpha$ , which lists the distances from the  $\alpha/\beta$  set that are also in the  $\alpha/\alpha$  set, and  $\alpha/\beta \notin \alpha/\alpha$ , which lists the distances from  $\alpha/\beta$  that are not in  $\alpha/\alpha$  (false positives). The same applies to the  $\beta/\alpha$  and  $\beta/\beta$  data sets.

calculated from the NMR model structures), 69.3% are long-range for the  $\alpha$  structure and 50.6% for the  $\beta$  structure. This again highlights the power of long-range NOEs to distinguish between different topologies.

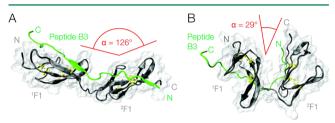
While a structure that violates 10–20% of the experimental data, as seen here when the experimentally measured data for one type of structure is back-calculated from the other type of structure, would not be considered a good solution in structure determination, what is more important to realize is that 80–90% of the experimentally measured data are not informative about the global topology; that is, only a very small fraction of the experimental data truly define the overall structure of the protein. This makes it even more important that sufficient

experimental data are measured and used in structure determination.

A further example highlighting the serious problems posed by the limited number of long-range NOEs typically used in the determination of the structure of large multimodular proteins is provided by the structure of the  $^1F1^2F1$  module pair of fibronectin in a complex with the bacterial peptide B3. The three-dimensional structure of the  $^1F1^2F1-B3$  complex has been determined using solution NMR, which produced a set of NOE distances that contains much information regarding the structure of the individual  $^1F1$  and  $^2F1$  modules and their interaction with B3. However, due to the lack of intermodule NOE distances, the spatial arrangement of the two fibronectin modules cannot be determined unequivocally from the

available NOE data set. Here, we illustrate how MD simulations can provide insight into the relative orientation of the  $^1F1^2F1$  module pair when in complex with B3 that is not obtainable purely from the experimental data.

With the aim of probing the relative orientation of the two modules in the <sup>1</sup>F1<sup>2</sup>F1–B3 complex, four MD simulations were performed starting from either NMR model structure 1 or 15: two 20 ns unrestrained MD simulations and two 10 ns MD simulations in which NOE distance bounds were imposed as time-averaged restraints. NMR model structure 1 represents an elongated conformation and structure 15, a bent conformation of the <sup>1</sup>F1<sup>2</sup>F1–B3 complex (Figure 8).



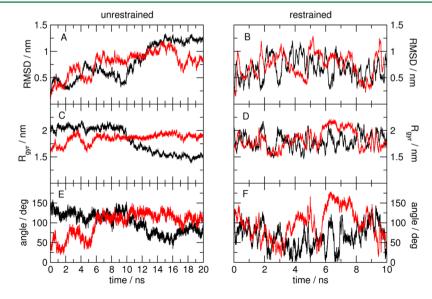
**Figure 8.** NMR structures of the  $^1\text{F1}^2\text{F1}\text{-B3}$  complex (PDB entry 109A). Fibronectin is shown in gray/black and the bacterial peptide B3 in green. (A) The first NMR structure of the 15 published NMR structures. The two F1 modules are in an elongated orientation with the intermodule angle  $\alpha = 126^\circ$ . The intermodule angle  $\alpha$  is defined as the angle between the axis of the D strands of the two F1 modules. The axis of the D strand passes through the center of the  $C_\alpha$  atoms of the two cysteine residues present in that strand (45CYS and 47CYS on  $^1\text{F1}$  and 92CYS and 94CYS on  $^2\text{F1}$  shown in yellow). The first 14 NMR structures have an angle similar to that in structure 1. (B) In contrast, NMR structure 15 shows a bent conformation of the two F1 modules with  $\alpha = 29^\circ$ .

In Figure 9, the RMSDs of the simulated trajectories relative to the initial structure, the radius of gyration, and the intermodule angle are presented as a function of time for the unrestrained and restrained MD simulations. For the unrestrained MD simulations started from NMR model structure 1, the RMSD values show a pronounced increase

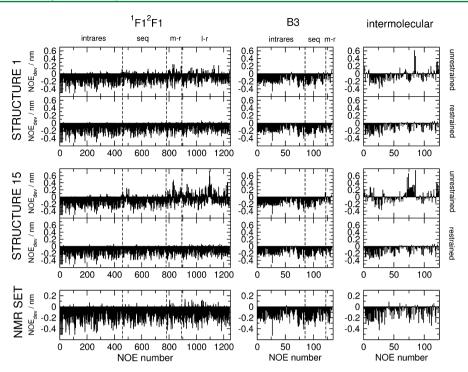
after 10 ns. As indicated by the radius of gyration at this time, the <sup>1</sup>F1<sup>2</sup>F1-B3 complex becomes more spherically compact, and the change in the intermodule angle reveals bending of the complex. The RMSD values for the unrestrained MD simulation started from NMR model structure 15 gradually increase over the course of the simulation. After some initial fluctuations in the first 5 ns, the corresponding radius of gyration increases from 1.6 to 2.0 nm, indicating an elongation of the <sup>1</sup>F1<sup>2</sup>F1-B3 complex. The change in the orientation of the two F1 modules is again strongly reflected in the analysis of the intermodule angle, which increases from 35 to 125°. It is important to note that the individual modules remain stable during the course of both unrestrained simulations, as shown by the undistorted secondary structure of the two F1 modules. Both preserve the native fold, which consists of a double-stranded and a triple-stranded antiparallel  $\beta$  sheet (Figure S2, Supporting Information).

Changes observed in the radius of gyration and in the intermodule angle suggest that significant variations in the orientation of the <sup>1</sup>F1 and <sup>2</sup>F1 modules in the <sup>1</sup>F1<sup>2</sup>F1–B3 complex are possible. However, since the reorientation of the modules seems to occur on the time scale of several nanoseconds, much longer unrestrained simulations are required to obtain statistically significant data on the dynamics of the <sup>1</sup>F1<sup>2</sup>F1–B3 complex.

In order to bias the sampling of the <sup>1</sup>F1<sup>2</sup>F1–B3 complex to the relevant regions of its conformational space, we repeated the MD simulations including the experimental NOE upper distance bounds as time-averaged restraints in the simulations. These trajectories revealed an interesting picture of the <sup>1</sup>F1<sup>2</sup>F1 intermodule orientation. The RMSD, radius of gyration, and intermodule angle in both restrained simulations display repetitive fluctuations, indicating that restraining the protein to the observed NOE distance bounds causes the complex to sample different bent and elongated conformations (Figure 9). Although the long-range proton—proton NOE distances do not bridge the two different modules, the intermodular orientation is apparently strongly influenced by the long-range NOEs within <sup>1</sup>F1<sup>2</sup>F1 as well as by the long-range NOEs between



**Figure 9.** Time-series of the atom-positional RMSD of the heavy atoms of the backbone from the energy-minimized starting structure (panels A and B), the radius of gyration ( $R_{gyr}$  panels C and D), and the intermodule angle (panels E and F) for unrestrained (left) and restrained (right) MD simulations started from NMR structure 1 (black) or NMR structure 15 (red) of the  ${}^{1}F1{}^{2}F1-B3$  complex.



**Figure 10.** Deviations from the experimentally derived NOE upper distance bounds NOE<sub>dev</sub> as a function of the NOE sequence number for the unrestrained and restrained simulations started from NMR structure 1 (top panels), NMR structure 15 (middle panels), and for the set of 15 NMR structures (bottom panels) for  ${}^{1}F1{}^{2}F1$ , B3, and between  ${}^{1}F1{}^{2}F1$  and B3. The vertical dashed lines indicate the boundaries between intraresidue (n:n), sequential ( $n:n \pm 1$ ), medium range (m-r,  $n:n \pm m$ , with m = 2, 3, 4) and long-range (1-r,  $n:n \pm m$ , with m > 4) NOEs, where n indicates the residue sequence number.

 $^{1}$ F1 $^{2}$ F1 and B3. For this reason, the conformational changes observed in the  $^{1}$ F1 $^{2}$ F1-B3 complex were analyzed by comparing the  $r^{-3}$  averaged proton-proton NOE distances from the simulations with the corresponding values derived from experimental NMR data.

Average violations of the NOE upper bound distances calculated over the entirety of the unrestrained or restrained MD simulations started from NMR model structures 1 or 15 and from the set of 15 NMR model structures are shown in Figure 10. Interestingly, 97% of all of the NOE distances in the unrestrained MD simulation started from NMR structure 1 and 91% of those in the unrestrained MD simulation started from NMR structure 15 are either satisfied or violate the upper bound by less than 0.1 nm, with 0.1 nm representing the uncertainty assumed to be inherent to experimental upper bound determination. However, a number of long-range NOE distances are violated by more than 0.1 nm in both unrestrained simulations. For the MD simulation started from NMR model structure 1, the largest violations are associated with the intermolecular proton pairs  $H_{\alpha}(Fn:100Gly)-H_{\delta}(B3:21Lys)$  and  $H_{\delta}(Fn:99Arg)-H_{\nu}(B3:23Lys)$ . In the MD simulation started from NMR structure 15, the largest violations arise from the intramolecular fibronectin proton pairs  $H_r(Fn:90Trp)-H_q(Fn:105Thr)$ ,  $H_r(Fn:90Trp) - H_{\theta}(Fn:105Thr), H_{\eta}(Fn:90Trp) - H_{\theta}(Fn:105Thr),$ and  $H_{\epsilon}(Fn:90Trp)-H_{\alpha}(Fn:105Thr)$  as well as from the intermolecular proton pairs  $H_{\alpha}(Fn:100Gly)-H_{\delta}(B3:21Lys)$  and  $H_{\zeta}(Fn:90Trp) - H_{\alpha}(B3:15Thr).$ 

Applying time-averaged distance restraining significantly improves the agreement with the experiment. As evident from Figure 10, the NOE distance violations in both restrained simulations are even smaller than the violations calculated for the set of 15 NMR structures. These results indicate that the entire set of NOE distances can only be satisfied if several

<sup>1</sup>F1<sup>2</sup>F1-B3 conformations with different intermodule orientations contribute to the ensemble average. Since the observed interdomain dynamics of the <sup>1</sup>F1<sup>2</sup>F1-B3 complex cannot be captured using standard NMR structure refinement, which searches for a single structure in keeping with all experimental data, the MD simulations presented here provide important insight into the conformational flexibility of the <sup>1</sup>F1<sup>2</sup>F1-B3 complex and offer a possible explanation for the presence of the bent conformation in the family of NMR structures deposited in the PDB.

# CONCLUSIONS

We have discussed here three ways in which MD simulations can help resolve difficulties in the derivation of protein structures from experimental NMR data. MD simulations can aid the interpretation of experimental NMR data in terms of structure by providing a model with which to supplement the limited amount of structural information typically acquired experimentally. Care must be taken that the simulation methodology is able to overcome sampling restrictions due to high energy barriers between different conformations and properly accounts for the averaging of observable quantities inherent to experimental data. This was highlighted by the cases of residues 44 and 96 of FKBP, for which conformational ensembles fully in keeping with the experimental NMR data were only obtained when time-averaged local elevation biasing was used. An additional illustration of this point was given by GCN4p16-31, for which the NMR model structures obtained using a standard, single-replica simulated annealing structure refinement protocol are highly helical but do not fit all of the experimental data, whereas the ensemble of structures obtained from an MD simulation incorporating time-averaged NOE restraints in conjunction with local elevation biasing of the

<sup>3</sup>*J* values are less helical and sample a broader range of conformational space, yet fit all of the experimental data on average.

MD simulations can also help to detect inconsistencies in experimental data. This was demonstrated using the <sup>3</sup>*J* couplings measured for the side chains of residues 8 and 43 of FKBP as examples. MD simulations of a model dihedral angle restrained or biased with these <sup>3</sup>*J* values not only revealed that it is impossible to generate a distribution of dihedral angle values that satisfies all of the experimental data but also indicated which items of data are most likely incorrect.

Finally, MD simulations also provide a means of shedding light on the inherent underdetermination of NMR model structures that arises due to the small number of NMR observables usually measured for a protein relative to its number of degrees of freedom. The calculation of NOE distances from the native folds and crossover homology model structures of two proteins with highly similar sequences but different native topologies,  $G_A$  and  $G_B$ , showed that approximately 90% of the NOE distances do not uniquely determine the overall structure. In a further example, the relative domain orientations of the  $^1F1^2F1-B3$  complex were poorly defined by the NOE data. The ensemble of structures produced by restrained MD simulations, which exhibited significant conformational fluctuation, provided a better fit to the experimental data than the set of NMR model structures obtained using standard structure refinement.

In summary, the examples presented here illustrate that the interpretation of experimental NMR data in terms of protein structure can be significantly improved by using unrestrained, time-averaged restrained, or local elevation biased MD simulations which account for the average nature of measured observables and allow proper sampling of the relevant degrees of freedom.

## ASSOCIATED CONTENT

## **S** Supporting Information

Figure S1 depicts the estimates of the  $\alpha$ -helical population of GCN4p16-31 derived from the C $\alpha$  chemical shifts measured experimentally and calculated from the set of NMR model structures and from the simulated ensemble. Figure S2 shows the secondary structure content of  $^1F1^2F1$  as a function of time for the unrestrained simulations started from NMR structures 1 and 15. This material is available free of charge via the Internet at http://pubs.acs.org.

# **■** AUTHOR INFORMATION

## **Corresponding Author**

\*E-mail: jozi@igc.phys.chem.ethz.ch.

# **Present Address**

\*Centre for Theoretical Chemistry and Physics, Institute of Natural Sciences, Massey University Albany, Private Bag 102904, North Shore, Auckland 0745, New Zealand

## Notes

The authors declare no competing financial interest.

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#### DEDICATION

We dedicate this work to Prof. Wilfred F. van Gunsteren in celebration of his 65th birthday and thank him for the advice and support he has given us and for sharing with us his knowledge and experience in the fields of NMR structure refinement and molecular dynamics simulations.

#### ABBREVIATIONS

EM, energy minimization; Fn, fibronectin; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; PDB, protein data bank; RDC, residual dipolar coupling;  $R_{\rm gyr}$ , radius of gyration; RMSD, rootmean-square deviation; SASA, solvent accessible surface area; SI, sequence identity; SPC, simple point charge

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