

Dynamite: a simple way to gain insight into protein motions

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A public web-based facility to infer, analyse and graphically represent the likely modes of a protein motion, starting from a static structure, is presented. This facility is based on the use of *CONCOORD* to generate an ensemble of feasible protein structures that are subsequently analysed by principal component analysis to identify probable concerted motions. The user is returned the ensemble of feasible structures, together with associated analyses, including animations and graphical representations of both the principal component of the ensemble covariance and indicators of strongly correlated pairwise atomic motions. Whilst users are warned that completely reliable inferences about protein motion may be beyond even substantially more rigorous tools for exploring configurational space, it is hoped that the service will allow a much wider community to benefit from the insights that simple dynamic data may offer.

1. Introduction

A full understanding of the structure–function relationship for a protein requires insight into dynamic properties as well as static structure. The principal function of certain proteins, including transporters and motor proteins, can only be understood on the basis of molecular motions (*e.g.* the coupling of proton transport to ATP synthesis in the F_0F_1 ATPase; Abrahams *et al.*, 1994). However, even for classes of protein for which mechanical action is not directly involved in function, molecular motions are still important. For example, the Src kinase regulatory mechanism that couples phosphorylated C-terminal tail binding to the maintenance of an inactive conformation has been suggested to relate to a tight coupling of the motions of SH2 and SH3 domains in the auto-inhibited form that is mitigated upon Src kinase tail dephosphorylation (Young *et al.*, 2001). This hypothesis, suggested by analysis of a molecular-dynamics simulation, was used to design mutagenesis experiments that probed and confirmed a proposed route of intramolecular communication.

Many software tools already exist to allow users to predict and analyse the likely motions of a protein to provide the type of results mentioned above [*e.g.* *GROMACS* (Lindahl *et al.*, 2001), *CHARMM* (Brooks *et al.*, 1983) and *CONCOORD* (de Groot *et al.*, 1997)]. For the most part, however, they require a high degree of technical competence in computational dynamics and visualization software. Whilst this level of competence could be achieved by most researchers given enough time, we have undertaken to deskill the process in order to allow a much larger population to use dynamics data in their research. We have produced *Dynamite*, a publicly accessible web-based server to generate and analyse likely modes of protein motion. This server-based approach

compliments the *Interactive Essential Dynamics* program (*IED*) provided by the McCammon group (Mongan & McCammon, 2003). *IED* is installed locally by a user and provides a graphical user interface to control the calculation of essential dynamics, using *GROMACS* to perform a molecular-dynamics simulation and subsequent analysis of the trajectory. As well as offering a different user interface, *Dynamite* performs a different set of analyses to those performed by *IED* and supports molecular-graphics routes to interpret the output.

1.1. Design goals for *Dynamite*

Dynamite has been designed to be simple to use, so that users should be able to access the functionality of the underlying programs without installing them on their local machines. This simplifies the user experience and removes much of the design cost associated with making a program compatible with multiple platforms. *Dynamite* has been written to be usable by anyone in the field of protein research, even if they have no dynamics experience, and to this end requires only an input coordinates file and offers selection of few parameters beyond specification of the type of analyses and representations that are requested. Ruggedness was another important goal. When analyses of the type provided by *Dynamite* are conducted manually, it is often found that minor problems have to be resolved during the process. For example, the implementation of the server has to handle difficult cases, which may have non-standard residues, may have atoms missing from the coordinate set or may simply be too large for computer memory. The base level for this ruggedness is that it should be able to trap and recognize errors, so that it can report back to the user/system manager the cause of problems. A secondary goal has been that *Dynamite* should be able to provide a reasonable response upon encountering common problems. For example, where inference of the essential dynamics for all atoms of a protein would involve analysis of a matrix of a size too large to be accommodated in computer memory, the expert layer of the server will automatically choose to restrict analysis to a subset of atoms, e.g. C α atoms.

2. Materials and methods

2.1. Overall scripting structure

The program performs the following functions: (i) input of initial protein structure and options, (ii) pre-screening of data for anomalies, (iii) generation of an ensemble of feasible structures from the starting structure, (iv) analyses of the ensemble, (v) generation of movies illustrating the results of the analyses and (vi) the return of results to the user.

To achieve these functions, *Dynamite* orchestrates a number of existing protein-dynamics packages, including *CONCOORD*, *GROMACS* and *VMD* (Humphrey *et al.*, 1996). *Dynamite* incorporates an expert/administrative layer that shepherds individual jobs through the correct sequence of routines to generate the requested analyses, deals with any

that have stalled or in some other way failed and notifies users of completion. In addition, *Dynamite* assembles a number of newly written programs that provide specific analyses not available from existing packages. *Dynamite* has been written in Python, an object-oriented programming language that lends itself well to the control of scripted programs and is able to interact directly with the Python interface of *VMD*. The overall process has been broken down into three stages: (i) the generation of an ensemble of probable protein configurations, (ii) the application of various analytical tools to identify concerted protein movements and (iii) the illustration of both global concerted motions and specific pairwise correlated atomic displacements.

2.2. Ensemble generation

The probable modes of motion available to a protein can be inferred from an analysis of a comprehensive ensemble of configurations that a protein is able to explore. One way to generate such an ensemble is by molecular dynamics (MD, reviewed recently by Moraitakis *et al.*, 2003). In this case, each frame of the ensemble is related to its predecessor through a time relationship; *i.e.* the positions and velocities of atoms in frame $N + 1$ are derived from their values in frame N by application of Newton's laws, with forces drawn from an empirical model of interatomic interactions. Initial coordinates are derived from the parent structure and initial velocities are randomly assigned from a Maxwellian distribution consistent with the simulated temperature. The resulting trajectory effectively represents a movie of the movement of the protein. Whilst this approach has the advantage of being a simulation of a physical situation, it is computationally intensive; it can take many months to generate trajectories corresponding to simulated periods of a few tens of nanoseconds. This length of simulation is, moreover, generally not long enough to explore the whole of the configuration space available to that protein, so that only a high-frequency subset of possible protein motions can be inferred.

An interesting alternative is to use non-Newtonian methods of ensemble generation. *CONCOORD* takes this approach and we have adopted it as a fast way to generate ensembles that explore configuration space more fully. This approach works in two phases. In the first phase, *CONCOORD* derives from the parent structure a table of constraints for the various interatomic distances (covalent, ionic *etc.*) that define a configuration of that protein. The permitted domain for each interatomic distance depends on an empirical model of the significance of different categories of interaction. Thus, if a covalent bond is recognized in the parent structure then a constraint is introduced that will reproduce this bond in all members of the derived ensemble to within a small tolerance. Similarly, a charge–charge electrostatic interaction in the parent structure will be reproduced in all ensemble members, although in this case a slightly greater tolerance is permitted. In the second phase, *CONCOORD* generates an ensemble of structures (typically 500) that preserve the interatomic distances of the parent structure. For each structure in the

ensemble, *CONCOORD* performs the following. Firstly, the atoms from the reference structure are randomly positioned in a box. Where pairs of atoms do not satisfy their specified distance constraint they are caused to move with respect to each other. This process is repeated until all constraints are satisfied. The ensemble generated in this way has some of the character of a dynamics trajectory, with the temporal order of frames scrambled. The obvious disadvantages of this method are (i) that the ensemble does not describe the physical evolution of the protein with time and (ii) that each frame in the ensemble does not necessarily correspond to a physically accessible configuration. Nevertheless, previous studies have found that ensembles generated by this method can closely resemble those derived from MD, while requiring several orders of magnitude less time to generate. Validation of this kind is presented by de Groot *et al.* (1997). In addition, we have tested whether the *Dynamite*-scripted implementation of *CONCOORD* reproduces the results of an MD simulation by comparing the results of *Dynamite* and *GROMACS* analyses of the protein MDM2. This work is presented below.

2.3. Covariance analysis and ‘correlation webs’

One of the simplest analyses performed addresses the question of which atoms appear to undergo concerted motions. This is achieved by an analysis of the covariance matrix derived from the ensemble of structures. If we list the coordinates of each atom for a frame and iterate two variables (i, j) over those coordinates for each frame t of the ensemble then the covariance is given by

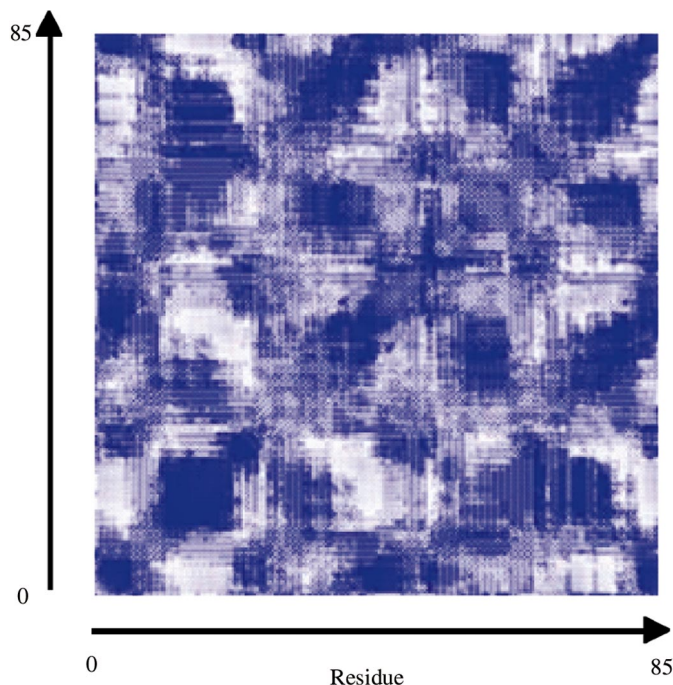


Figure 1
Graphical representation of the covariance matrix for the *CONCOORD* ensemble of MDM2/apo. The pairwise covariance expressed in (1) of the coordinates of each C_{α} of each residue is plotted with a darker blue representing higher covariance.

$$C_{ij} = \langle [r_i(t) - \langle r_i \rangle][r_j(t) - \langle r_j \rangle] \rangle_t \quad (1)$$

Note that the frames are enumerated by the symbol t because it would be traditional to perform this analysis over an MD trajectory in which each frame is associated with a particular time. The fact that the frames are not temporally related does not affect the analysis and t remains as a convenient label for the frames. This matrix is illustrated in Fig. 1.

This matrix is consolidated to form a per-atom normalized covariance (PANC) matrix by summing the x, y and z covariance components for each atom and normalizing,

$$C'_{i,j} = \frac{C_{x_i x_j} + C_{y_i y_j} + C_{z_i z_j}}{|(C_{x_i x_i} + C_{y_i y_i} + C_{z_i z_i})(C_{x_j x_j} + C_{y_j y_j} + C_{z_j z_j})|^{1/2}} \quad (2)$$

The renormalization of the matrix is such that the self-covariance of an atom, $C'_{i,i}$ is 1. Significant PANC values substantially away from the leading diagonal of the matrix identify parts of the protein that are not close in the primary structure but that do in fact tend to reconfigure in a concerted fashion.

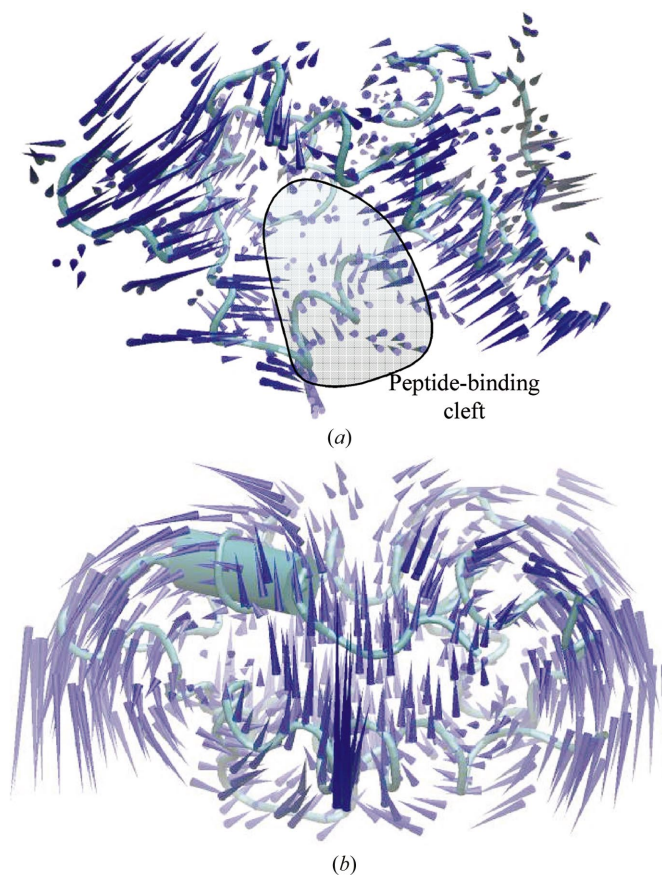


Figure 2
Porcupine plot of the principal mode of conformational variability calculated from a *CONCOORD* ensemble. (a) The principal mode of motion for MDM2/apo is dominated by a closing of the p53 cleft, indicated by the convergence of the cones on the two lips of the cleft. (b) Orthogonal view of the principal mode of MDM2/p53. This view is dominated by a pair of antiparallel vortices at each end of the molecule, indicative of a two-lobed flexing.

Young *et al.* (2001) suggest an interesting way to visualize this data. Quite simply, a line is drawn on a three-dimensional representation of the molecule to connect any two atoms i and j such that $C'_{ij} > \text{threshold}$. We have arbitrarily chosen a threshold of 0.7. This approach yields an image in which correlated regions are linked by a web, as if their motion were constrained by a network of elastic rods. This information can give a strong feeling for the movement of the molecule. In the reference cited, good use is made of comparing the webs formed in this way from simulations of different functional states of a protein in order to compare the resulting dynamic properties of the protein.

2.4. Principal components analysis and porcupine plots

Principal components analysis (Garcia, 1992) takes a data set and reduces its dimensionality. Applied to the current situation, we derive a matrix Λ by diagonalizing C_{ij} with a transformation matrix \mathbf{T} , so that $\Lambda = \mathbf{T}^T \mathbf{C} \mathbf{T}$. The columns of \mathbf{T} are then the eigenvectors \mathbf{v}_i of the motion, with the first column being the most significant motion, and the diagonal elements of Λ are the eigenvalues of the decomposition. A cone drawn from the atomic coordinate of an atom, with height and direction derived from the components of \mathbf{v}_1 that relate to that atom, gives a graphical representation of the motion held in \mathbf{v}_1 , the first eigenvector. We refer to this representation as a porcupine plot (Tai *et al.*, 2002). Examples are shown in Fig. 2. In these examples, the size of the cones has been scaled to make them visible when the whole molecule is imaged.

Equivalent information can be illustrated by generating a movie of N frames to illustrate the motion represented by an eigenvector. We take frame 1 as the average structure of the molecule within the ensemble. Frame N has the atoms displaced by a vector defined by components of \mathbf{v}_1 . The intermediate frames are produced by simple interpolation between these two extremes. There is a danger to this interpolation, in that the intermediate frames are almost certainly non-physical. We stress that they offer only an impression of the character of a probable preferred mode of protein motion.

2.5. Molecular-dynamics simulation

For the purpose of comparison, we have generated MD trajectories for the protein MDM2 both alone and in complex with a peptide derived from p53. *GROMACS* was used to simulate several runs up to 10 ns in length based on the GROMOS96 43a force field. In line with common practice, in each case there was a brief energy-minimization phase followed by a dynamics run with atomic positions restrained before the production dynamics run. The trajectories generated in this way were

analysed and compared with analyses of the *Dynamite/CONCOORD* ensemble.

3. Results

Dynamite has been tested with a number of proteins. We present here the *Dynamite* analysis of MDM2, an oncoprotein that binds to the p53 transcription factor (Momand *et al.*, 1998). Binding of MDM2 causes p53-directed gene transcription to be inhibited since the p53/MDM2 complex has lower affinity for DNA and is subject to nuclear export. MDM2, moreover, has E3-ubiquitin ligase activity that enables it to target p53 to the 20S proteasome for degradation. The transforming quality of MDM2 derives from its ability, when overexpressed, to hyperdestabilize p53, since p53 plays an

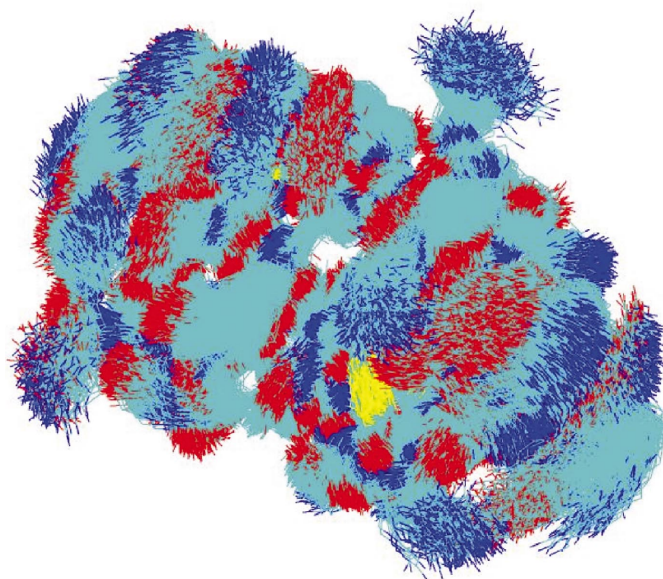


Figure 3
The superimposed ensemble of structures generated by a *CONCOORD* run on MDM2/p53.

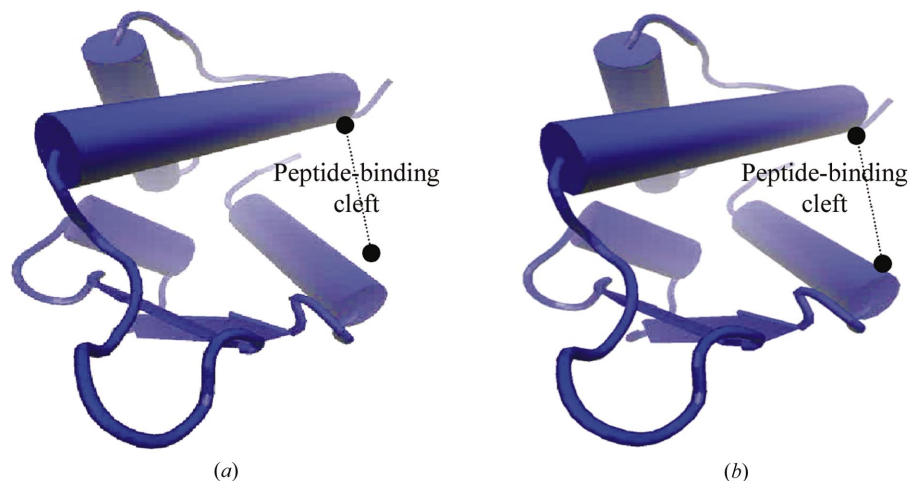


Figure 4
(a) First and (b) last frames from the movie illustrating the principal mode of MDM2/apo conformational variability inferred from *CONCOORD* analysis. Scale bars of equal length in each panel highlight the closure of the cleft that results from motion along the principal eigenvector.

essential role at the end point of several checkpoint pathways that control progress through the eukaryotic cell cycle. The *mdm2* gene is amplified or overexpressed in several human cancers (Vargas *et al.*, 2003), enabling the cancerous cells to overcome p53-mediated checkpoint surveillance that would otherwise result in cell-cycle arrest or apoptosis. Taken together, these facts suggest that the interaction between p53 and MDM2 is a suitable target for structure-based inhibitor design as a potential route to anticancer therapy (Lane, 1999). To date, structures have been reported for the p53-interaction domain of MDM2 in complex with a p53-derived peptide (Kussie *et al.*, 1996) and with a family of small-molecule inhibitors (Vassilev *et al.*, 2004). Although we have been able to reproduce the former crystals, we have not been able to crystallize the apo enzyme so as to enable high-throughput ligand-binding studies by crystal soaking. For this reason, we wished to test whether the absence of a ligand from the p53-binding domain might be responsible for introducing additional flexibility into the p53-binding domain, explaining the apparent difficulty in crystallization.

The peptide-bound structure of MDM2 (PDB code 1ycr) was used to produce parent structures either that retained the peptide structure (termed MDM2/p53) or from which the peptide had been deleted (termed MDM2/apo). These structures were submitted to the *Dynamite* server and the resulting *CONCOORD*-generated ensemble and the graphical representations of the corresponding analyses are given below.

3.1. Ensemble

The ensemble of *CONCOORD*-derived structures was returned as a text file. The main reason to return this text file is to allow the user to perform their own analyses on the data and to verify that *Dynamite* has returned a sensible result. For the purposes of this paper we have manually converted this data into a graphical image, shown in Fig. 3.

3.2. Movie along the first eigenvector

Fig. 4 shows two frames from the first eigenvector movie of MDM2/apo. When watching the movie it is clear that there is an opening and closing motion of the site where p53 had been bound. The user can elect to receive both the MPEG movie and a text file that lists the coordinates of each atom for each frame of the movie. This latter option allows the comparison of several movies if the user has some basic visualization skills. For example, Fig. 5 shows frames from a movie that was prepared by hand from the two PDB-format trajectories superimposed and aligned frame by frame. This figure emphasizes the increased amplitude of closing motion of the p53-binding site of MDM2 when p53 is removed. Clearly, if the user wished to quantify the magnitude of this motion they could do so by measuring relevant interatomic distances at the start and end points of the motion.

Biologically, the conclusion from these movies is that when the peptide is removed from the MDM2/p53 the structure shows an increased capacity to undergo breathing motions that narrow the peptide-binding site. This result in turn

suggests that there may be a large degree of induced fit of MDM2 to p53.

3.3. Porcupine plots

Fig. 2(a) shows a porcupine plot for eigenvector 1 of MDM2/apo. Examination of the 'quills' for the regions that border the peptide-binding cleft indicate a closing motion, necessarily consistent with the impression offered by the movie representation. A similar plot for MDM2/p53 (not shown from this viewpoint) does not show such a clear rearrangement, consistent with a reduced amplitude for the breathing motion in the peptide-bound complex. Fig. 2(b) shows an alternative viewpoint of MDM2/p53, generated by a *VMD* script from data returned by the *Dynamite* server. This figure illustrates a pair of 'vortices', one at each end of the protein, an appearance that is characteristic of a motion whereby two approximately rigid lobes flex about a connecting hinge.

3.4. Covariance web

Fig. 6 shows a covariance web for MDM2/p53. As would be expected, a mesh of lines interconnects the atoms within secondary-structural elements, consistent with locally correlated motions of the atoms within these elements, which therefore behave as approximately rigid bodies. In addition to such observations that are consistent with chemical intuition, less readily predicted higher order correlations also appear to occur. For example, movement of the peptide appears to correlate most closely with movements of the bottom-left region of MDM2, as seen from the orientation of Fig. 6. This effect is more apparent in a rotating view of the covariance web than from any single oriented view and correspondingly it is a movie of a rotating picture of the web that is returned by *Dynamite*. We are exploring the use of VRML as an alternative means of returning this analysis while allowing the user to select a preferred viewing orientation, but also intend to improve the interface with molecular-graphics packages such as *VMD* and *CCP4MG* (Potterton *et al.*, 2002).

Interestingly, the apparently 'tighter' interaction of p53 with the 'lower' surface of MDM2 suggests that this region might be the more appropriate part of MDM2 to target in the design of inhibitors of the p53-MDM2 interaction.

3.5. Validation

Real validation of the *Dynamite* protocol would require comparison of *Dynamite* results with an objectively true picture of protein motions. Although no experimental technique provides such a picture, techniques such as NMR offer some insights into the magnitude and character of motions that occur in a protein in solution. Interestingly, a comparison of the NMR spectra of the p53-binding domain of MDM2 alone and in complex with a p53-derived peptide is consistent with the *Dynamite* prediction that the MDM2/apo structure might undergo substantial changes in structure and dynamics upon binding to the p53-peptide (Schon *et al.*, 2002).

An alternative approach to validating *Dynamite* is to compare its results with those derived from a method of exploring configurational space that is more physically rigorous than *CONCOORD*, namely MD simulation. A full comparison of predicted modes of motion from two different techniques of generating ensembles is complicated and highlights the significant problems associated with focusing upon a single eigenvector as we have with *Dynamite* (van Aalten *et al.*, 1997). Although in the following discussion several favourable comparisons were discovered between the predicted modes of motion as inferred from MD and *CONCOORD* ensembles, attention is drawn in the output of the server to the fact that such a simple treatment can potentially be misleading.

Fig. 7 presents the reconfiguration of MDM2 that is predicted by MD to occur upon ligand release. This structural change is consistent with the preferred mode of motion of MDM2/apo that is predicted by *CONCOORD* (Fig. 2a).

For both MDM2/p53 and MDM2/apo, breathing motions are observed around the peptide-binding cleft. Of the two states, MDM2/apo demonstrates a substantially greater

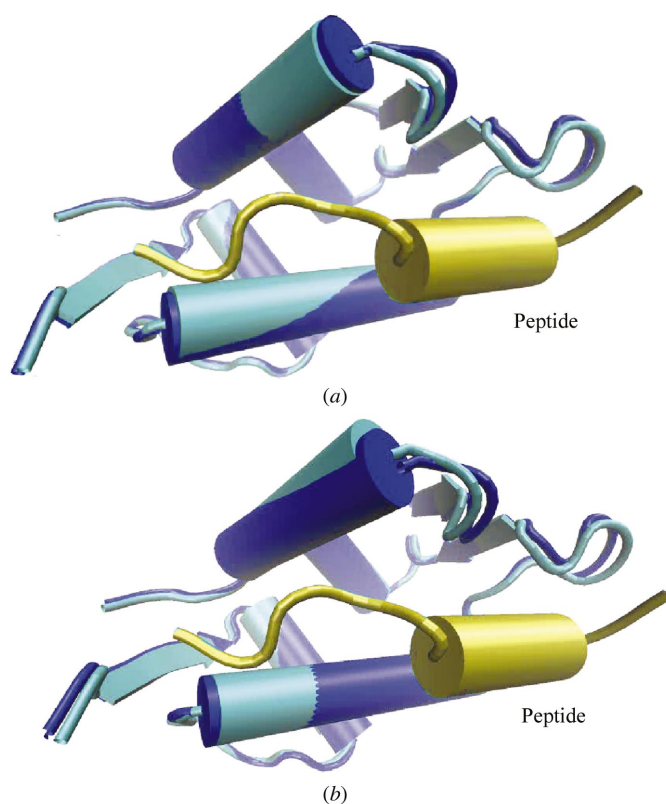


Figure 5

Superimposed first and last frame coordinates of MDM2/apo (turquoise) and MDM2/p53 (blue). In addition to generating the movie of the motion along the first eigenvector, *Dynamite* also provides the atomic coordinates of this motion for analysis by hand. This figure shows (a) the superimposed mean structures from the ensembles of MDM2/apo and MDM2/p53 (equivalent to the first frame of the returned movies) and (b) the superimposed mean structures of MDM2/apo and MDM2/p53 offset according to the first eigenvectors of the covariance matrix (equivalent to the last frame of the returned movies). This figure emphasizes that the principal difference between the two ensembles relates to the envelope of configurations that they explore rather than to their average structure.

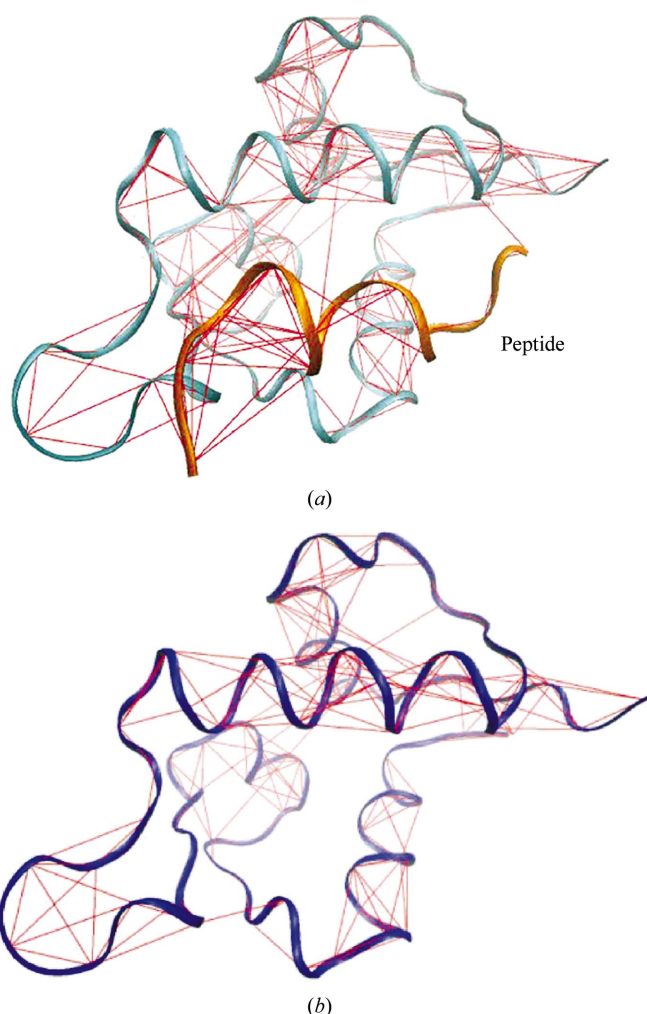


Figure 6

'Covariance web' for (a) MDM2/p53 and (b) MDM2/apo. The lines represent C^α atoms related by a PANC of greater than 0.7 and highlight regions of the molecule that move together. Lines that connect the peptide to the protein may identify the more significant regions of molecular recognition.

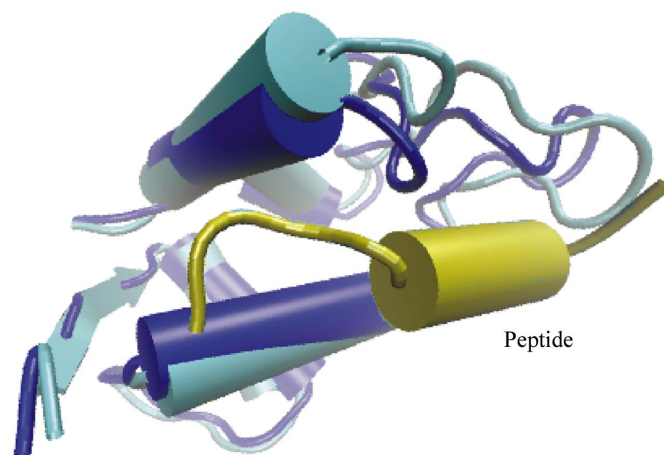


Figure 7

Reconfiguration of MDM2/p53 when the peptide is removed and the structure is simulated by MD. A significant closure of the peptide-binding cleft is observed in the apo structure (blue) compared with the peptide-bound form (cyan).

tendency to reconfigure in this way. Whilst the porcupine plots (Fig. 8) of the first eigenvector of the MD ensemble appear more complex than those from the *Dynamite* analysis (Fig. 2), they are not inconsistent with the conclusions drawn from them.

The similarity between the motions predicted by MD and *CONCOORD* is further supported by a comparison of covariance webs of ensembles generated by the two methods. Fig. 9 shows a comparison of covariance webs of MDM2/apo as evaluated from MD-based and *CONCOORD*-based ensembles. The webs from different ensemble-generating techniques are qualitatively similar (yellow lines predominate). The covariance threshold applied to the *CONCOORD* analysis is 0.7, while that applied to the MD analysis is 0.39,

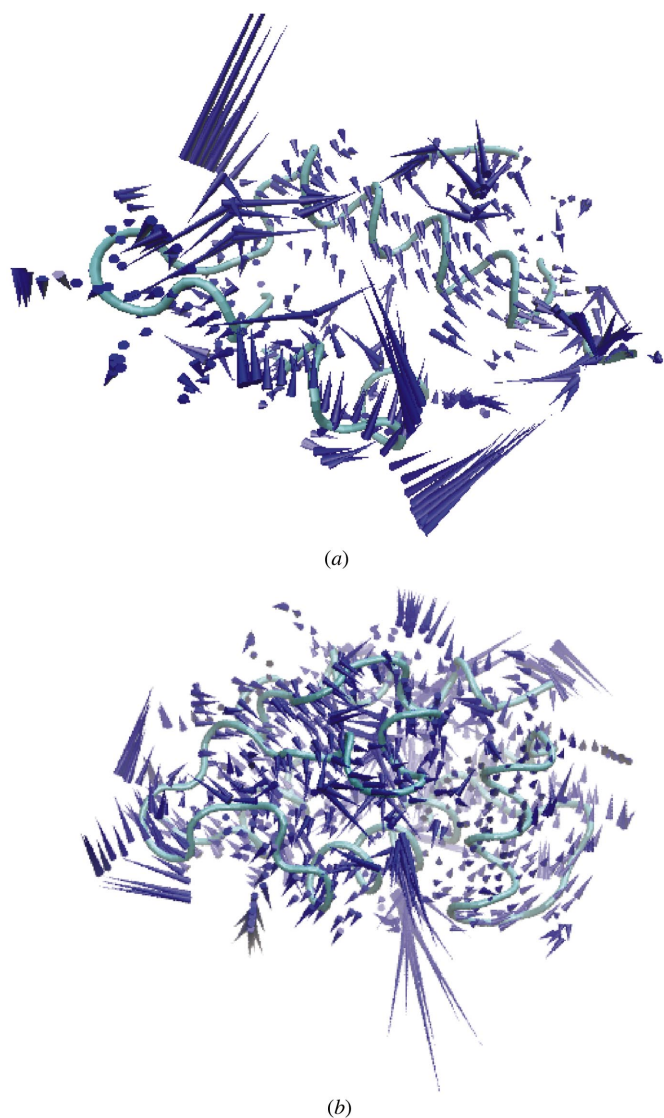


Figure 8
Porcupine plot of the principal mode of conformational variability calculated from an MD ensemble. (a) Although some aspects of the breathing motion of MDM2/apo indicated in Fig. 2(a) are recaptured from a 10 ns MD simulation, the porcupine plot here indicates that the motion may be more complex. (b) Orthogonal view of the principal mode of MDM2/p53. As in the *CONCOORD* analysis of MDM2/p53 in Fig. 2(b), this view is dominated by a pair of antiparallel vortices at each end of the molecule, indicative of a two-lobed flexing.

reflecting the fact that fewer strongly correlated motions are found in the MD simulation.

Qualitatively, it appears that the longer the MD simulation is performed, the more closely the results resemble those deduced from *CONCOORD* analysis. By comparison of Figs. 10(a), 10(b) and 10(c), it can be seen that the covariance web inferred from a 10 ns simulation resembles the *CONCOORD* covariance web more closely than the web inferred from a 5 ns simulation does. It is tempting to extrapolate from this that *CONCOORD* is achieving its ambition of more efficiently sampling configurational space while retaining physical plausibility.

4. Discussion

Dynamite is a free web-based service to provide data and analyses of probable protein motions. It needs only an input PDB file to work and functions without the user needing experience of molecular dynamics. In this paper, MDM2 has been used to illustrate the kinds of results that can be provided.

It is important to understand that these analyses are not rigorous for a number of reasons discussed in the text above, not least that only the first eigenvector of motion is returned. We hope, however, that *Dynamite* will broaden workers' appreciation of the impact dynamics have on the function of their subject proteins, suggesting further analyses and experiments.

The results for MDM2 presented above suggest that MDM2 binds to p53 by induced fit and that removal of p53 peptide from the binding cleft on MDM2 leads to a partial collapse of the cleft. Additionally, we find that the peptide moves in concert with one half of MDM2 more than the other. This result suggests that targeting the surface of MDM2 in this area may be particularly effective in disrupting the binding of p53.

Principal components analysis visualized with 'porcupine' plots indicates that a large part of the bulk motion of MDM2 can be described by a bilobal flexing of the molecule.

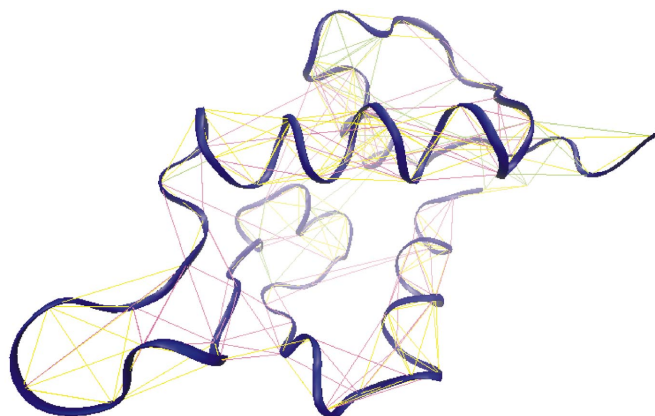
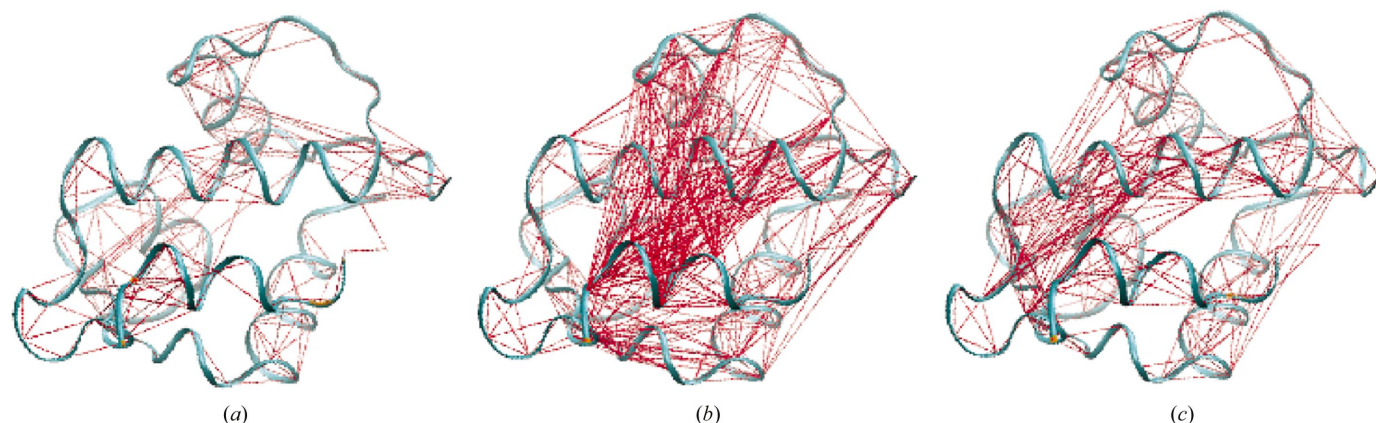


Figure 9
Comparison of covariance webs evaluated from MD and *CONCOORD* ensembles for MDM2/apo. Yellow lines are correlated motions predicted by both methods, magenta are those predicted by MD only and green those predicted by *CONCOORD* only.

**Figure 10**

Covariance webs predicted from ensembles generated by (a) *CONCOORD*, (b) a 5 ns MD simulation and (c) a 10 ns MD simulation.

We have provided some validation of the non-Newtonian procedure employed in generating the ensembles used for the analysis. We observe that the key features of the dynamics predicted by *Dynamite* (based on *CONCOORD*) are also seen in a molecular-dynamics analysis run on *GROMACS*.

The extent to which a physically meaningful ensemble of structures can be generated depends on both the algorithm used to generate candidate members and the force field employed to screen them. In order to explore alternative approaches to both of these aspects, we intend to implement an internal coordinate mechanism (ICM) module as an alternative to *CONCOORD* for this phase of protein-motion prediction. As well as providing carefully calibrated potentials for use in screening candidate ensemble members, ICM works with a lower-dimensional description of protein structure to effectively remove the need for high-frequency sampling of a Newtonian trajectory (Abagyan *et al.*, 1994).

Further development will include the extension of the expert layer to recognize and cater for the analysis of non-trivial input coordinate sets (*e.g.* multiple conformations, tightly bound metal ions, nucleic acids and non-proteinaceous ligands). We also intend to return data through means that will simply allow the user to control representation and orientation. At least one way will be through refining the interface between *Dynamite* output and laboratory visualization tools such as *VMD* and *CCP4MG*.

Dynamite took only a few hours to produce the ensemble, analysis and movies presented in this paper. We hope that its ease of use and relative short turnaround time will enlarge the population of protein scientists that can use dynamics data to guide their investigations. *Dynamite* is accessible through the URL <http://dynamite.biop.ox.ac.uk/dynamite>.

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