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Functional concerted motions in the bovine serum retinol-binding protein

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Summary

The large concerted motions in the apo/holo bovine serum retinol-binding protein were studied using molecular dynamics simulation and 'essential dynamics' analysis. Initially, concerted motions were calculated from conformational differences between various crystal structures. The dynamic behaviour of the protein in the configurational space directions, described by these concerted motions, is analysed. This reveals that the large backbone dynamics of the protein is not influenced by the presence of retinol. Study of free retinol dynamics and retinol in the retinol binding site reveals that the protein binds retinol in a favourable conformation, as opposed to what has been previously described for the bovine cellular retinol-binding protein.

Introduction

The retinoid compounds are biologically important with involvement in vision [1], embryonic development, cell differentiation and neoplasia [2]. In the bloodstream, retinoid compounds are transported as retinol by the serum retinol-binding protein (sRBP). In the holo form, sRBP is bound to another protein, transthyretin (TTR), which prevents it from being filtered out in the kidney [3]. It is hypothesized that there exists a complex retinol transport system [4]: sRBP binds to a membrane receptor which induces sRBP to release retinol, and this receptor then transports retinol across the membrane where it is taken up by the cellular retinol-binding protein (cRBP) which then targets its ligand to specific intracellular locations.

Both the structure and dynamics of cRBP have been extensively studied; the apo- and holo-forms exhibit significant differences in conformational flexibility [5, 6]. In particular, it has been shown that retinol adopts a strained conformation when bound to cRBP [7]. This has led to hypotheses of how cRBP would take up retinol and how certain loops in the protein might be involved in protein-protein contacts, for example, with the membrane receptor. Similar questions can be asked about sRBP. How does the protein take up retinol? Would there be any changes in dynamic behaviour of specific loops which could be important for binding to TTR and the membrane receptor?

The retinol-sRBP complex is also a suitable model for studying hydrophobic interaction. It has been shown by X-ray crystallography that there is little structural difference (0.3 Å root mean square difference on C_α atoms) between the apo- and holo-proteins [8]. Retinol is a molecule with relatively few degrees of conformational freedom. Neither retinol nor sRBP contain metallic ions. Experiments have also been performed to determine the equilibrium binding constant of retinol to sRBP [9]. These features make them ideal candidates for simulation studies of hydrophobic interaction. A simulation study to unbind the retinol from the protein would enable us to gain insight into hydrophobic interaction between larger molecules.

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However, before this is attempted, it would be advantageous to learn more about the dynamics of the ligand and the receptor protein. In the case of retinol and cRBP, for example, favouring certain motions of the protein could lead to 'opening up' of the binding pocket, and the subsequent release in the conformational strain on retinol would lead to its unbinding [6, 7]. Could a similar mechanism be in action in the sRBP?

MD simulation work on the apo- and holo-forms of the human serum retinol-binding protein has been performed [10]. This work, however, suffers from several drawbacks. The coordinates of the apo-form were not determined experimentally. The simulation times were very short; both forms of the protein were equilibrated for 30 ps, while the production run for the apo-form and holo-form lasted, respectively, 40 ps and 30 ps. Lastly, the simulations were carried out *in vacuo.*

In this work, we carry out much longer MD simulations of these molecules in water, with experimental coordinates for retinol and both forms of the protein. We use the conformational differences between various sRBP structures to study their dynamics. The structure of sRBP has been studied extensively [8, 11] and various apo and sRBP-retinoid structures are available. Additional molecular dynamics (MD) simulations are performed to supplement this investigation of sRBP dynamics, and are furthermore used to study dynamic differences between apo/holo sRBP and free/protein-bound retinol.

Methods

Essential dynamics

It has been shown recently that it is possible to calculate functionally relevant concerted motions from the structural variation found in a cluster of crystal structures [12] from the same protein. The approach is similar to that described for calculating concerted motions from an MD trajectory using 'essential dynamics' (ED) [13]. ED is based on the diagonalisation of the covariance matrix, built from atomic fluctuations relative to their average positions:

$$C_{ij} = \langle (x_i - \langle x_i \rangle)(x_j - \langle x_j \rangle) \rangle \qquad (1)$$

where x are the x-, y-, z-coordinates of the atoms, $\langle x \rangle$ the average positions of the coordinates and the average is calculated over all structures (i.e., all crystal structures or all structures generated by MD simulations), after they have been superimposed onto a reference structure to remove overall translational and rotational motion. Here, only C_α atoms are used as it has been shown that this subset of atoms already captures most of the conformational changes in the protein [13, 14]. This covariance matrix is then diagonalised, yielding a set of eigenvalues and eigenvectors. The eigenvectors are directions in a $3N$ dimensional space (where N is the number of atoms), and motions along single eigenvectors correspond to concerted fluctuations of atoms. The eigenvalues represent the total mean square fluctuation of the system along the corresponding eigenvectors. The eigenvectors are sorted by the size of their corresponding eigenvalues, the 'first' eigenvector being the one with the largest eigenvalue. In the case of proteins, there are always only a few ('essential') eigenvectors with large eigenvalues. Hence, the overall internal motion of the protein can be adequately described using only a few degrees of freedom [13]. It has been shown that this approach yields a description of large concerted atomic motions which appear to be linked to the function of the protein [6, 12–14]. When crystal structures are used for ED, the resulting eigenvectors are based only on experimental data. Comparison with eigenvectors calculated from MD trajectories usually shows a high overlap, confirming the validity of using MD to study protein dynamics [12, 15]. Here, we used all crystal structures currently in the database, with or without ligand. In total 11 structures were used: 1RLB (2 chains) [16], 1BRP [17], 1BRQ [17], 1ERB [18], 1FEL [19], 1FEM [19], 1FEN [19], 1HBQ [8], 1HBP [8] and 1RBP [11].

Simulation details

MD simulations of apo- and holo-sRBP were performed using a version of GROMOS optimised for execution in a parallel computing environment. The simulations were started from the apo (1HBQ [8]) and holo (1HBP [8]) bovine sRBP structures. With conservation of crystallographically determined water molecules, the proteins were solvated in truncated octahedral boxes filled with SPC [20] water molecules. Ions were added to ensure total electronic neutrality. The structures were minimised, and simulations were started with a 5 ps run at 300 K, taking initial velocities from a Maxwellian distribution. Temperature and pressure coupling [21] were applied with time constants of 0.1 ps and 0.5 ps, respectively. SHAKE was

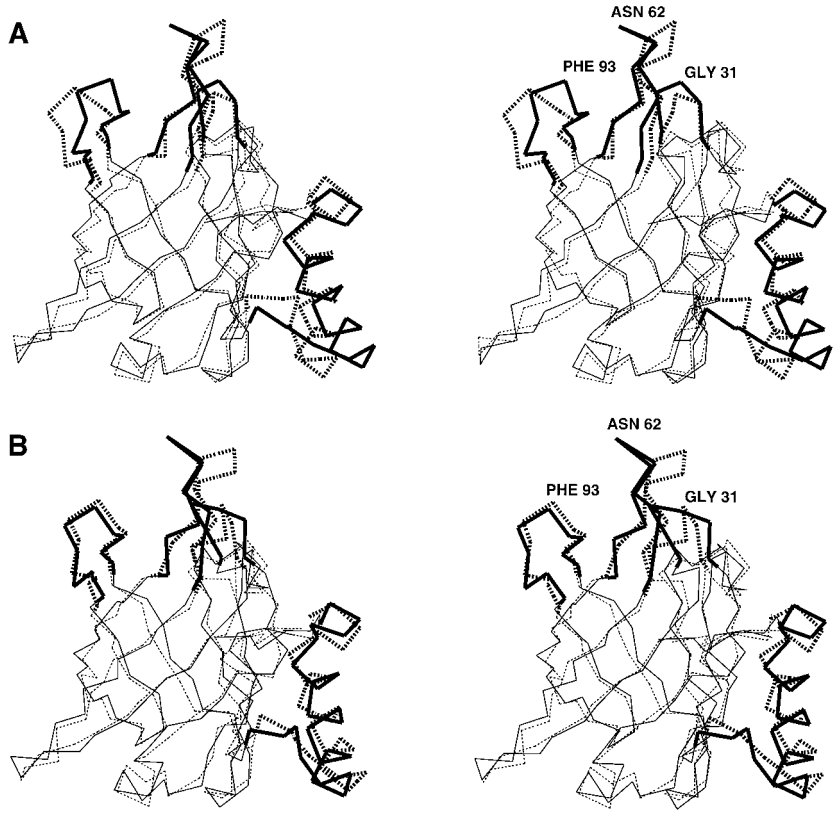

Figure 1. Stereo views of protein conformations projected at −1.0 nm (solid) and +1.0 nm (dotted) along crystal eigenvectors 1 (A), and at −0.8 nm (solid) and +0.8 nm (dotted) along crystal eigenvectors 2 (B). The regions where the difference between the two structures is most prominent are outlined in bold, and three amino acids are labelled.

used to constrain bond lengths [22]. The time step was 2 fs. A long range electrostatic cutoff of 10 Å was used, together with a short range 8 Å cutoff. The simulations were then continued for 600 ps, of which the last 400 ps were stable as estimated by various geometrical properties, calculated with the WHATIF trajectory analysis module [23].

The simulation of free retinol has been described elsewhere [7]. Briefly, this is a 1 μs simulation *in vacuo*, the results of which are in agreement with various solution experimental data [7]. Free retinol dynamics was compared to dynamics of retinol bound to sRBP using all atoms except the terminal hydroxyl group. We have not included the hydroxy tail in this analysis, because we plan to compare the ED of retinoids in future work. For such comparisons one can only use a 'common' backbone, thus we omitted the terminal atoms (in case of retinol, the hydroxyl group).

Results

Concerted motions from sRBP crystal structures

Using the 11 crystallographic sRBP structures described in the Methods section, we evaluated the concerted atomic motions using the essential dynamics method [13]. The motions described by eigenvectors 1 and 2 are shown in Figure 1. There are two main areas of motion. The loops around residues 35, 65 and 95, which form the entrance to the β-barrel, move together, controlling the diameter of the solvent tunnel towards the center of the β-barrel. In concert with this there is a tilting of the helix in the first eigenvector, whereas in the second eigenvector there appears to be a vibrational motion along the helical axis. For clarification, these eigenvectors will be called the 'crystal ED eigenvectors'. The spread of the X-ray structures in the space spanned by the first and second crystal ED eigenvectors is shown in Figure 2.

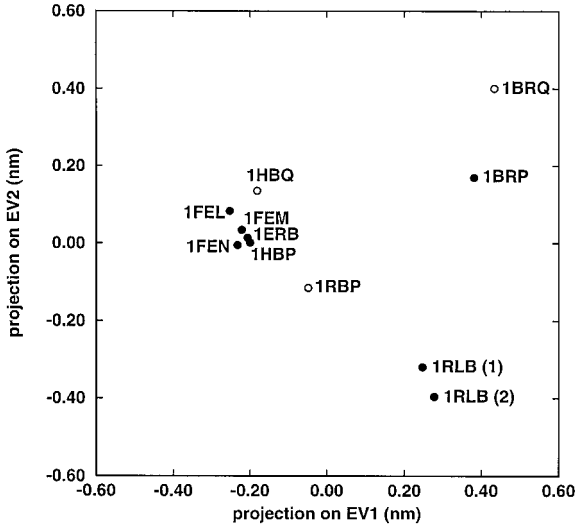

Figure 2. Projection of all 11 X-ray structures onto eigenvectors 1 and 2 determined from them ('crystal eigenvectors'). Open circles denote the apo-proteins, while closed circles denote the holo-proteins. 1RLB contains two distinct chains, and they are considered separately.

Properties of the apo/holo sRBP simulations

The stability of the apo/holo sRBP simulations was assessed by calculating various geometrical properties as functions of simulation time. The stability of structural deviation from the starting structure, hydrogen bonds, and radius of gyration are shown in Figure 3. The solid line shows the values for the apo-sRBP, while the broken lines shows those for the holo-sRBP.

It can be seen that the root mean square structural deviation from the starting structure is about 2 Å for the apo-sRBP, and about about 2.2 Å for the holo-sRBP. These are within acceptable limits. The number of hydrogen bonds is about 130 per protein molecule, with the apo-protein possessing slightly more hydrogen bonds than the holo-protein. The radius of gyration of the protein is about 1.53 nm for the apo-sRBP, and about 1.55 nm for the holo-sRBP. All three properties are stable, which indicates that the simulations represent fluctuations around a stable average.

By projection of the apo/holo sRBP trajectories on the crystal ED eigenvectors (Figure 4), a number of observations can be made.

Firstly, both apo-sRBP and holo-sRBP have a significant amount of motion in the space defined by the first two eigenvectors determined from the crystal structures. This means that the concerted motions as calculated from the crystal structures can be reproduced by MD simulations.

Secondly, the motions along these crystal ED eigenvectors are similar (i.e., the clouds of points have similar shapes and sizes) for apo-sRBP and holo-sRBP. This seems to indicate that there is no significant change in dynamics upon release/uptake of retinol. The only difference is that, apparently, the two simulations visit different areas of the two-dimensional essential space, which indicates that there is a small static conformational change.

Additional support for this comes from the following analysis: we performed an ED analysis on the combined MD trajectory to calculate a set of eigenvectors, and then projected the MD movement onto these eigenvectors. Note that these eigenvectors are not the crystal ED eigenvectors, because they are calculated from ED analysis of the MD trajectory. They will be called the 'simulation ED eigenvectors'. The results are shown in Figure 5. The MD movement projected onto simulation ED eigenvector 1 is different for the apo- and holo-protein (A). For other simulation ED eigenvectors, it is impossible to distinguish the projections for the apo- and holo-protein (B–D). Only projections onto four simulation ED eigenvectors are shown in these panels. The projections onto other simulation ED eigenvectors are similar to eigenvectors 2–4.

We also calculated overlaps between simulation ED and crystal data ED eigenvectors. We assumed that 5% of the total eigenvectors represent the 'essential subspace'. It is found that the first 5% of eigenvectors (25 eigenvectors) of simulation ED reproduce the first crystal ED eigenvector for 32%. The same number for the second eigenvector is 23%. There is definitely a concentration of motion in the essential space; otherwise, 5% of the eigenvectors would have given only 5% of the overlap. This is further evidence that the concerted motions as calculated from the crystal structures can be reproduced by MD simulations.

Lastly, there is space in the simulation ED space that is not covered by the experimental data, and there is space in the experimental conformations not visited by the MD simulations. This is inevitable because of the limitations in sampling in MD, and also the small number of experimental structures available. A recent paper examines these aspects in greater detail [12]. One also has to bear in mind that the crystal structure is only a time average as well.

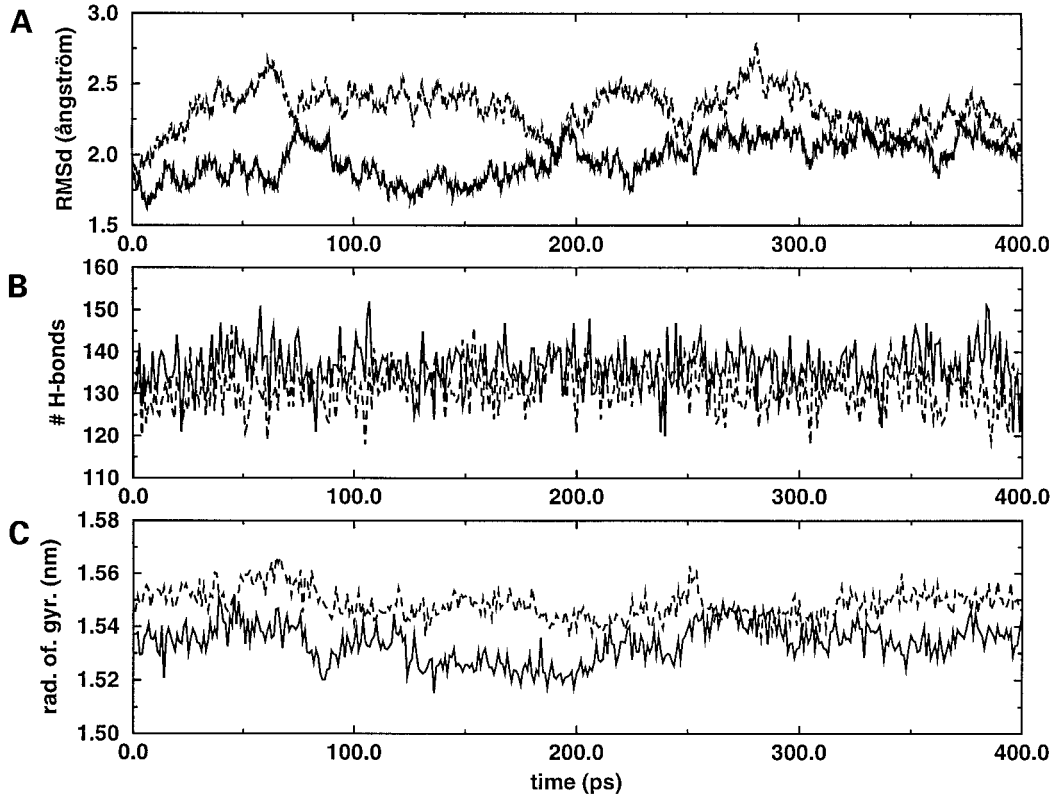

Figure 3. Root mean square deviation from the starting structure (A), backbone-backbone hydrogen bonds (B) and radius of gyration (C) as a function of simulation time. Solid line = apo-sRBP, dashed line = holo-sRBP.

Dynamics of free retinol and in the sRBP binding pocket

It has been observed previously that retinol binds to cRBP in a strained conformation and that this strain is relieved when retinol moves towards the exit portal of the cRBP binding pocket [7]. Here we study the dynamics of retinol bound to sRBP and compare it with the conformational freedom of free retinol. Although this free retinol simulation was performed *in vacuo*, the results obtained could be compared to experiments in solution, and the properties determined were similar [7].

Using the free retinol simulation trajectory, we evaluated the three largest eigenvectors using ED methods. Note that these eigenvectors were calculated from the retinol alone, and are different from the eigenvectors described in previous sections. We shall call these three eigenvectors the 'free retinol eigenvectors' to distinguish them from the others. The probability distribution of the free retinol MD simulation projected onto free retinol eigenvector planes 1–2, 1–3 and 2–3 is shown in Figures 6A, 6B and 6C. Some conformations of retinol are strongly preferred over others. The white areas represent relaxed structures of retinol, which have the ring rotated about $+60°$ or $-60°$ with respect to the plane of the tail (described mainly by eigenvectors 1 and 2). Cyclohexene ring inversion (described mainly by eigenvector 3) is another possible structural transition in retinol with two relaxed conformational states.

In Figures 7A, 7B and 7C, we take the bound retinol trajectory from the holo-sRBP simulations, and then project the equivalent movement of retinol onto the free retinol eigenvectors. On eigenvector plane 1–2, bound retinol prefers the region where $0.1 < \text{ev1} < 0.4$, $-0.1 < \text{ev2} < 0.4$. This corresponds to a highly preferred region of free retinol movement. However, the free retinol ED space where $\text{ev1} < 0$ is not traversed by the bound retinol. On eigenvector plane 1–3, the movement of bound retinol is centered around $\text{ev1} = 0.3$, $\text{ev2} = 0.1$, but the free retinol has a much larger movement, with two preferred areas centered around $\text{ev1} = 0.4$, $\text{ev3} = -0.1$ and $\text{ev1} = -0.4$, $\text{ev3} = 0.1$. On eigenvector plane 2–3, bound retinol occupies the

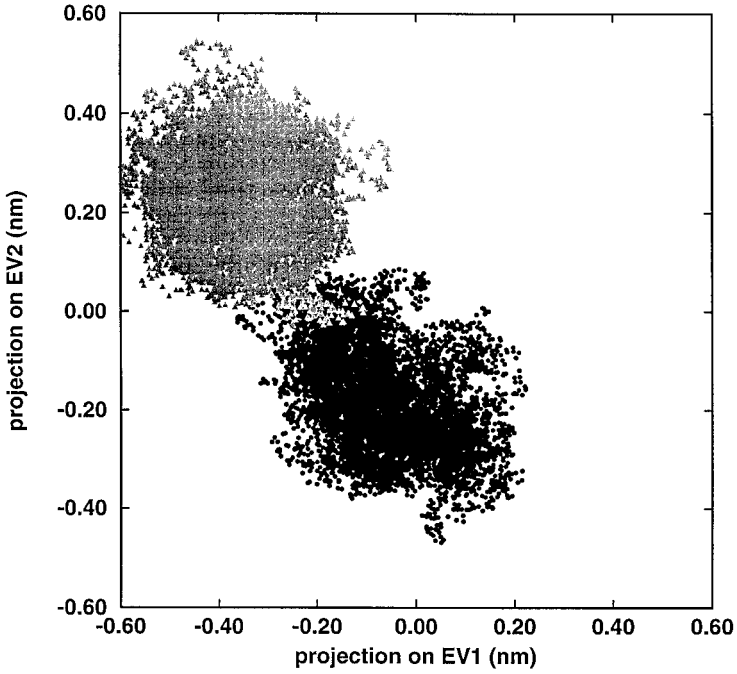

Figure 4. Projection of the apo-sRBP and holo-sRBP simulations onto the first and second crystal ED eigenvectors (eigenvectors calculated from the cluster of crystal structures). The black circular points represent apo-protein structures, while the grey triangular points denote the holo-protein structures. This figure can be compared with Figure 2.

region where $-0.1 < \text{ev2} < 0.3$, $0 < \text{ev3} < 0.2$. This corresponds to only part of the highly preferred regions of free retinol.

Thus, we observe that the movement of bound retinol occupies only part of the ED space of free retinol. In addition, the movement space of bound retinol is usually a preferred space of free retinol. Moreover, the movement of bound retinol does not exceed the movement space of free retinol. This shows that bound retinol in holo-sRBP occupies a smaller ED space of motion, limited to the more preferred states, and it does not adopt any conformation not observed in the case of free retinol; bound retinol is not in a strained conformation.

We would also like to investigate the correlation between the movement of retinol and the movement of the protein. We carry out the following procedure: the holo-sRBP MD frames are projected onto the holo-sRBP eigenvectors, and the retinol trajectory extracted. This 'correlated retinol' trajectory thus describes the large correlated motions of the retinol-protein system. Thereafter, we project this correlated retinol trajectory onto the free retinol eigenvectors. The results are displayed in Figures 8A, 8B and 8C, and they show the amount of movement of bound retinol that is correlated with the protein. It can be seen that it is very small, compared to the total motion of retinol bound to sRBP (see Figures 7A, 7B and 7C).

We can thus conclude that the movement of bound retinol bears a low correlation to that of the holo-sRBP. In addition, bound retinol does not adopt any strained conformations, and its ED space corresponds to the preferred ED space of free retinol.

Discussion

The study of ligand-protein recognition is almost as old as enzymology and molecular pharmacology itself, dating back to the original lock-and-key model of Fischer [24], and being updated in the 1950's by the proposals that proteins adapt their shape to the ligand upon binding [25]. This latter, so-called induced fit theory, seems to accord well with most of the data available so far [26, 27], in that some accommodation of the protein to the incoming ligand takes place. In some instances, there is even a moveable lid covering the binding site for the ligand [28] which moves out of the way in order to expose the binding site.

Equally important, but relatively neglected, is the issue of the accommodation of the ligand to the

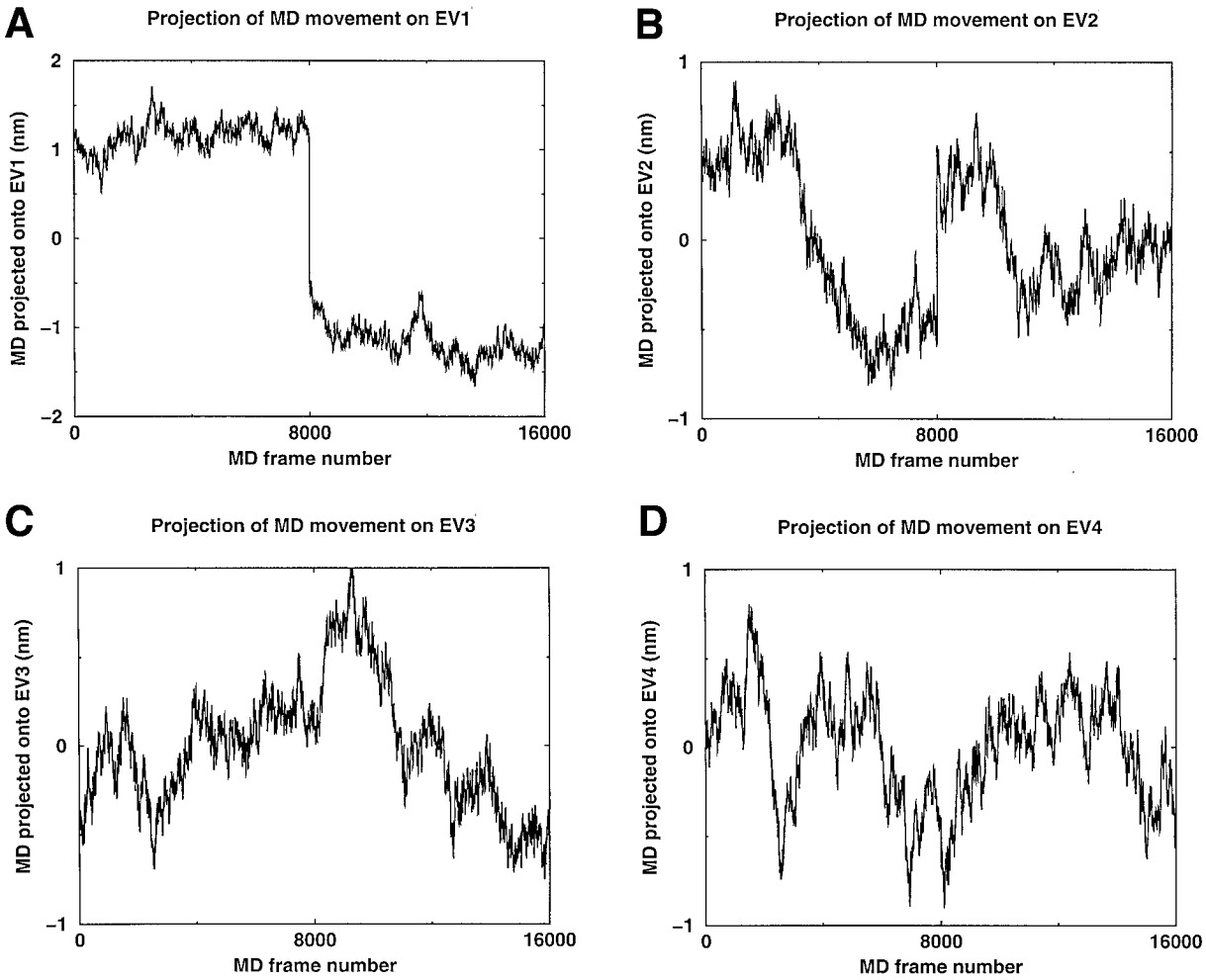

Figure 5. Projection of the MD movement onto the simulation ED eigenvectors (eigenvectors obtained from ED analysis of the combined MD trajectory). The MD movement projected onto simulation ED eigenvector 1 is shown in panel A. The projections onto simulation ED eigenvectors 2, 3 and 4 are shown in panels B, C and D, respectively. The first 8000 MD frames show the movement of the apo-protein, while the last 8000 MD frames show that of the holo-protein.

protein. Most of the ligand modelling and design software [29–34] works on the assumption that the ligand is a static entity while only a few more recent ligand design programs, notably DOCK [35] and AUTODOCK [36], take into account ligand flexibility and conformational change upon binding. The first well-documented case of a crystallographically determined ligand-protein complex structure, dihydrofolate reductase complexed to methotrexate (4DFR), clearly showed that the ligand was distorted in the complex [37]. Nevertheless, it was at least ten years later that this concept was incorporated into design programmes.

The study of receptor-ligand dynamics can give us a better understanding of receptor-ligand binding, and has important applications in drug design. Past efforts to investigate the motion of both the ligand and the receptor include that of Kern et al., who performed a 300 ps MD simulation of adenylate kinase [38] to compare the structure of the complex in vacuum and in aqueous solution. Rognan et al. carried out MD simulations on six different peptides bound to the MHC molecule [39], and elucidated the properties important for binding. However, neither work compared the differences between the bound and free molecules.

Recent research has started to simulate the unbinding process, and in so doing, to elucidate the dynamics and energetics of ligand-receptor binding. Grubmüller et al. [40] explored the rupture force of the streptavidin-biotin complex using molecular mechan-

ics calculations. However, the binding pathway was known in advance, so this method cannot be generally applied. Other researchers aim to outline the binding pathway using various methods, in the hope of unbinding the ligand using the pathway found. Peters et al. [28] used MD and Brownian dynamics simulations to explore the opening of the active site lid in a lipase. They discovered that the timescale of lid-opening was of the order of 100 ns. Lüdemann et al. [41] combined MD with thermal motion pathway analysis to elucidate the putative unbinding trajectory of camphor to cytochrome P-450cam.

Bearing in mind the significance of retinoids in vision [1], embryonic development, cell differentiation and neoplasia [2], we consider that it is important to elucidate the mechanism by which retinoids are recognised by their different transport proteins and to find out the unbending trajectory of the ligand. Ultimately we hope to unbind the ligand and explore the behaviour of water molecules around the receptor and the ligand on unbinding.

It has been shown that retinol adopts a strained conformation when bound to cRBP [7]. Releasing this strain along the main ED eigenvector would cause retinol to unbind from cRBP. We would like to know if this strategy could be applied in the case of sRBP, and also to compare and contrast this recognition between the two cases of the serum and the cellular transport proteins.

To this end, we apply 'essential dynamics' (ED) methods to crystal structures to identify two major eigenvectors that account largely for the motion of the protein. A comparison of the ED space spanned by these eigenvectors shows that the concerted motions as calculated from the crystal structures can be reproduced by the MD simulations.

Having thus validated our methods, we find that the motion along these eigenvectors are similar in the cases of apo-sRBP and holo-sRBP (see Figure 4). Thus it appears that there is no significant change in the dynamics of the protein upon release and uptake or retinol. However, the two MD simulations take up different areas of the ED space, indicating that there is a small static conformational change.

We also compare the motion of the retinol molecule *in vacuo* with its motion inside the sRBP binding pocket. The bound retinol prefers some conformations more strongly than others but does not assume a strained state. The movement of bound retinol is not correlated with the movement of the protein. These results are contrary to what has been observed in the

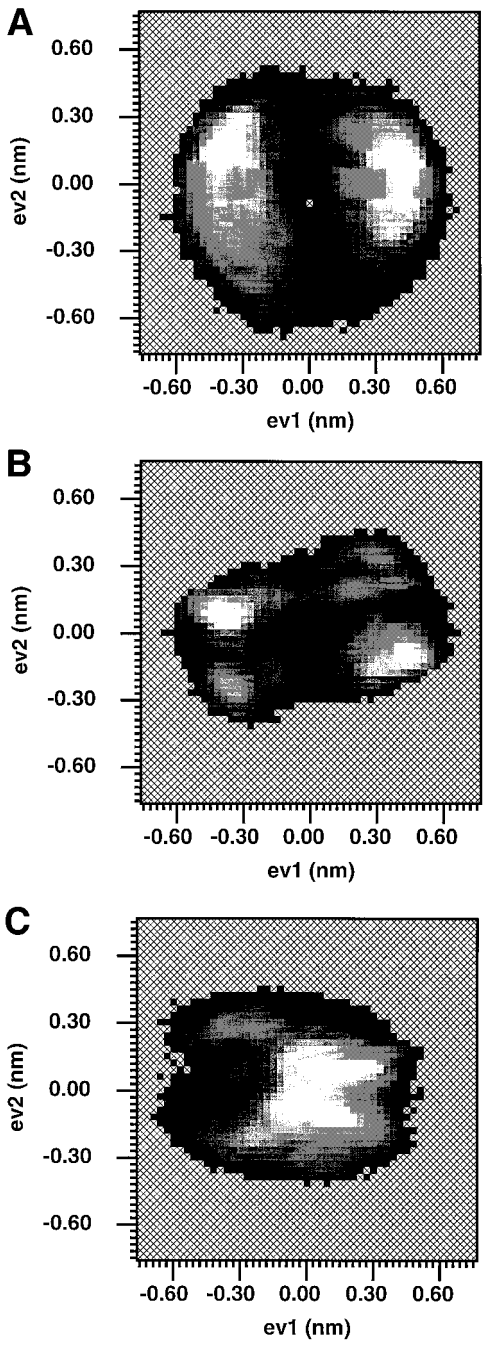

Figure 6. Projection of the free retinol MD structures onto planes spanned by combinations of free retinol eigenvectors 1–3. A = eigenvector plane 1–2, B = eigenvector 1–3, C = eigenvector 2–3. A grid is superimposed onto each plane and the gridpoints are coloured by a greyscale representing the frequency by which they are visited during the simulation (white = visited > 15 times, black = visited once, crosses = never visited).

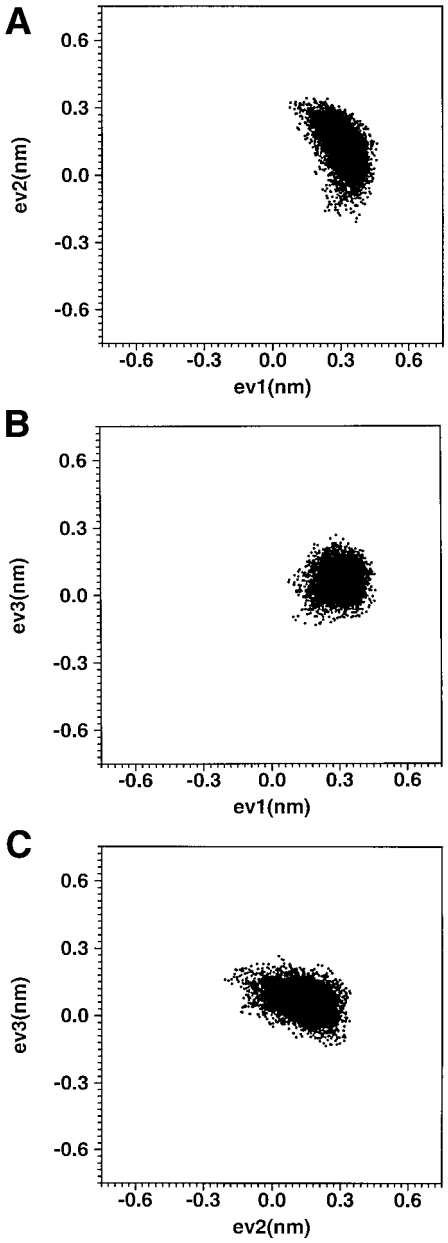

Figure 7. Projection of the retinol structures from the holo-sRBP simulation onto free retinol eigenvector planes 1–2 (A), 1–3 (B) and 2–3 (C).

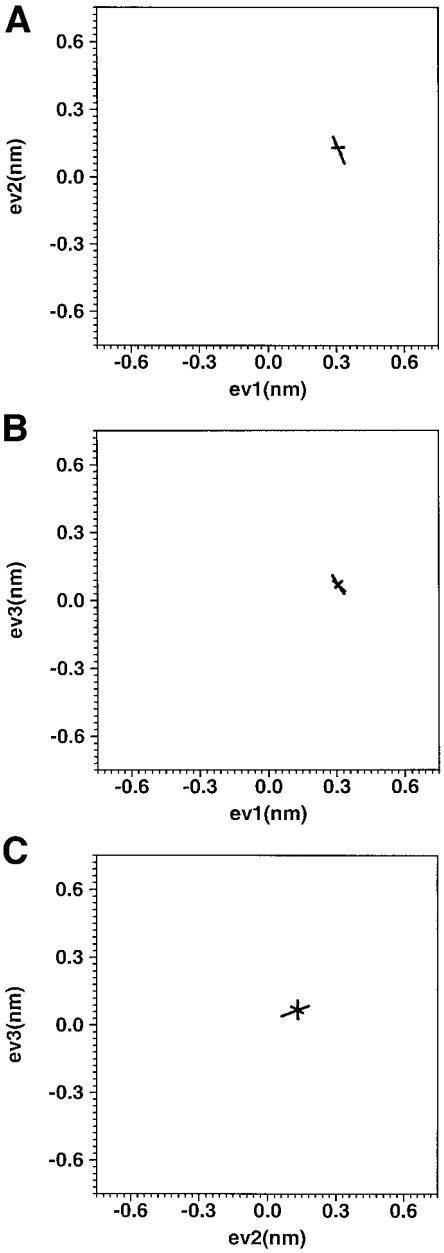

Figure 8. Projection of 'correlated retinol' trajectory onto the free retinol eigenvector planes 1–2 (A), 1–3 (B) and 2–3 (C).

case of cRBP. Favouring the motion of cRBP along the main eigenvectors induces the retinol to unbind by relaxing the strain. This has been attempted in molecular dynamics simulations as a more natural way of unbinding ligands, and observing the changes in the structure and dynamics of the biomolecules and of the solvent.

In this work, we have shown that the binding of retinol to sRBP does not involve much conformational change on the part of the ligand, nor of the protein. We have also demonstrated the validity of using crystallographic data and MD simulations as a reliable method of studying motions in complex biomolecules. These methods are rigorous, robust, and of general inter-

est in other protein-ligand systems for elucidating the mechanism of binding.

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