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A possible route for the release of fatty acid from fatty acid-binding protein

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A simulation of the release of fatty acid from intestinal fatty acid-binding protein was attempted, starting with the crystallographic model and using molecular-dynamic processes at different temperatures. The release of the ligand was observed only at high temperature, which perhaps makes the process unreliable in detail. Nevertheless, the overall behaviour of the protein, also confirmed by the simulation performed at room temperature, strongly supports the idea that the fatty acid leaves the protein through an opening formed by α-helix II and turns βC-βD and βE-βF. Additionally, it suggests a role for the lack of hydrogen bonds between the main chains of β-strands D and E: this feature, observed in all the protein structures of this family which have currently been determined, seems to provide the structure with great flexibility, allowing the barrel to open and close without disruption of the hydrogen-bond network.

INTRODUCTION

The cellular lipophilic-binding-protein family is a group of small cytosolic proteins (molecular masses 13000–15000 Da) able to bind small hydrophobic molecules, such as long-chain fatty acids, retinol and retinoic acid. At present, the crystal structures of nine members of the family have been determined at different resolutions: rat intestinal fatty acid-binding protein (I-FABP) [1], human muscle FABP [2], chicken liver basic FABP [3], bovine myelin P2 [4], adipocyte lipid-binding protein [5], *Manduca sexta* FABP [6], bovine heart FABP [7], cellular retinol-binding protein [8] and cellular retinol-binding protein II [9]. All these structures share a characteristic motif: a 'clam shell' made up of ten antiparallel β strands (named from A to J) and two short α helices (Figure 1). I-FABP is the member of the family which has been best characterized so far: the structure with bound palmitate has been refined to a resolution of 2.0 Å [10] and that of the apo-protein to 1.2 Å [11]. In the holo structure, the fatty acid is buried in the interior of the β-barrel, with the carboxylate group interacting with Arg-106, Gln-115 and two solvent molecules, and its hydrocarbon portion is surrounded by hydrophobic residues. In the other crystal structures where the ligand is visible, the general motif of binding is the same, except that the carboxylate group of fatty acid makes a different pattern of hydrogen-bond interactions with side-chain residues of the protein. While it has been shown in the case of I-FABP that the apo protein is nearly identical with the holo [11], it is not well understood how the ligand can enter and exit the protein. A feature common to all the structures currently solved is the absence of hydrogen bonds between the main-chain atoms of strands D and E. This sort of gap is nevertheless not large enough to allow for the exit of fatty acid. The region where quite a large opening between the internal cavity and the external solvent occurs is the area delimited by α-helix II and turns βC-βD and βE-βF [11]. Nevertheless, this opening in I-FABP is quite narrow, about 5.5 Å in diameter. Consequently, the exit or entrance of a fatty acid molecule without some rearrangements in the protein would appear to be unlikely. Besides, it has been shown that fatty acid analogues containing bulky groups, e.g. 11-dansylamino-undecanoic acid, can bind to liver FABP [12]. Fluorescence-based data do not explicitly indicate that the ligand is inside the cavity. Nevertheless, if this is the case, the binding of the fluorescent probe requires an even larger change in the protein. It has also been suggested that fatty acid could be released through a portal located on the opposite side of the barrel [10]. However, this would require a more drastic conformational change in the protein structure. For the reasons previously given, a molecular-dynamics computer simulation of the release of fatty acid from FABP was attempted and is described in this paper. The model of I-FABP was used in the simulation, as its structure is the best defined among the members of the family.

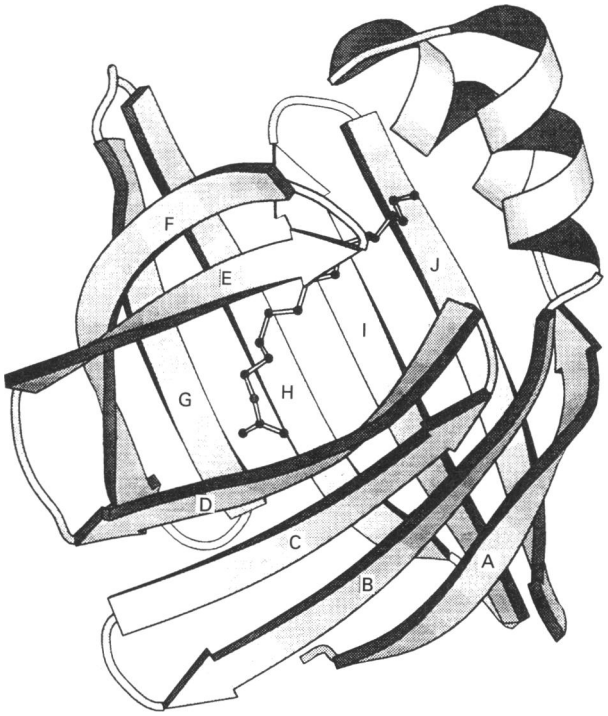

Figure 1 Diagrammatic representation of the FABP molecule, with the strands labelled from A to J

Distance between strands D and E prevents the formation of hydrogen bonds among atoms of the main chain. All the other strands are, on the contrary, connected by main-chain hydrogen-bond interactions.

Abbreviations used: FABP, fatty acid-binding protein; I-FABP, rat intestinal FABP.

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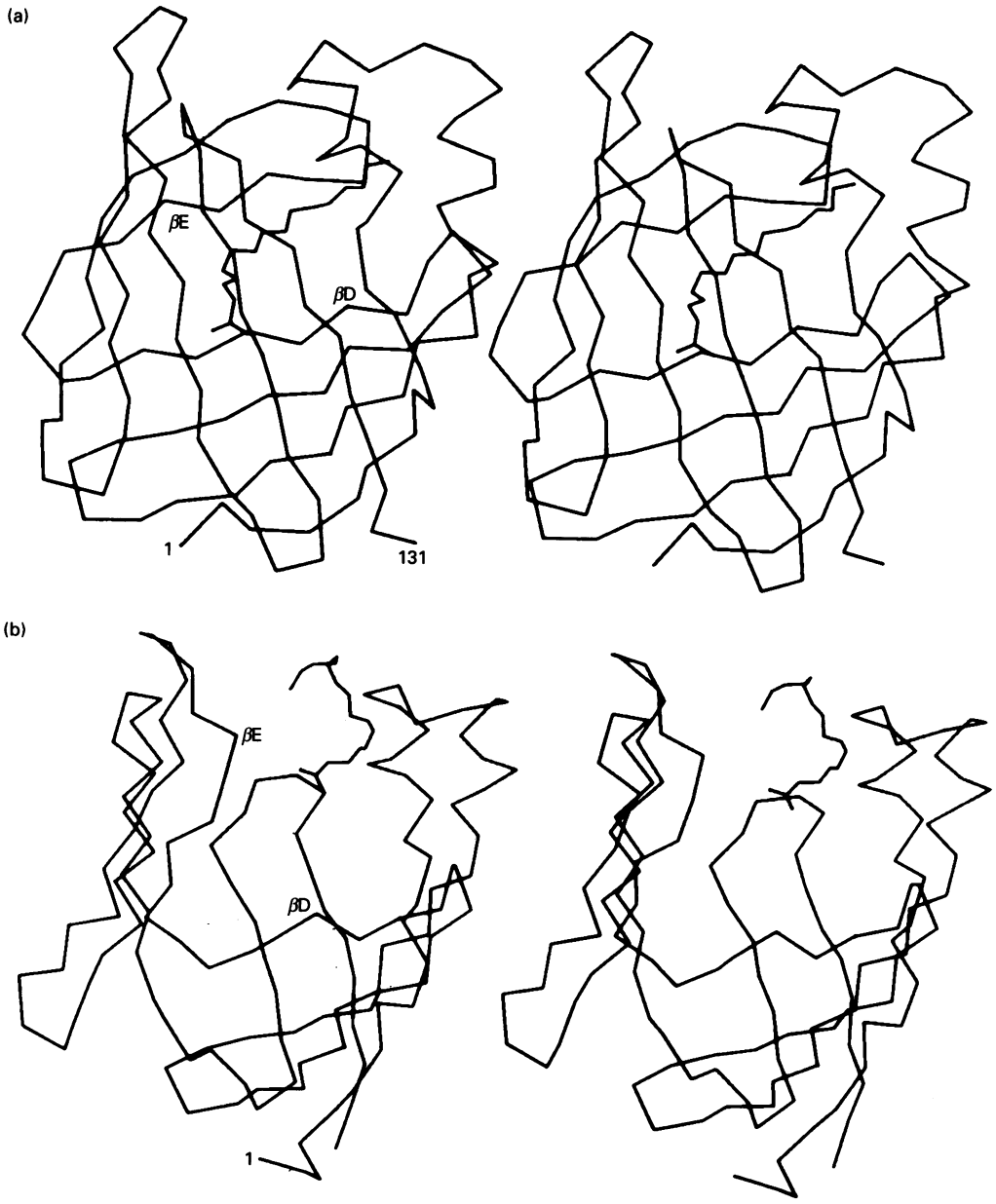

Figure 2 Stereo drawing of the α-carbons of (a) the I-FABP structure obtained by X-ray diffraction and (b) the model resulting from simulation A

(**a**) The I-FABP structure was obtained by X-ray diffraction (ref. [10]; the numbering system is that followed in the reference), with the palmitic acid bound; and (**b**) shows the model resulting from simulation A, at 1500 K. The fatty acid is exiting from the hole delimited by α-helix II and loops βC-βD and βE-βF.

EXPERIMENTAL

The extraction of fatty acid from FABP, either by organic solvent or by affinity chromatography, is a slow process when compared with the times used in molecular-dynamics simulations: on proteins they can be performed for some picoseconds, the time allowed by the present generation of fast computers. The release of fatty acid from FABP is connected with the general barrier-crossing problem [13]. For this reason it appears difficult to simulate the process using classical molecular dynamics. One way of overcoming this difficulty consists of increasing the temperature of the system: a high temperature allows the molecule to access a large number of conformational states and, in particular, to access them in a short time. On the other hand, the physical meaning of a molecular model at a temperature of several hundred degrees Kelvin is highly questionable. It must anyhow be considered that the temperature in such kinds of simulation can be assimilated to a probability coefficient more than to a physical quantity [14]. An analogous procedure was recently used to trap the molten globular state of lysozyme [15]. We performed a molecular-dynamics simulation, increasing the temperature of the system until the phenomenon we expected to observe was verified. It could be thought that the conformation of the molecule in that situation approaches the transition state of the system. That conformation was subsequently taken as the starting point for a simulation at room temperature, or for a slow annealing from a relatively low temperature, in order to check whether the system was able to regain the native starting structure. We cannot trust the atomic details of the process just described; nevertheless some general

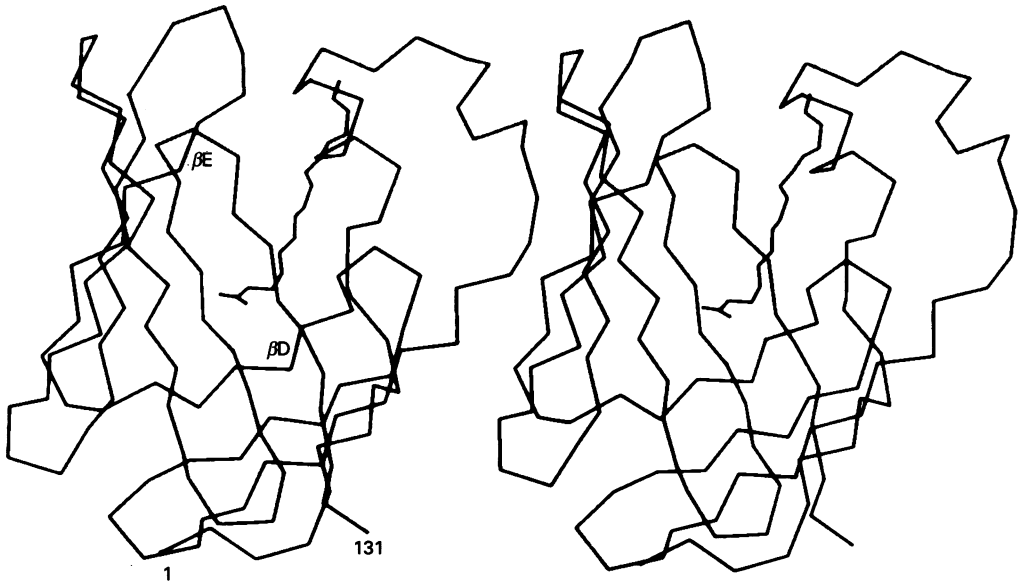

Figure 3 Stereo drawing of the model of I-FABP at the end of simulation B, at 1000 K

The opening is narrower than in the model of Figure 2b, not only because the temperature is lower, but also possibly because there are more solvent molecules around the protein. The fatty acid is in the process of exiting.

features of the dynamics can approximate the real behaviour of the system under study.

Four simulations were performed in different conditions. In the first (simulation A), the starting point was the crystal structure of I-FABP, with the fatty acid bound and the crystallization water (60 molecules) present. The system was subjected, after equilibration, to Langevin dynamics, increasing the temperature from 300 K to 1500 K in steps of 25 K. A time step of 0.5 fs was used and the system was equilibrated for 200 steps at every increase of temperature. In the second model (simulation B), the crystal structure of I-FABP with the bound fatty acid was included in a sphere of radius 25 Å, centred on the centre of mass of the protein and filled with solvent made up of 635 water molecules and 281 propanol molecules. This mixture was chosen in an attempt to create a medium favourable to ligand extraction, but at the same time not too unfavourable to the protein. The system was heated from 300 K to 1000 K with $\Delta T = 25$ K; every step was equilibrated for 2000 steps of 0.5 fs each, for a total time of 28 ps. In the third simulation (simulation C), the model so obtained, deprived of palmitic acid, was subjected to dynamics at room temperature (10 ps at 300 K), to check whether it was possible to regain the native conformation. In the fourth model (simulation D), a dynamics simulation at room temperature for 36 ps with the following protocol was performed: a 2 ps dynamics was followed by 80 steps of minimization, then the dynamics was resumed, and so on. The previous procedure was used in order to reduce the structural stress introduced by a long dynamics run and to keep the molecule close to a local minimum.

In all the simulations, the temperature was controlled by a weak coupling to an external bath ($t = 12$ fs). For non-bonded and electrostatic interactions, a cut-off of 7.5 Å was used. The dielectric constant was set to 1.0 and held constant for all simulations. Only hydrogen atoms connected to polar groups were treated explicitly. Dynamics and minimizations were carried out using the program X-PLOR V3.0 [16]. Minimization was performed with the conjugate-gradient algorithm and stopped when the average of the gradient of the total energy was less than 0.0001. The version of the CHARMM force field included in the X-PLOR program was used; in particular, parameters for water were those of the TIP3p model, while for palmitate and propanol they were those of an aliphatic side-chain and of a carboxylic and an alcoholic group appropriately modified. To avoid the solvent escaping at high temperature, periodic boundary conditions were introduced including the protein in a large artificial P1 crystal cell. The results were visualized with the program INSIGHT II (Biosym Technologies of San Diego, San Diego, CA, U.S.A.) on a Silicon Graphics IRIS 4D turbo workstation. Atomic co-ordinates 2IFB were taken from the Protein Data Bank [17].

RESULTS AND DISCUSSION

As observed before, the procedure used to mimic the release of fatty acid from FABP does not allow us to discuss in detail the process. Nevertheless, the behaviour of the protein is quite reasonable and consistent with the crystal structure. The model of I-FABP at high temperature, at the end of simulation A, is shown in Figure 2, compared with the native conformation. The main changes involve the two regions at the extremity of the barrel: the area including α-helix II (residues 25–31) and loops βC-βD and βE-βF and, on the opposite side, the area delimited by loops βB-βC, βF-βG, and βH-βI. The root-mean-square deviation between the Cα's of the native structure and the model resulting from the simulation is 6.2 Å. The palmitic acid, that was buried in the cavity in the native protein, is now on the surface, close to loop βE-βF and in contact with the solvent. Figure 3 shows the result of simulation B: the situation overall is similar to the previous one, but as this time the final temperature is lower, and possibly due to the large amount of solvent surrounding the protein, the structure is less 'open' and the fatty acid has not yet come out. The root-mean-square deviation from the starting model is 3.6 Å. However, the features of the high-temperature models are the same: loops βC-βD and βE-βF and α-helix II tend to fall apart and the consequence is a widening of the small hole already present. This makes possible not only the release of fatty acid, but also the concurrent entry of solvent molecules. On the opposite side of the barrel, loops βB-βC and

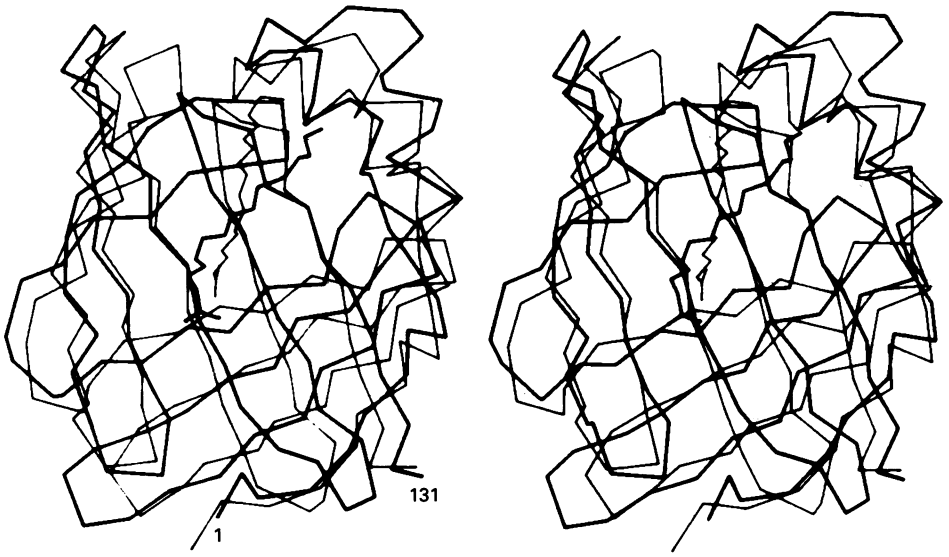

Figure 4 Stereo drawing of the model of I-FABP after a molecular-dynamics simulation at room temperature for 36 ps, with minimization every 2 ps (thin line), superimposed on the crystal-structure model (thick line)

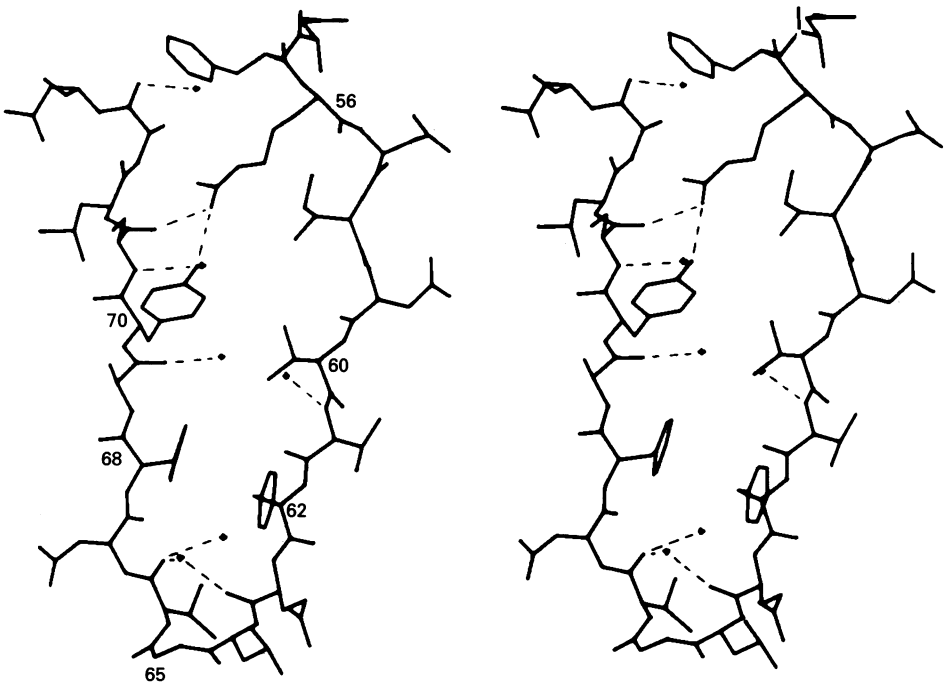

Figure 5 Stereo view of residues in strands βD (residues 56–63) and βE (residues 66–72) in I-FABP (crystallographic co-ordinates from ref. [10])

Small crosses represent water molecules and broken lines signify hydrogen-bond interactions.

βF-βG also move slightly, but the hole thus formed is not comparable in size with that just described. Moreover, side chains pointing to the the interior of the barrel prevent the exit of the ligand by that route. The co-ordinates just described were taken as the starting point for simulation C. After a 10 ps simulation, the protein model presents a root-mean-square deviation from the Cα's of the crystallographic model of 2.6 Å. The model is more similar to the crystal structure than to model B, but it has not yet regained the 'native' conformation. The final step of simulation D, performed without increasing the temperature, is illustrated in Figure 4. It is interesting to notice that, after a simulation which is quite long for the scale of molecular dynamics, the behaviour of the macromolecule is similar to that observed at higher temperatures: loops βC-βD and βE-βF are starting to fall apart and the hydrocarbon tail of the fatty acid tends to move toward the exterior of the barrel cavity. Of course the release of the fatty acid cannot be observed with our model, not only because of the time scale used, but also perhaps because the medium that surrounds the protein is not favourable enough for the ligand.

It is noticeable that the movements just described occur without disruption of the barrel structure: the hydrogen-bond inter-

actions between adjacent strands are substantially preserved in all models. This is made possible by the fact that strands D and E are not connected by hydrogen bonds among the main-chain atoms: the interactions present in I-FABP involve only side-chains and solvent molecules (Figure 5). This feature is common to all nine of the structures of this family currently known and is quite puzzling: it has been suggested that it could be the result of a favoured folding pathway [5]. From our simulation, strands D and E appear to act as a zip: some small rotations involving residues in loop βD-βE, and in particular the quite flexible Gly-65, allow the structure to open up at the expense of breaking some hydrogen bonds between side-chains, but without a disruption of the β-barrel. Interestingly, only three residues are conserved in the structures of this family solved so far and one of them is Gly-65. Another one is Phe-62, which makes an hydrophobic interaction with Phe-68, which is also conserved, except in human liver FABP, where it is Cys. Moreover, strands D and E are in general quite well preserved. It is possible to speculate that the weak interactions of side-chains of the two strands, particularly Phe-62 and Phe-68, could serve to stick them together in a more flexible and less tight way than could be made by hydrogen bonds.

In conclusion, the FABP folding makes it a very stable structure, owing to the network of hydrogen bonds among the strands, but at the same time the lack of them between strands D and E gives to the structure great flexibility, allowing the barrel to open and close with minor structural modifications to the rest of the protein.

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REFERENCES

1 Sacchettini, J. C., Gordon, J. I. and Banaszak, L. J. (1988) J. Biol. Chem. **263**, 5815–5819
2 Zanotti, G., Scapin, G., Spadon, P., Veerkamp, J. H. and Sacchettini, J. C. (1992) J. Biol. Chem. **267**, 18541–18550
3 Scapin, G., Spadon, P., Mammi, M., Zanotti, G. and Monaco, H. L. (1990) Mol. Cell. Biochem. **98**, 95–99
4 Jones, T. A., Bergfors, T., Sedzik, J. and Unge, T. (1988) EMBO J. **7**, 1597–1604
5 Xu, Z., Bernlohr, D. A. and Banaszak, L. J. (1992) Biochemistry **31**, 3484–3492
6 Benning, M. M., Smith, A. F., Wells, M. A. and Holden, H. M. (1992) J. Mol. Biol. **228**, 208–219
7 Müller-Fahrnow, A., Egner, U., Jones, T. A. Rüdel, H., Spener, F. and Saenger, W. (1991) Eur. J. Biochem. **199**, 271–276
8 Cowan, S. W., Newcomer, M. E. and Jones, T. A. (1993) J. Mol. Biol. **230**, 1225–1246
9 Winter, N. S., Bratt, J. M. and Banaszak, L. J. (1993) J. Mol. Biol. **230**, 1247–1259
10 Sacchettini, J. C., Gordon, J. I. and Banaszak, J. (1989) J. Mol. Biol. **208**, 327–339
11 Scapin, G., Gordon, J. I. and Sacchettini, J. C. (1992) J. Biol. Chem. **267**, 4253–4269
12 Wilkinson, T. C. I. and Wilton, D. C. (1987) Biochem. J. **247**, 485–488
13 Chandler, D. (1990) J. Phys: Condens. Matter **2**, SA9–SA13
14 Metropolis, N., Rosenbluth, A. W., Rosenbluth, M. N., Teller, A. H. and Teller, E. (1953) J. Chem. Phys. **21**, 1087–1092
15 Mark, A. E. and van Gunsteren, W. F. (1992) Biochemistry **31**, 7745–7748
16 Brünger, A. T., Kuriyan, J. and Karplus, M. (1987) Science **235**, 458–460
17 Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) J. Mol. Biol. **112**, 535–542