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# A Dynamic Model for the Allosteric Mechanism of GroEL

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GroEL-assisted protein folding is regulated by a cycle of large coordinated domain movements in the 14-subunit double-ring assembly. The transition path between the closed (unliganded) and the open (liganded) states, calculated with a targeted molecular dynamics simulation, shows the highly complex subunit displacements required for the allosteric transition. The early downward motion of the small intermediate domain induced by nucleotide binding emerges as the trigger for the larger movements of the apical and equatorial domains. The combined twisting and upward displacement of the apical domain determined for a single subunit is accommodated easily in the heptamer ring only if its opening is concerted. This is a major source of cooperative ligand binding within a ring. It suggests also that GroEL has evolved so that the motion required for heptamer cooperativity is encoded in the individual subunits. A calculated model for a di-*cis* 14-subunit assembly is found to be destabilized by strong steric repulsion between the equatorial domains of the two rings, the source of negative cooperativity. The simulation results, which indicate that transient interactions along the transition path are essential for GroEL function, provide a detailed structural description of the motions that are involved in the GroEL allosteric cycle.



The molecular chaperonin GroEL mediates protein folding in the bacterial cell (Clarke, 1996; Ellis & van der Vies, 1991; Fenton & Horwich, 1997; Gething & Sambrook, 1992; Hartl, 1996; Martin & Hartl, 1997; Sigler *et al.*, 1998). GroEL consists of two rings of seven identical subunits stacked back-to-back with dyad symmetry (Figure 1(a)). Non-native proteins appear to bind first to the portion of the apical domain at the end of the central channel (Buckle *et al.*, 1997; Chen & Sigler, 1999; Chen *et al.*, 1994; Joachimiak, 1997). GroEL can recognize a wide range of protein substrates due to the intrinsic flexibility of the active site (Buckle *et al.*, 1997). Upon binding of ATP and the heptameric co-chaperonin GroES, a large closed cavity (the *cis* assembly) is created into which the unfolded peptide is released so that it can refold in a shielded environment, referred to as the ''Anfinsen cage'' (see Figure 1(a)). When ATP is hydrolyzed to ADP in the *cis* ring and ATP is bound in the *trans* ring, the native (or still misfolded) protein, ADP and GroES are released and the corresponding assembly is formed in the *trans* ring (Rye *et al.*, 1997, 1999). The Anfinsen cage is created from the unliganded (''closed'') structure by large, nearly rigid body movement of the intermediate and apical domains of the *cis*-ring subunits (Figure 1(b)) (Boisvert *et al.*, 1996; Braig *et al.*, 1994; Chen *et al.*, 1994; Llorca *et al.*, 1997; Roseman *et al.*, 1996; Xu *et al.*, 1997). These movements are coupled to the binding of ATP and GroES in a process with high positive cooperativity (Gray & Fersht, 1991). The behavior of the *trans* ring is out of phase with the *cis* ring, due to the negative cooperativity

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Abbreviations used: TMD, targeted molecular dynamics.

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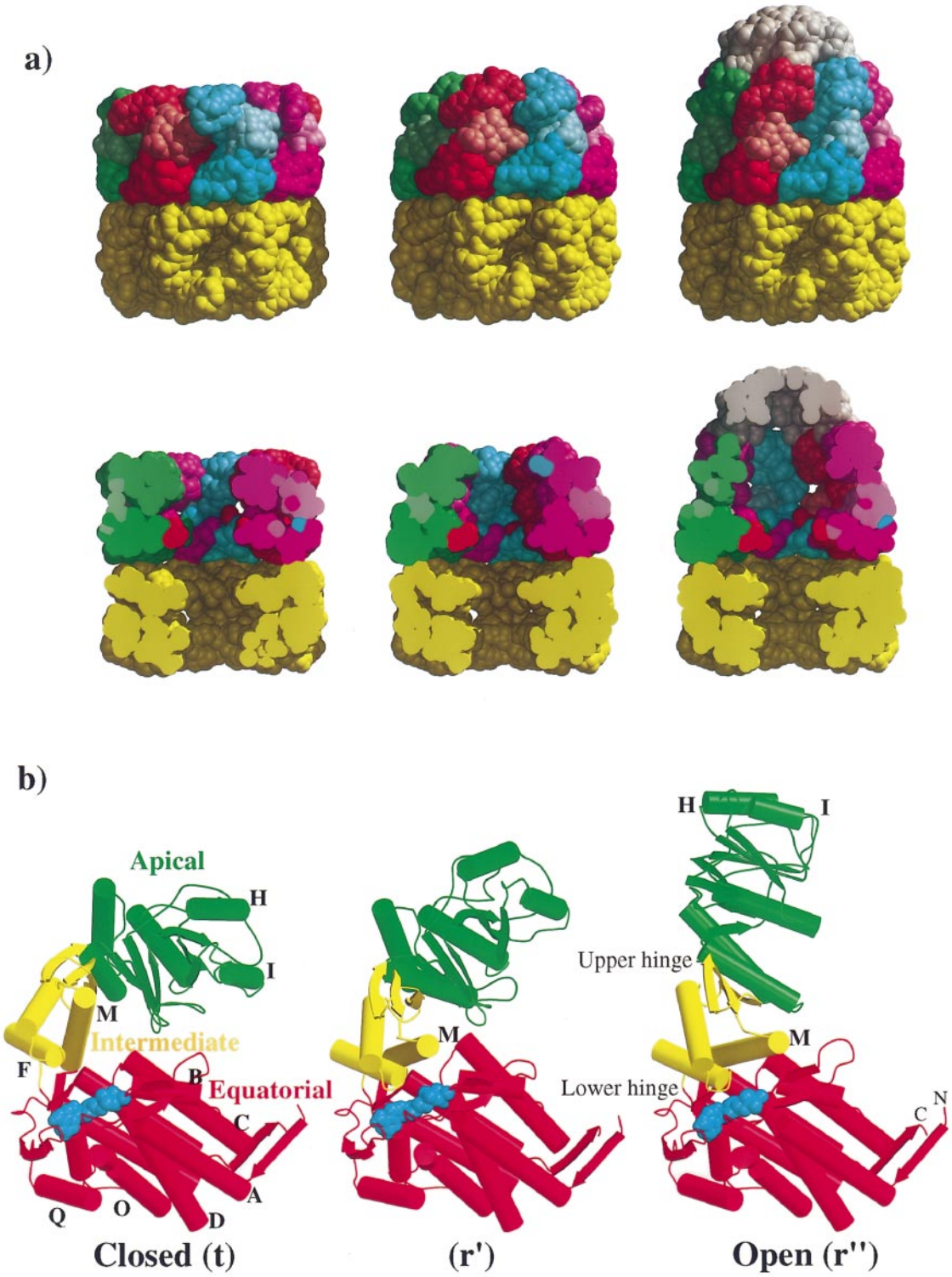


**Figure 1.** The overall architecture and conformational change of GroEL. The closed ATPγS crystal structure (Boisvert *et al.*, 1996), the intermediate r′ structure (from the TMD simulation), and the GroEL-GroES-$(ADP)_7$ structure (Xu *et al.*, 1997) are shown from left to right. (a) van der Waals space-filling models (6 Å spheres around $C^{\alpha}$ atoms). Upper panels show the overall dimension; lower panels show the interior of the complex by a cut-through view. Different colors are used to indicate the subunits in the upper ring. The domains are distinguished by shading: apical, medium hue; intermediate, light hue; equatorial, dark hue. The lower ring is uniformly in yellow and GroES is uniformly in gray. (b) Representations of a subunit of GroEL corresponding to the structures shown in (a). The color coding is: apical, green; intermediate, yellow; equatorial, red. The nucleotide is shown as a blue space-filling model. Some of the key secondary structural elements are marked. The view of the subunits is roughly from inside out of the GroEL central cavity. Drawings in (a) are made with MIDAS (UCSF) programs and in (b) with MOLSCRIPT (Kraulis, 1991) and RASTER 3D (Bacon & Anderson, 1988).

between the rings (Yifrach & Horovitz, 1994, 1995).

Although aspects of the conformational changes in GroEL have been elucidated by studies of stable structures (Boisvert *et al.*, 1996; Braig *et al.*, 1994; Llorca *et al.*, 1997; Roseman *et al.*, 1996; Xu *et al.*, 1997), it is necessary to follow the motions from one state to another to understand the interactions involved. We have used the targeted molecular dynamics (TMD) method (Schlitter *et al.*, 1993) to determine the transition paths from the ''closed'' (Boisvert *et al.*, 1996) to the GroES-bound ''open'' structure (Xu *et al.*, 1997). The trajectory has the spatial and temporal resolution necessary to reveal the structural basis of the ordered allosteric transition that is essential for the function of GroEL. The present calculations provide the first unified dynamic description of the essential elements of the GroEL allosteric cycle.

### The two-stage transition

The conformational transition of a GroEL subunit (see Figure 2(a)) can be divided into two stages; the first one is associated with the binding of nucleotide (t to r′) and the second one involves the binding of the co-chaperonin GroES (r′ to r″).

The first stage begins with the downward ''folding'' motion of the intermediate domain around the ''lower hinge'' towards the equatorial domain (see (2) in Figure 2(b)). This motion, which is induced by the interaction of nucleotide ($Mg^{2+}$-ADP in the crystal structure) with residues of the F and M helices of the intermediate domain, occurs during the first 5% of the transition. The downward domain motion is nearly equal to the 25° rotation observed in the final crystal structure ((6) in Figure 2(b)) and it closes off the nucleotide binding pocket for effective hydrolysis of ATP. Thus, the catalytically active conformation is reached at an early stage, independent of the binding of GroES, in accord with the suggestion that nucleotide binding is fast compared to the formation of the fully open *cis* structure (Burston *et al.*, 1995; Jackson *et al.*, 1993; Yifrach & Horovitz, 1998b). The t to r′ transition is completed by a small, mainly upward, pseudo-rigid body motion of the apical domain. Looking down from the top of the GroEL ring, there is also a small clockwise twist of the apical domain relative to the equatorial domain around the upper hinge; this can be seen in Figure 2(b) ((2) to (3)) from the change in the orientations of the H and I helices (Figure 1(b)). The final structure of the first stage has a partly opened and twisted apical domain and a fully folded down intermediate domain (Figure 1, middle and Figure 2(b) (3)), which leads to an enlargement of the GroEL cavity. Even at this stage (r′), the hydrophobic side-chains of the H and I helices (Fenton *et al.*, 1994) are less available to interact with the substrate than in the t structure, in accord with the fact that substrate binding affinity of GroEL is lower after the binding of nucleotide (Staniforth *et al.*, 1994). The clockwise twist of the apical domain is accompanied by a counterclockwise twist of the equatorial domain, which is an essential element of the negative inter-ring cooperativity (see below).

The second stage of the GroEL conformational transition, designated as that from r′ to r″ (Ma & Karplus, 1998), consists primarily of apical domain motions. There is a continuation of the clockwise twisting of the apical domain about the upper hinge which results in a nearly 90° rotation (Xu *et al.*, 1997), as is evident from the further change in the orientation of helices H and I in (4) to (6) of Figure 2(b). In addition, the apical domain undergoes an upward tilt of nearly 60° (Roseman *et al.*, 1996; Xu *et al.*, 1997). The trajectory shows that the large twist of the apical domain is possible only after the downward motion of the intermediate domain, which would otherwise clash with the apical domain. The twist and upward motion of the apical domain are essential for GroES binding; the H and I helices are reoriented to interact with the GroES mobile loops (Fenton *et al.*, 1994). The calculated order of events is consistent with the result (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990) that GroES does not interact with GroEL in the absence of nucleotide. Thus, the allosteric binding of nucleotide and GroES is regulated by a sequential series of stereochemical interactions. If GroES bound to GroEL before binding of nucleotide, GroEL would be locked in an ATP-deficient non-functional state. The r″ apical domain position is unstable by itself and GroES appears to be required to induce this state. Thus, the coupling of the intermediate and apical domains motion and the requirement for GroES binding lead to an ordered two-stage mechanism for the allosteric transition (as illustrated schematically in Figure 2(a)).

A helical hairpin of the apical domain, i.e. the K/L protrusion (Boisvert *et al.*, 1996; Braig *et al.*, 1994; Ma & Karplus, 1998; Xu *et al.*, 1997), has moved into its final position after 60% of the simulation path has been completed. This motion is mediated by favorable interactions between Asp359 and Ser358 at the tip of the hairpin of the apical domain and Lys80 on the upper surface of the equatorial domain. Although this helical hairpin protrudes outward to the side of the GroEL ring in the closed structure (Boisvert *et al.*, 1996; Braig *et al.*, 1994), it makes up a substantial portion of the wall of the GroEL cavity of the open GroES-bound structure (Xu *et al.*, 1997). To avoid a large hole in the wall of the open structure (not shown), it is necessary that the hairpin move into its final position ahead of the main twisting of the apical domain.

The simulated path for the inverse transition from the open to the closed structure is found to correspond very closely to the reverse of the sequence of events shown in Figure 2. This is in accord with recent data (Horowitz *et al.*, 1999)

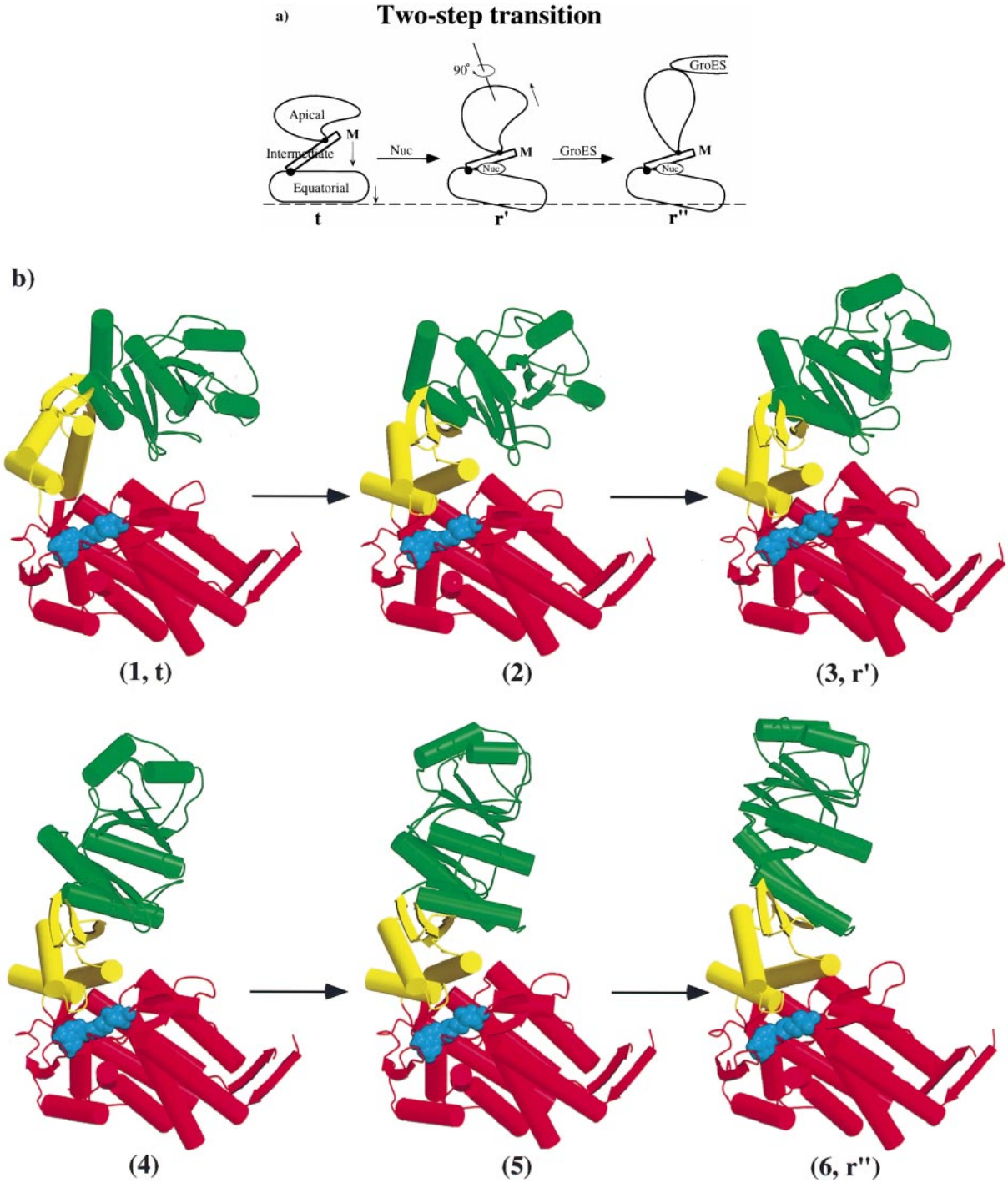


**Figure 2.** Calculated transition path. (a) Schematic representation of the two-stage transition. (b) A set of structures on the path: (1) through (3) correspond to the first stages associated with nucleotide binding, i.e. the t to r′ transition; (4) through (6) correspond to the second stage involving GroES binding, i.e. the r′ to r″ transition. Structures (1) (Boisvert *et al.*, 1996) and (6) (Xu *et al.*, 1997), labeled t and r″, respectively, are the X-ray structures. The other structures are from the TMD simulation. The equatorial domain is superimposed as a reference in the various structures and so it has an essentially fixed orientation. The view of the subunit is the same as that of Figure 1(b) and the twist of the apical domains, especially in the second stage, is towards the reader. To appreciate the full complexity of the motion, see http://yuri.harvard.edu/ ~jma .

demonstrating that ADP dissociation occurs after GroES release; i.e. the upward movement of the intermediate domain that exposes the nucleotide binding site occurs near the end of the transition to the closed form.

During the transition, certain interactions that are not present in the crystallographic end structures appear between the apical domain and the two other domains of the same subunit. Of particular interest are two salt-bridges involving the apical

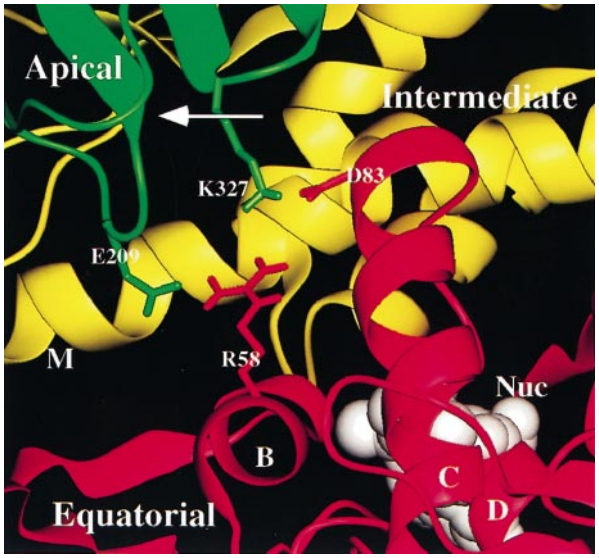


**Figure 3.** Transient ionic interactions observed in the structure r′ (see the text). The side-chains of Glu209 and Lys327 of the apical domain that interact with Arg58 and Asp83, respectively, of the equatorial domain are shown. The white arrow indicates the direction of motion of the apical domain in going from r′ to r″. The view is in a similar direction as the subunit shown explicitly on the right-hand side of the upper ring in Figure 5(a).

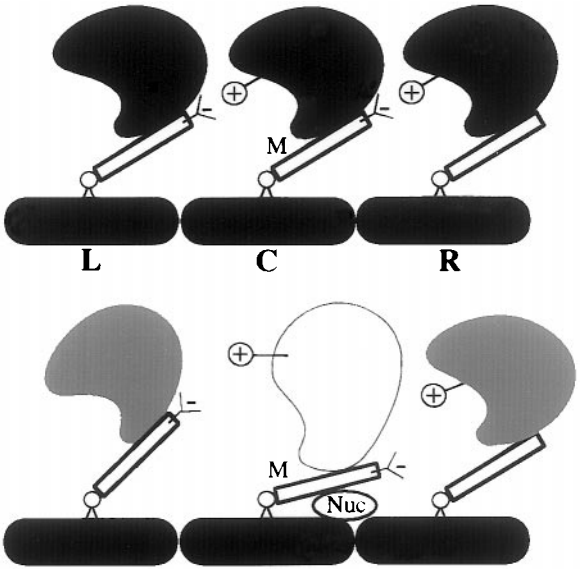


**Figure 4.** Schematic illustration of the dominant interactions between subunits leading to positive cooperativity within the ring (see the text). The nucleotide is assumed to be bound only to the central subunit (C) for the purpose of illustration. The Arg197, Glu386 salt-bridge and the effect of the steric interactions are indicated. Helix M represents the intermediate domain. The lightened shading of the apical domains indicates the altered nucleotide binding affinity. The schematic representation corresponds to a view looking in from outside of the GroEL ring.

and equatorial domains (Boisvert *et al.*, 1996; Lorimer & Todd, 1995); i.e. E109 and K327 of the apical domain interact with R58 and D83, respectively, of the equatorial domain (see Figure 3). They contribute to the coupled motions of the two domains, although the salt-bridges are ultimately broken in completing the r′ to r″ transition. This is in accord with the result that an engineered cysteine disulfide bond between D83 and K327 prevents the full transition (Murai *et al.*, 1996).

## The allosteric mechanism

### *Intra-ring interactions and positive cooperativity*

Two types of interactions between the subunits make the dominant contribution to the positive homotropic allostery within a ring; they are illustrated schematically in Figure 4. The first of these arises from the steric (van der Waals) interference which would result if one subunit moves to the ''open position'' (C) and its right-hand neighboring subunit (R) remain in the ''closed'' position (Ma & Karplus, 1998). The other interaction is electrostatic, due to an intersubunit salt-bridge which is present in the t structure and broken in the r′ and r″ structures.

When nucleotide binds to a subunit (subunit C in Figure 4) and it undergoes the conformational change from t to r′ described above, both the left-hand (L) and right-hand (R) neighbor are affected. The repulsive (steric) interaction between C and R can be resolved if R moves simultaneously with C toward the partially open r′ structure. Also, after a transient opening motion to state r′, L no longer collides with C. The salt-bridge couples Glu386 at the N terminus of helix M of the intermediate domain of C and Arg197 on a loop at the bottom of the apical domain of R. The downward movement of helix M and the upward movement of the apical domain leads to the dissociation of the salt-bridges with both the R and L subunits. Thus, the steric and electrostatic interactions act in concert and tend to produce a coupled conformational transition.

A model calculation for the GroEL heptamer shows that it is energetically costly for one subunit to move without the others. However, if all the subunits simultaneously follow the simulated transition path from t through r′ to r″ (Figure 2), there are no large steric clashes along the entire pathway. The results provide strong support both for the significance of the single subunit calculations and for the role of steric interactions in the cooperative transition. The results also indicate that the second stage of the transition, i.e. that from r′ to r″, is intrinsically cooperative, though it takes place only in the presence of GroES.

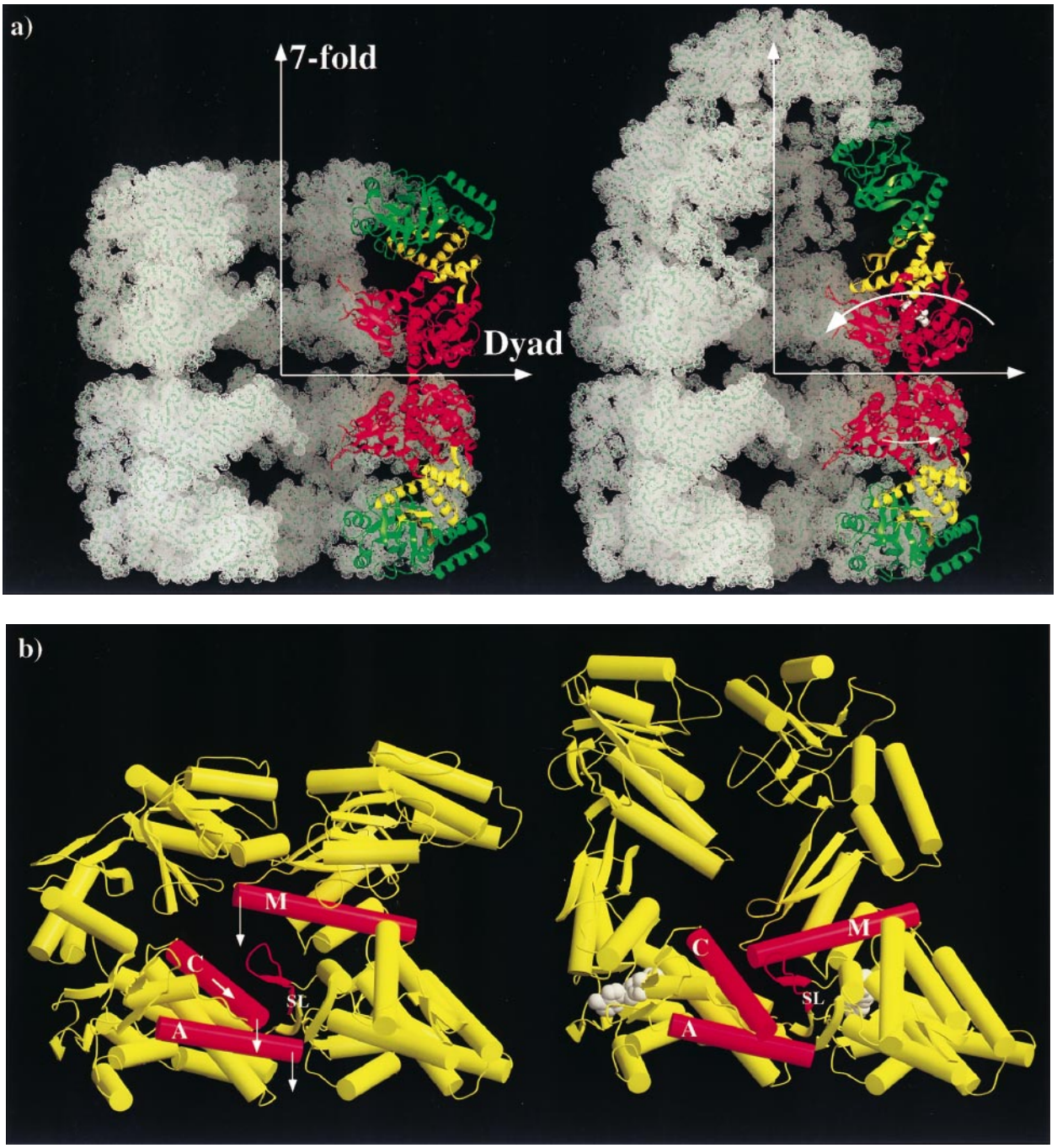


**Figure 5.** The mechanism of inter-ring negative cooperativity. (a) Ligand-free structure (Braig *et al*., 1994, 1995) on the left and ligand-bound asymmetrically open structure on the right (Xu *et al*., 1997). The view is perpendicular to the dyad axis. The direction and size of the white arrows indicate the direction and relative extent of the rotation of the equatorial domains in the two rings. Only two dyad-related subunits are shown explicitly. The nucleotide (ADP) is indicated by a white space-filling model. The overall complexes are shown in the terms of a dot surface based on the $C^{\alpha}$ atoms. For clarity, the subunits blocking the view of the explicit ones are not included. The two small horizontal helices in the equatorial domain near the word ''dyad'' are the Q helices (see the text). (b) Two adjacent subunits from the crystal structures in (a) shown in a view looking out from the inside. The key structural elements are in red; they are helices A, C, M, and the stem-loop. The inclination of the equatorial domain is shown to be stabilized by the downward motion of helix M. The arrows indicate the direction of the motion of the helices. The arrow along the axial direction of the C helix indicates the axial translation of this helix reported previously (Boisvert *et al*., 1996; Xu *et al*., 1997). Such a motion is an integral part of the rigid body inward inclination of the equatorial domain. (c) The view is into the dyad axis, and the observed subunits are in the front. Upper panel: on the left, the asymmetric crystal structure (Xu *et al*., 1997), on the right, the modeled di-*cis* complex (see the text). The lower panels show the detailed interactions around the Q helices, as indicated by the arrows in the upper panels. The broken lines indicate the middle point between the two rings in the ligand-free complex (Braig *et al*., 1994, 1995), which we used as a reference for the ring interface. The clash of two contacting points in the lower right panel is evident. (d) A schematic illustration of the steric interactions between the three equatorial domains shown in (c). The unliganded subunits are shown as shaded.

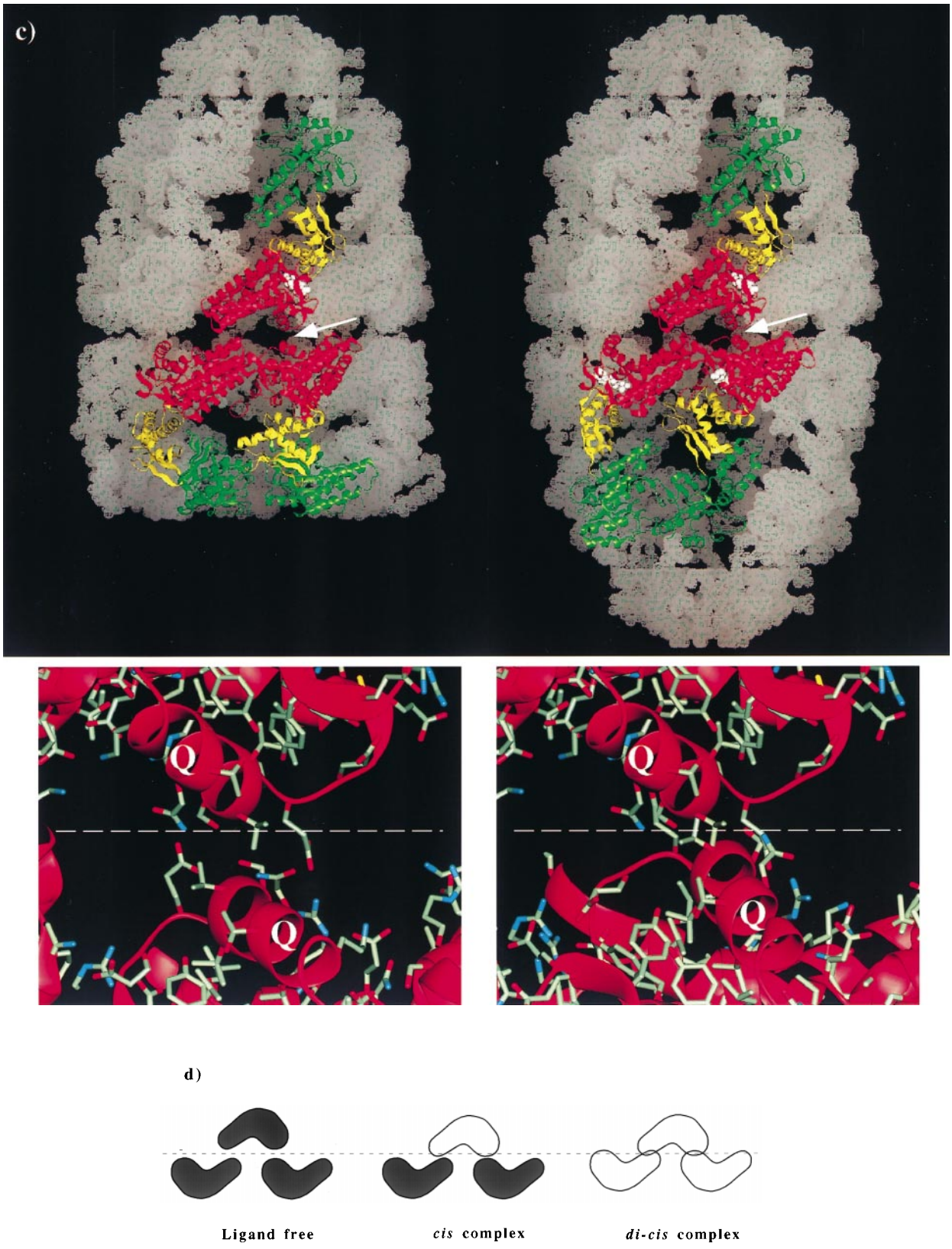


**Figure 5** (*legend shown opposite*)

The importance of the salt-bridge was demonstrated by the mutation Arg197Ala, which abolishes positive cooperativity (Yifrach & Horovitz, 1994). Apparently the steric clashes due to individual subunit motions are reduced significantly, since the structure expands when the salt-bridge is deleted by mutation (White *et al.*, 1997). Also, a recent protein engineering study (Yifrach & Horovitz, 1998a) has shown that this salt-bridge is broken in the transition state between the closed and open conformation. The result is in accord with the simulation, in that the downward folding of the intermediate domain, with its concomitant motion of helix M, is the earliest event in the subunit conformational change, as described above. Several other salt-bridges, which also affect cooperativity, were shown to be still present in the transition state by the protein engineering experiment (Yifrach & Horovitz, 1998a); e.g. the intra-subunit salt-bridge between Arg501 (equatorial) and Glu409 (intermediate), which is near the lower hinge between the two domains, is not broken by the domain movements, even in the open (r″) structure.

A molecular dynamics simulation of two subunits, in which the right-hand one follows the TMD path while the left-hand one is not constrained, shows that, in the absence of nucleotide in the latter, there is a tendency for persistence of the Arg197, Glu386 salt-bridge. The motion of the apical domain in the right-hand subunit induces a transient upward displacement of helix M in the left-hand subunit (see Figure 4), which enhances access to its nucleotide binding pocket. This suggests that the tertiary structural changes of the subunits are not completely in phase; i.e. there may be some deviations from a fully concerted Monod-Wyman-Changeux model (Monod *et al.*, 1965); see also Horovitz & Yifrach (2000). Such behavior is consistent with the biochemical studies by (Todd *et al.*, 1993) that indicate the interactions among subunits are heterogeneous, especially at low ligand concentration.

### *Inter-ring interactions and negative cooperativity*

The important factors giving rise to the negative cooperativity between rings are shown in Figure 5. An essentially rigid-body reorientation of the equatorial domains with respect to the ring interface is induced by nucleotide binding, as indicated schematically in the structures r′ and r″ of Figure 2(a) (de Groot *et al.*, 1999; Ma & Karplus, 1998; Xu *et al.*, 1997). The rotation of the equatorial domain (see arrows in Figure 5(a)), results in downward displacements of the two ''tips'', one involving the inner part of helix Q and the other, the C-terminal end of helix D (see Figure 1(b)), both of which contact the equatorial domain in the *trans* ring (Braig *et al.*, 1994). Figure 5(b) shows that the downward movement of the intermediate domain (on the right) induced by nucleotide binding requires the equatorial domain in the neighboring subunit (on the left) to adjust its orientation to avoid a clash. There results a ''ternary complex'' between helix M, the stem-loop and the equatorial domain. As a consequence, motion of the equatorial domain must occur early in the conformational transition (t to r′) although it is likely to be augmented by the presence of GroES (r′ to r″). The former is supported by the fact that negative cooperativity is observed for nucleotide binding alone (Bochkareva & Girshovich, 1994; Burston *et al.*, 1995).

To determine how negative cooperativity between the rings is induced by the equatorial domain rotation, the crystal structure (Xu *et al.*, 1997), in which only one ring (the *cis* ring) is liganded (structures r″ and t for the *cis* and *trans* ring, respectively), was compared with a model in which the *cis* assembly is present in both rings (the di-*cis* model, with both rings having structure r″) (Figure 5(c)). Such a di-*cis* present approximates the nearly symmetric ''football'' structure that has been observed by cryo-electron microscopy at high nucleotide concentration with GroES bound to both rings (Llorca *et al.*, 1997; Roseman *et al.*, 1996). In the asymmetric (r″, t) crystal structure, there are close contacts between each *cis* ring equatorial domain and the two *trans* ring equatorial domains that interact with it (Figure 5(c), left lower panel), but there is no steric strain. In the di-*cis* model (r″, r″), by contrast, there are severe steric clashes between the three equatorial domains. The clashes of the tip involving helix Q are particularly severe (Figure 5(c), right lower panel). Therefore, the negative cooperativity, which inhibits binding of nucleotide simultaneously to both rings, is not due to the small structural change induced in the *trans* ring on binding of ATP to the *cis* ring. Instead, it arises from the steric energy penalty resulting from the simultaneous structural change induced by binding of nucleotide to both rings. This is analogous to the cooperative mechanism in hemoglobin, where steric stress is present in the T structure only after $O_2$ is bound and the heme group assumes the liganded conformation (Gelin & Karplus, 1977; Paoli *et al.*, 1997).

## Concluding discussion

The simulation of the pathway followed by the chaperonin GroEL in the transition of a subunit from the closed (t) structure to the ADP-bound structure in the absence of GroES (r′) and the fully liganded (ADP plus GroES) structure (r″) provides a dynamic model for the allosteric mechanism. The architecture of a GroEL ring is such that the transition from the closed to open structure can take place if all seven subunits follow the calculated single subunit pathway in an essentially concerted manner. Thus, the motion required for the cooperative transition of the heptamer is encoded in the structure of the monomer. The cooperativity of the transition may play a role also in GroES binding when some of the mobile loops of GroEL that

interact with GroES in the X-ray structure are originally interacting with the partly folded protein substrate.

The small intermediate domain emerges as a key element in coupling the motion of the apical and equatorial domains so as to achieve the ordered allosteric transition required for GroEL function. Nucleotide binding to a ''protein-loaded'' GroEL results in folding down of the intermediate domain as the first step in the allosteric transition. The intermediate domain displacement is required for upward motion of the apical domain in the same subunit, for breaking of the Glu386 to Arg197 salt-bridge to one neighboring subunit and for the rotation of the equatorial domain of the other neighboring subunit. The first two are essential for intra-ring cooperativity and the third for inter-ring anti-cooperativity. The intra-ring cooperativity arises because the concerted apical domain motions of the subunits avoid the steric clashes that would result if one subunit opened while the others remain closed. The release of the constraint due to the inter-subunit salt-bridge facilitates this coupled motion. The homotropic interactions of nucleotide with GroEL in the first stage of the transition primes the assembly for a heterotropic interaction with GroES in the second stage. Specifically, the upward motion of the apical domain involves a twist that orients the hydrophobic groups to interact with the GroES mobile loops. In the absence of the nucleotide-induced structural changes, GroES does not bind; i.e. the formation of an empty cage is prevented by the stereochemical regulation intrinsic to the GroEL structure. The inclination of the equatorial domain, which is initiated in the first stage and completed in the second, inhibits a corresponding transition in the second ring because strong steric repulsions between the two rings would result. This is the source of the inter-ring negative cooperativity.

The dynamic analysis makes clear how the structure of the subunits and their response to ligand perturbations have evolved to produce the finely tuned mechanism for GroEL-assisted protein folding (Coyle *et al*., 1999; Shtilerman *et al*., 1999; Wang & Weissman, 1999) by an asymmetric reciprocating two-stroke cycle (Lorimer, 1997), in which one ring loads reactant while the product is discharged from the opposite ring (Rye *et al*., 1999; Xu & Sigler, 1998). To extend the present model, it would be useful to compare ATP and ADP as a ligand, and to examine the effect of the GroEL transition on a partly folded protein, in accord with experiments that indicate the opening motion can induce unfolding (Buckle *et al*., 1997; Chatellier *et al*., 1999; Robinson *et al*. 1994, 1998; Shtilerman *et al*., 1999). The current study illustrates a general approach applicable to the structural transitions of other nucleotide triphosphate driven complexes, including molecular motors (Howard, 1997).

## Methods

The trajectory for the transition between two known structures of a single subunit was simulated with the TMD simulation method (Schlitter *et al*., 1993) interfaced with the CHARMM program (Brooks *et al*., 1983). The method uses the standard molecular mechanics potential (Neria *et al*., 1996) and adds a time-dependent constraining force which is proportional to the difference between the conformation of the moving structure and the target structure at each time-step. The ATPγS-bound closed structure (PDB code 1DER) (Boisvert *et al*., 1996), whose tertiary and quaternary structures are essentially identical with those of unligated structure (Braig *et al*., 1994, 1995), was used as the initial structure to provide a starting point for the TMD simulation with a bound nucleotide. The conformational transition was achieved by slowly ''pulling'' the system towards the target, the GroES-bound open structure (PDB code 1AON) (Xu *et al*., 1997). The side-chains of two mutations, R13G and A126V, in the crystal structure of ATPγS-form were modeled as wild-type. The nucleotide was modeled as ADP and two metal ions (one $Mg^{2+}$, one $Ca^{2+}$) were also included. The coordinates for the $Ca^{2+}$, which is present in the ATPγS structure (Boisvert *et al*., 1996), was modeled into the open structure. The two terminal β-strands and the stem-loop in the two neighboring subunits, which make the dominant intersubunit contacts with the equatorial domain (Braig *et al*., 1994), are included in the simulation. A shell of explicit solvent molecules (670 water molecules) was included in the simulation, and a modified TIP3P water model (Jorgensen, 1981; Neria *et al*., 1996) was used. The dynamic constraint was applied to all the atoms but not the water molecules. Such a procedure has been demonstrated as an effective way of simulating the conformational changes in the presence of explicit solvent molecules (Ma & Karplus, 1997). The total simulation time was 500 ps; a 1 ns run showed no qualitative difference in the path, suggesting that the artifacts of the short simulation time are small. To supplement the dielectric effect of the solvent, a distance-dependent dielectric constant ($\varepsilon = r$) was used, and the charges on charged residues were reduced by a factor of 0.6. The temperature was kept near 300 K by coupling the system to a heat bath (Berendsen *et al*., 1984) with a relaxation time of 0.2 ps. A modified leap-frog method (Allen & Tildesley, 1980; Schlitter *et al*., 1993) was used for the integration of the equations of motion with a time-step of 1 fs; all the bonds involving hydrogen atoms were fixed by SHAKE algorithm (Ryckaert *et al*., 1977).

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