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Conformational change in the activation of lipase: An analysis in terms of low-frequency normal modes

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Abstract

The interfacial activation of *Rhizomucor miehei* lipase (RmL) involves the motion of an α-helical region (residues 82–96) which acts as a "lid" over the active site of the enzyme, undergoing a displacement from a "closed" to an "open" conformation upon binding of substrate. Normal mode analyses performed in both low and high dielectric media reveal that low-frequency vibrational modes contribute significantly to the conformational transition between the closed and open conformations. In these modes, the lid displacement is coupled to local motions of active site loops as well as global breathing motions. Atomic fluctuations of the first hinge of the lid (residues 83–84) are substantially larger in the low dielectric medium than in the high dielectric medium. Our results also suggest that electrostatic interactions of Arg86 play an important role in terms of both the intrinsic stability of the lid and its displacement, through enhancement of hinge mobility in a high dielectric medium. Additional calculations demonstrate that the observed patterns of atomic fluctuations are an intrinsic feature of the protein structure and not dependent on the nature of specific energy minima.

Keywords: activation mechanism; conformational change; interfacial activation; normal mode analysis; *Rhizomucor miehei* lipase

Lipases (EC 3.1.1.3) are enzymes that catalyze the hydrolysis of lipids and are used widely in the dairy industry and in washing powders (Vulfson, 1994). The activity of lipases increases significantly at the interface of lipid and water (Sarda & Desnuelle, 1958). Crystal structures of *Rhizomucor miehei* lipase (RmL) in its *apo* and liganded conformations (Brady et al., 1990; Brzozowski et al., 1991) have revealed that the interfacial activation is associated with a conformational change in the lipase. The most striking feature of this conformational change is the movement of a "lid" (residues 82–96), where a short alpha helix (residues 85–91) is displaced about two hinge regions (residues 83–84 and 91–95) (Derewenda et al., 1992b).

In water, the lid covers the active site (Fig. 1A), hindering any substrate or solvent molecules from entering (Derewenda et al., 1992b) and is termed the "closed" conformation. When the lipid is bound, the lid rotates around two hinge regions so that the active site becomes exposed to solvent and adopts the "open" conformation (Derewenda et al., 1992a); it is this motion that is implicated in the interfacial activation of the enzyme. In the open conformation, the active site, a largely hydrophobic region, is exposed to the solvent (Fig. 1B), and the hydrophilic side of the amphipathic lid is buried in a polar cavity behind the lid.

Although the structures of both the closed and open conformations of RmL are known, the mechanism of the lid opening is unclear. Molecular dynamics (MD) and Brownian dynamics (BD) simulations have been used to explore the activation of RmL (Norin et al., 1993, 1994; Peters et al., 1996a, 1996b, 1997a, 1997b). Although MD simulations can provide detailed microscopic insights into processes, collective atomic motions, which lead to rigid-body displacements of the kind seen in RmL, occur typically on a time scale greater than nanoseconds and are outside the realm of conventional MD simulations (Wade et al., 1993, 1994; Peters et al., 1996a). One approach is to use modified MD methods such as "essential dynamics," which selectively enhances conformational sampling along specific directions of motion (Amadei et al., 1993; Peters et al., 1996b, 1997b). An alternative is to use normal mode analysis (NMA, Mouawad & Perahia, 1993; Hayward & Go, 1995; Marques & Sanejouand, 1995; Perahia & Mouawad, 1995; Thomas et al., 1996a, 1996b). In NMA the motions of the system (about an energy minimum) are obtained in a direct way by assuming that the potential energy function varies quadratically about an energy minimum (Janežič et al., 1995) and yields a complete description of the dynamics of the molecule within the harmonic approximation at a low computational cost. NMA has been used for investigating conformational changes in several proteins (Brooks

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Abbreviations: MD, molecular dynamics; NMA, normal mode analysis; RmL, *Rhizomucor miehei* lipase; RMS, root mean square; RMSD, RMS atomic positional displacement.

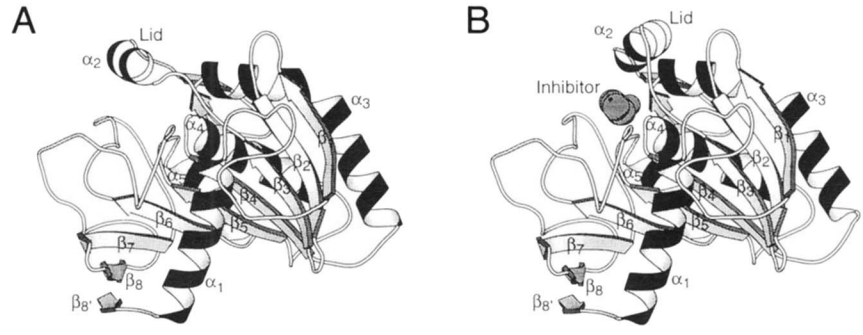

Fig. 1. Schematic representation of the closed (**A**) and open (**B**) conformations of *R. miehei* lipase (RmL). The secondary structure is labeled for reference in the text.

& Karplus, 1985; Gibrat & Gö, 1990). Recent advances in methodology have extended the application of this method to large proteins (Marques & Sanejouand, 1995; Mouawad & Perahia, 1996; Thomas et al., 1996a, 1996b).

The aim of this study was to investigate the conformational change of RmL in terms of harmonic motions about two limiting states, i.e., the closed and open conformations associated with ligand binding. Lipase activation is an interfacial (water–lipid) phenomenon. In this study, we do not attempt to model the water–lipid interface; rather, we consider the two phases individually. The two environments were represented implicitly via different continuum models representing the high and low dielectric properties of water and lipid, respectively (see Methods). The degree to which equilibrium vibrational modes of the open and closed conformations contribute to the transition path between these two states was investigated. The sensitivity of the results to the exact nature of the potential energy minimum was investigated by NMA of five additional minima of the closed RmL obtained from a MD simulation. Furthermore, the influence of the electrostatic interactions of a key residue in the lid, Arg86, was investigated by removing the partial atomic charges from its side chain. Arg86 was selected, as there is both experimental and theoretical evidence that this residue is important for activation. Chemical modification of arginine residues has been shown to lower the lipase activity significantly (Holmquist et al., 1993). Furthermore, dynamical simulations have shown that mutations of Arg86 effect the stabilization of the lid α-helix and the lid opening (Norin et al., 1993; Peters et al., 1996a).

Results

Frequency spectrum

The frequencies of the 47 lowest frequency normal modes (Fig. 2) are very similar for both conformations in a given dielectric medium. However, the effect of the dielectric medium on the frequency distribution is marked. An almost uniform shift to lower frequencies in "lipid" is observed, compared to the distribution in "water." The lowest frequencies are 5.2 cm^{-1} and 3.2 cm^{-1} for the closed conformation and, 4.6 cm^{-1} and 3.5 cm^{-1} for the open conformation in "water" and "lipid," respectively.

Atomic fluctuations

The overall motion was investigated by calculating the atomic root-mean-square (RMS) fluctuations for each residue in the protein for each of the 47 modes (see Fig. 3). The fluctuations are generally larger in "lipid" than in "water," reflecting the pronounced shift to lower frequencies. In general, the same loop and turn regions are mobile for both conformations in both environments, with the largest fluctuations in the lid region occurring in the lowest frequency modes.

Figure 4 shows the atomic fluctuations of Cα-atoms for the alpha and beta secondary structural elements averaged over all the 47 modes. Significantly, the largest fluctuation of the nonterminal secondary structure elements is found in the lid helix α_2 for both conformations, in both environments; the largest fluctuation is found in the closed conformation in "lipid."

Some interesting differences are evident in the behavior of the hinge regions flanking the lid. In the closed conformation the motion of the lid is dominated by fluctuations of the first hinge in "lipid," whereas in "water" this region is relatively immobile. This phenomenon is evident across all the modes (see Fig. 3). Similar behavior is also found in the open conformation, although it is not as evident in the contour levels used in Figure 3.

Modes associated with the conformational change

The degree to which the 47 lowest frequency modes, which form a subset of the complete (3N-6, where N is the number of atoms) normal mode basis, describes the conformational change was investigated (see Methods). Figure 5 shows the projection of the

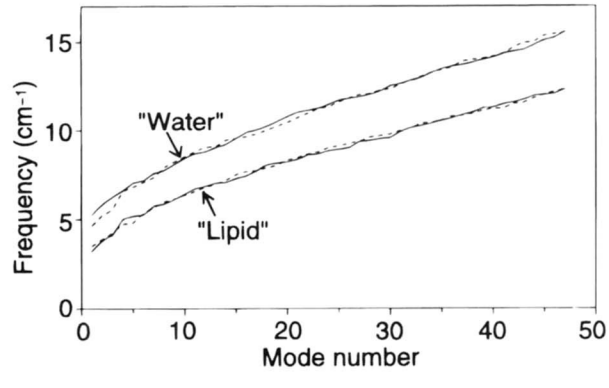

Fig. 2. Frequency spectra of the 47 lowest frequency normal modes of the closed (continuous line) and open (broken line) conformations of RmL in both "water" ($\epsilon = r_{ij}$ in Å) and "lipid" ($\epsilon = 4$) environments.

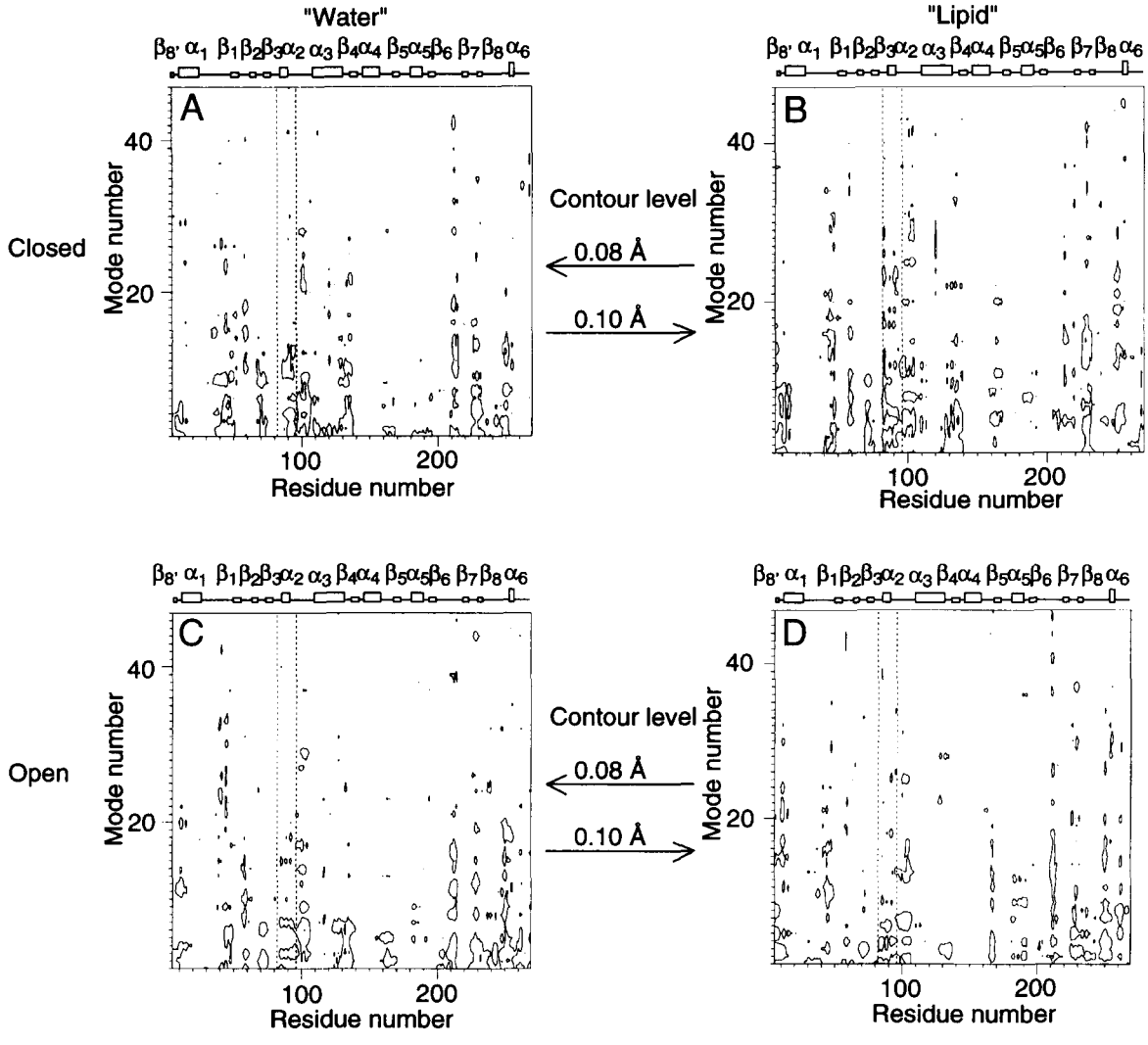

Fig. 3. Atomic RMS fluctuations at 300 K of the Cα-atoms of the closed conformation in "water" (**A**) and "lipid" (**B**), and of the open conformation in "water" (**C**) and in "lipid" (**D**). The extent of the secondary structure elements (see Fig. 1) is shown at the top of each figure. The lid region (residues 82–96) is shown with a dashed line. Fluctuations are contoured either at 0.08 Å ("water") or 0.1 Å ("lipid") level. Fluctuations are generally larger in "lipid" than in "water."

normal modes onto the conformational reaction coordinate (the difference vector between the open and closed conformations of RmL). As expected, no individual mode describes the lid motion completely, although a few make significant contributions in each case. Visual inspection of the normal mode trajectories confirmed that the lid is mobile along all the modes that have high scalar products. Of these, a few of the low frequency modes, which best described the local lid displacement, were selected for further analyses (the "active" modes). The character of the lid motion in terms of atomic displacements along some of the active modes is shown in Figure 6.

The total extent of the conformational change accounted for by all the 47 modes was also computed. For the closed conformation it is 46% in "lipid," but only 32% in "water," and for the open conformation it is 34% in "lipid" and 21% in "water." This indicates that within the harmonic approximation the lid motion is better characterized by motion about the closed rather than the open conformation; the best description is obtained from the modes of the closed conformation in "lipid." Further, the propensity for the conformational change is higher in "lipid" than in "water."

Domain motions

The structure of RmL was divided into three subdomains, which are located between residues 35–97 (subdomain 1), 98–160 (subdomain 2), and 161–241 (subdomain 3), by using the program ProDom (Gouzy et al., 1996). ProDom classifies regions of proteins as domains based upon sequence analysis and a protein domain data base.

An overall breathing motion of the enzyme was found in the lowest frequency mode of the closed conformation (both in "lipid" and in "water"). Furthermore, in the closed conformation, breathing was also associated with lid opening: the protein contracts when the lid opens and it expands when the lid closes. The radius

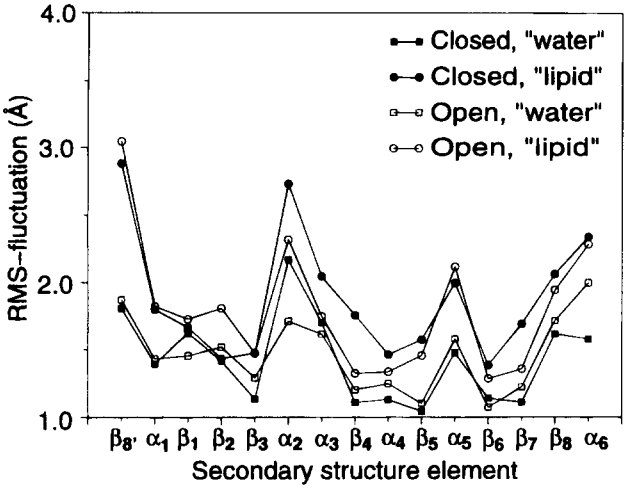

Fig. 4. Average atomic RMS fluctuations at 300 K of Cα-atoms in the alpha and beta secondary structural elements over the 47 lowest frequency modes.

of gyration decreases when the lid opens and increases when the lid closes, the difference being about 0.1%. The contraction associated with the lid opening can be seen clearly in the difference distance plot (Fig. 7), which shows that subdomains 1 and 3, and 2 and 3 move closer together, when the lid opens. The biological significance of the breathing motions is unclear, as this may simply reflect the mechanical characteristics of the protein (ben-Avraham, 1993).

Motions of loops

The normal mode trajectories were inspected visually to identify loops associated with lid motion. Five common loop motions were identified throughout the modes and their location is illustrated in Figure 8. The motions of loops 28–41 and 57–62 are coupled with lid opening and closure. Loop 57–62 moves closer to the lid upon opening. Another common motion in the active modes was that of loop 209–214, which moves toward the lid when the lid closes. These loops are in general highly mobile across all the computed modes (Fig. 3).

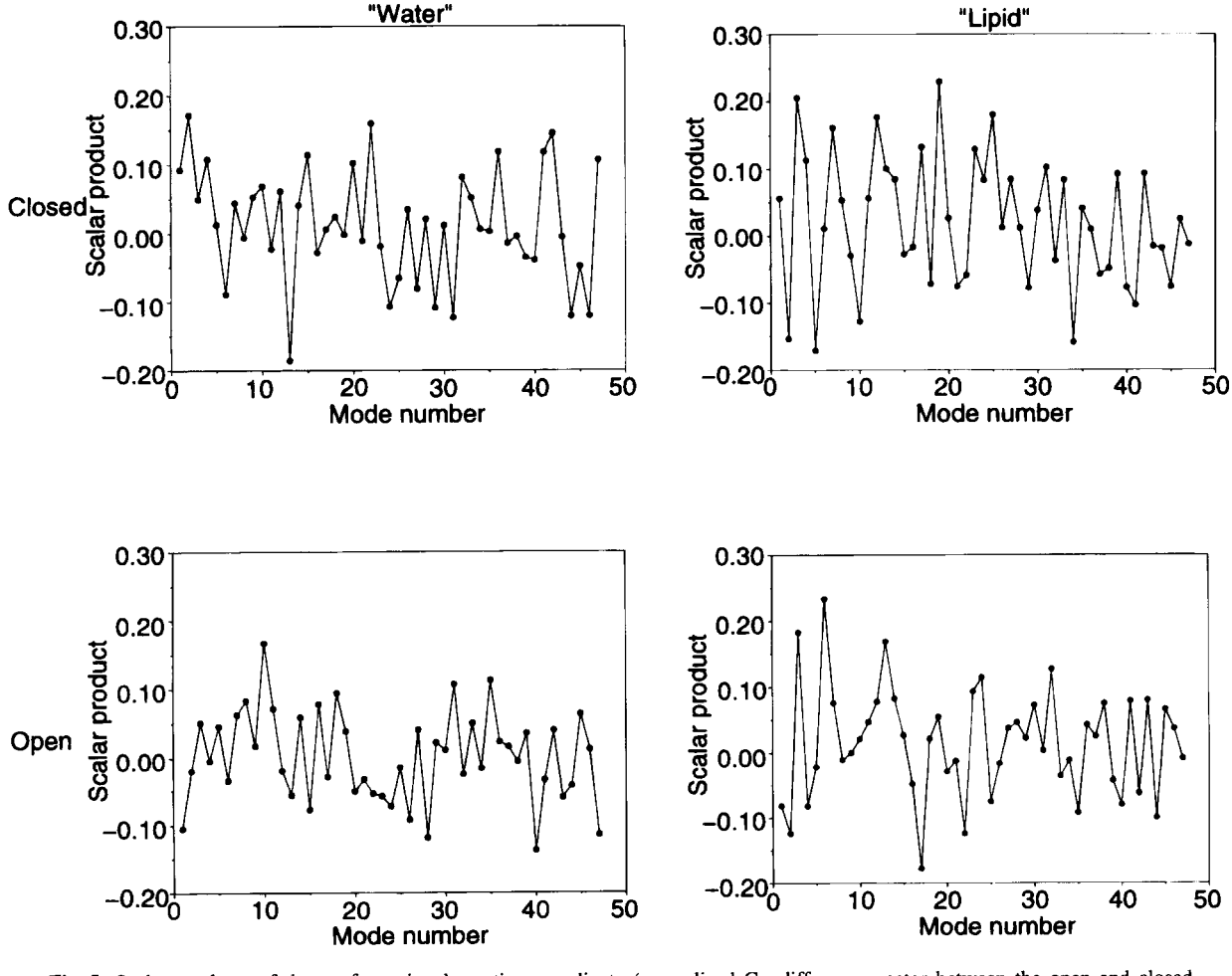

Fig. 5. Scalar products of the conformational reaction coordinate (normalized Cα difference vector between the open and closed conformations) and the Cα eigenvector of each vibrational mode. Values close to unity indicate modes that are aligned close to the direction of the conformational reaction coordinate; values close to zero indicate that the mode does not overlap with the reaction coordinate.

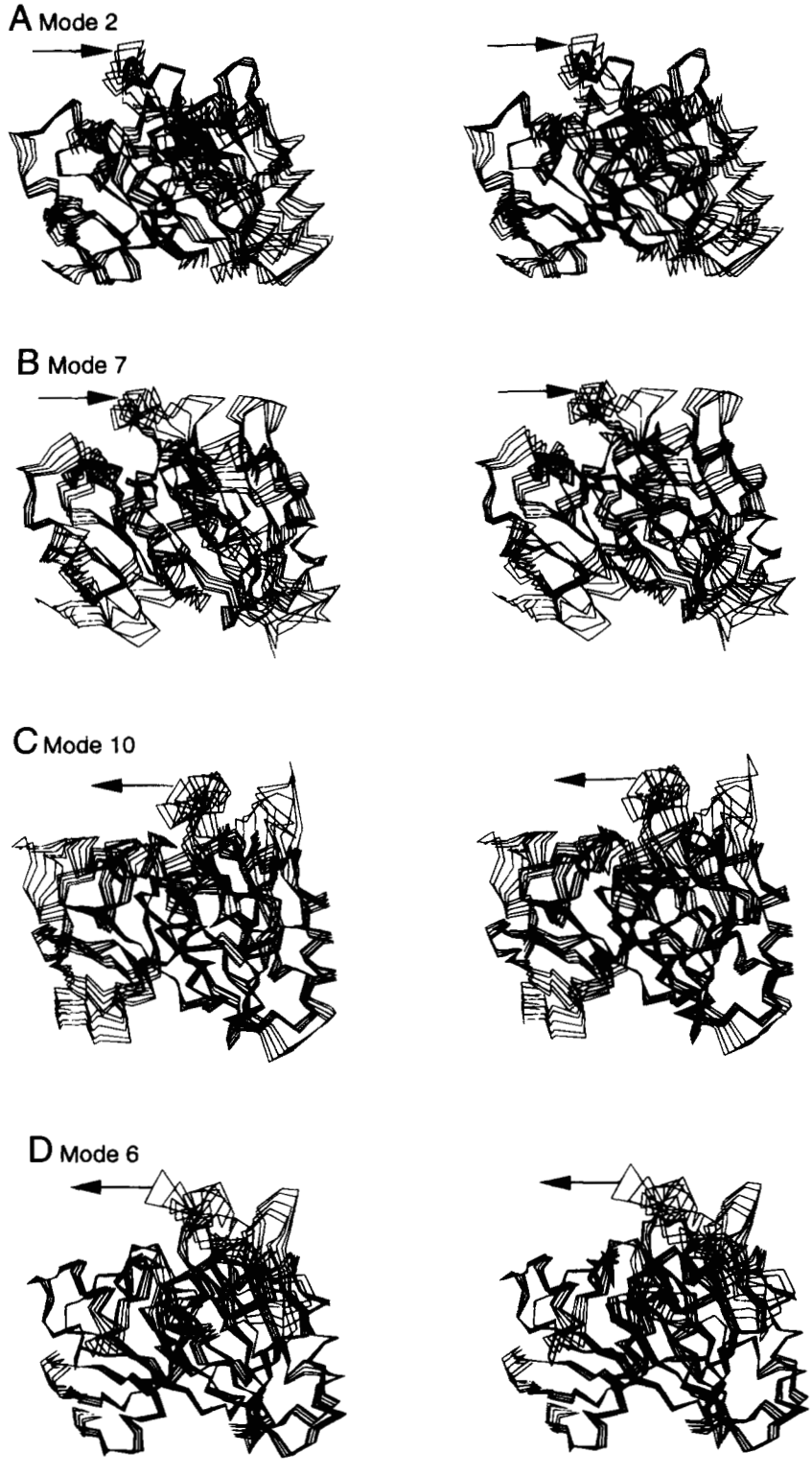

Fig. 6. Stereo pairs of the superposed Cα-coordinates for displacements along selected active modes that best describe the conformational change of the lid region (see Fig. 5). Trajectories for mass weighted RMS displacements of 2.0 Å are shown for the closed conformation in "water" (**A**) and in "lipid" (**B**), and for the open conformation in "water" (**C**) and in "lipid" (**D**). The arrow indicates the direction of the conformational change from closed to open (**A,B**) or open to closed (**C,D**).

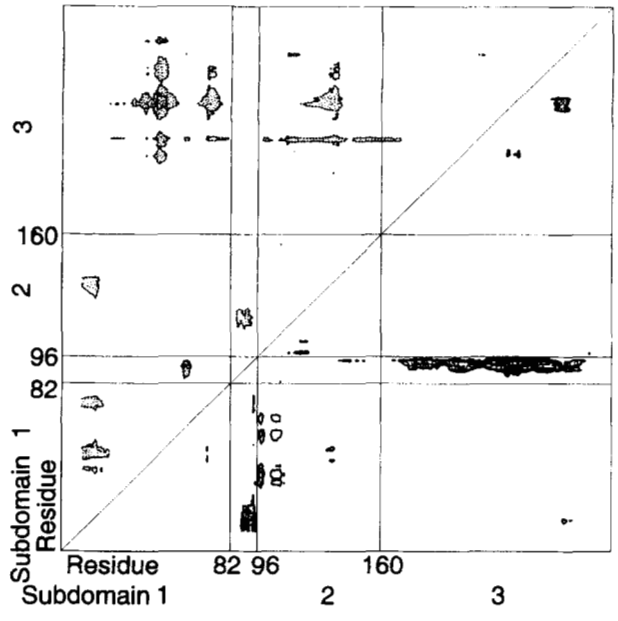

Fig. 7. Distance difference plot for the Cα coordinates of mode 4 (frequency 6.6 cm^{-1}) for the closed conformation in "water." Distances between residues that decrease as a result of stepping along the mode are shown in the upper triangle of the plot with a dashed line; distances that increase are shown in the lower triangle with a solid line. The lid is situated between residues 82 and 96. The extent of the three subdomains are marked on the plot with solid lines. Contour levels are drawn at values of 2.0 to 6.0 at 0.5 intervals of the standard deviation of the matrix elements (0.038 Å) for harmonic mode displacement amplitudes at 300 K.

Sensitivity of the normal mode results

The significance of our interpretations of the results from the NMA of the crystal structure was investigated by performing additional normal mode calculations of the closed RmL in five different energy minima from a MD simulation. The RMSD of the main chain atoms of the structures from the dynamics simulation and the initial crystal structure were in the range 0.96–0.98 Å. The trend of the atomic fluctuations across the structure is quite similar among the lowest modes, although the magnitudes vary. Although the atomic fluctuations of the lid region are smaller than found in the NMA of the crystal structure, it is clear from visual examination of the mode trajectories that the lid opening and associated loop and sub-domain motions and global breathing motions are qualitatively similar.

The effect of removing the charge from Arg86

To investigate the effect of specific electrostatic interactions in relation to the NMA results, selected calculations were performed with Arg86 uncharged (see Introduction and Methods). In the closed conformation in "water," the removal of charge from Arg86 results in significantly larger atomic fluctuations in the second hinge and the lid (see Fig. 9). In the two lowest frequency (highest displacement) modes, the second hinge moves markedly toward its position in the open conformation, whereas the first hinge remains immobile, leading to a deformation of the lid α-helix. In the open conformation in "lipid," the mobility of the lid and hinge regions are unaffected by neutralization of Arg86.

Discussion

Normal-mode analyses (NMA) of the closed and open conformations of *R. miehei* lipase (RmL) reveal that the conformational change associated with substrate binding can be described partially in terms of a few low-frequency harmonic vibrational modes in which the localized lid displacement is coupled to local loop motions as well as global delocalized motions of the whole protein.

In our study, only the 47 lowest frequency modes of the 7,800 (3N-6) possible vibrational modes were examined. However, there is strong evidence that it is the lowest frequency modes that qualitatively describe the functionally important large-scale motions of proteins (Brooks & Karplus, 1985; Gibrat & Gō, 1990; Marques & Sanejouand, 1995; Mouawad & Perahia, 1996; Thomas et al., 1996a, 1996b). We emphasize qualitative analysis because NMA is based on a harmonic approximation of an anharmonic potential energy surface (Hayward & Go, 1995; Roitberg et al., 1995).

Because the conformational change we have investigated is implicated in the interfacial activation of the enzyme, the effect of the different dielectric properties of the water and lipid environments were investigated by performing calculations in high and low dielectric media, via different continuum models of the dielectric constant. NMA in "lipid" ($\epsilon = 4$) and "water" ($\epsilon = r_{ij}$ in Å)

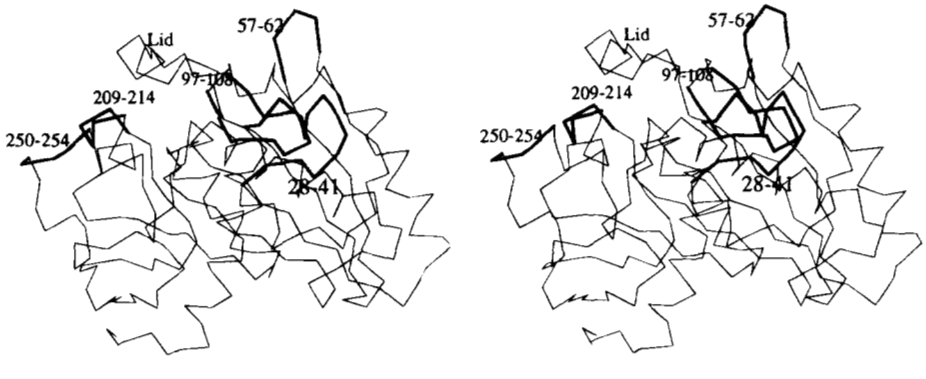

Fig. 8. Stereo pair of the Cα coordinates of the closed conformation of RmL. Loops in the vicinity of the active site with consistently large atomic fluctuations from the normal mode analyses are highlighted in bold.

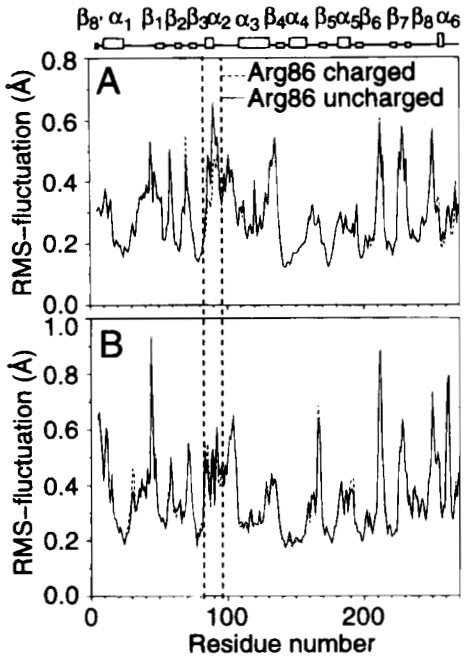

Fig. 9. Atomic RMS fluctuations at 300 K of the Cα-atoms from the normal mode analyses performed with charged and uncharged Arg86. (**A**) Closed conformation in "water" and (**B**) open conformation in "lipid." The extent of secondary structural elements and the position of the lid are marked as in Figure 3.

environments were compared in terms of the magnitude and pattern of atomic fluctuations and the degree to which the normal modes describe the reaction coordinate describing the conformational change between closed and open conformations.

These analyses reveal that the largest atomic fluctuations of the lid are found in the closed conformation in "lipid." This state also has the highest propensity for conformational change as measured by the overlap of its modes with the conformational reaction coordinate. In contrast, the open conformation in "water" has relatively low atomic fluctuations of the lid and a low propensity for transition to the closed conformation. These results suggest that a low dielectric medium promotes the transition from the closed to the open conformation.

The pattern of RMS fluctuations also reveals a remarkable difference in the mobility of the first hinge (residues 83–84) in the two different environments. Both in the closed and open conformations the first hinge is substantially less mobile in "water" than in "lipid" throughout the computed frequency spectrum. NMA of the closed conformation in "water" with an uncharged Arg86 shows significantly enhanced mobility of the lid and the second hinge. This indicates that electrostatic interactions of Arg86 play a role in the stabilization of the α-helical conformation of the lid in the closed conformation; similar observations have been made in dynamical simulations (Peters et al., 1996a). Further, neutralizing Arg86 in the open conformation in "lipid" has no effect on the mobility of the lid and hinge regions. Arg86 is one of three arginines (including Arg30 and Arg80), which form a network around the first hinge region. Clearly, further studies addressing the role of charged residues in the vicinity of the lid are required.

Lid opening and closing was found to be associated with localized loop motions and global contraction and expansion of the protein (breathing). Five loops in the vicinity of the active site were identified as being mobile throughout the modes. In modes that show breathing motions, the radius of gyration decreases by ~0.1% when the lid opens. This contrasts with the ~1% change in the radius of gyration observed in MD simulations of RmL in cyclohexane (Peters et al., 1996b). This difference is not surprising, because NMA describes harmonic vibrations that are localized to one energy minimum and are smaller in amplitude, whereas MD simulations sample several energy minima (Elber & Karplus, 1987). A similar pattern of loop and subdomain motions were identified in additional normal-mode calculations confirming that the reported motions are an inherent feature of the protein structure and not specific to a particular energy minimum (Janežič et al., 1995).

The present study was able to characterize partial opening and closing of the lid of RmL and associated loop and subdomain motions in terms of a few low frequency normal modes. However, detailed knowledge of the activation mechanism of RmL remains unclear. Our results suggest that characterization of specific electrostatic interactions of the lid region would help to identify key residues associated with lid stability and/or mobility, and could form a basis for mutation studies. Recent work has highlighted the importance of electrostatic modulation, particularly as a function of irregular surfaces and ionic concentrations, that typically characterize the interface of proteins and surrounding environments (Arakelian et al., 1993; Gilson, 1995). Further studies would ideally employ more detailed descriptions of the dielectric properties and polarizability of the solute, solvent, and the distribution of ions; however, to our knowledge, the incorporation of such models into NMA has not yet been reported. Moreover, this study uses a very crude (linear) estimate of the reaction coordinate for the lid motion that only approximately describes the gross conformational changes. A more accurate description of the transition pathway using reaction path methodologies (Fischer & Karplus, 1992) is currently in progress.

Methods

Preparation of models

Atomic coordinates for *R. miehei* lipase (RmL) used in this study were: for the closed (*apo*) conformation, PDB entry 3tgl (Derewenda et al., 1992b; resolution 1.9 Å), and for the open conformation (with a diethyl phosphate inhibitor bound), PDB entry 4tgl (Derewenda et al., 1992a; resolution 2.6 Å). RmL has 269 residues; however, the first four residues are not located in the crystal structures. Buried water molecules, as located using the program SQUID (Oldfield, 1992), were incorporated, resulting in 35 and 26 water molecules in 3tgl and 4tgl, respectively. The CHARMM force field (Brooks et al., 1983; Neria et al., 1996) was used to model the protein. Water molecules were modeled using the TIP3 (Jorgensen et al., 1983) potential. The inhibitor was removed from 4tgl, and polar hydrogens added to the molecules with the HBUILD (Brünger & Karplus, 1988) function of CHARMM. The protonation state of the residues was assigned assuming neutral pH, resulting in net charges of: Arg/Lys +1, Asp/Glu −1, His 0. Optimization of hydrogen bonding potentials around the side chains of Asn, Gln, and His was done using CHARMM (Plou et al., 1996), and the lowest energy structures were selected.

In the calculations, nonbonded interactions were truncated at 14 Å, with the electrostatic interactions SHIFTed and the van der Waals interactions SWITCHed between 10 and 14 Å; a nonbonded

list was generated for atom pairs within a radius of 15 Å (Brooks et al., 1983; Loncharich & Brooks, 1989). The dielectric properties of bulk solvent were modeled using continuum representations: for "water," a distance dependent dielectric constant ($\epsilon = r_{ij}$ in Å) was used and for "lipid," a fixed dielectric constant ($\epsilon = 4$) was used. NMA requires the system to be at a well-defined minimum on the potential energy surface (Brooks et al., 1995). Energy minimizations were performed using cycles of Steepest Descent and Adopted Basis Newton-Raphson (ABNR) algorithms (Brooks et al., 1983). During minimization, atoms were restrained using harmonic potentials (Bruccoleri & Karplus, 1986). The restraints increased in magnitude from the geometric center of the molecule in a series of radial shells each 5 Å thick (from 2 to 10 in steps of 2 kcal mol^{-1} $Å^{-1}$) and were reduced periodically until they were zero. Finally, the restraints were removed and the system minimized using ABNR until the gradient of the potential was less than 10^{-6} kcal $(mol\ Å)^{-1}$. In this way, four initial models were prepared: the open conformation in "water," and in "lipid"; the closed conformation in "water" and in "lipid." After minimizations, the RMSD from the crystal structure of the main chain (N, Cα, C) atoms was 0.96 Å for the closed conformation in water and 1.02 Å in lipid and 0.95 Å for the open conformation in water and 1.04 Å in lipid, respectively. In all minimizations the major RMS atomic positional deviations (>1.0 Å) occurred in loop regions.

Normal-mode analysis

Normal mode analysis was carried out using the iterative Diagonalization in a Mixed Basis (DIMB) method (Mouawad & Perahia, 1993; Perahia & Mouawad, 1995) to extract the 47 lowest frequency modes (excluding net translation and rotation modes of zero frequency) for the four initial models. Iterations were continued to a convergence criterion for the eigenvectors of each mode of 0.05 (for definition, see Thomas et al., 1996a, 1996b).

The motion along each mode was examined by calculating atomic fluctuations, radius of gyration, difference–distance matrices, and visualizing normal mode trajectories. The modes that best describe the lid motion were selected by calculating scalar products (projections) of the normalized Cα difference vector between the open and closed conformations (the linear conformational reaction coordinate) and the Cα eigenvector of each mode (Thomas et al., 1996a, 1996b). The latter was obtained by extracting the Cα components of the normal modes and reorthogonalizing this subspace. An absolute value of this scalar product (the "involvement coefficient" of Ma & Karplus, 1997) close to unity indicates that the mode and difference vectors are aligned along similar directions.

The sensitivity of the normal mode results was investigated by performing additional normal mode calculations of the closed RmL in "water." The potential energy surface of RmL was explored by a 320 ps 500 K MD simulation (data not shown). Structures were selected randomly at 220 ps, 245 ps, 270 ps, 295 ps, and 320 ps of the simulation for minimization and NMA.

Residue Arg86, located in the lid, has been implicated in the activation of the RmL (Holmquist et al., 1993). To investigate the effect of the electrostatic interactions of Arg86 on the normal modes of RmL, the partial charges of the side-chain atoms were set to zero, and two more normal mode calculations were performed: one for the closed conformation in "water," and one for the open conformation in "lipid." In both calculations, the minimized structures used for the corresponding NMA of the crystal structure were used as the starting structures for the minimization of the uncharged Arg86 systems. This was done to ensure that the new energy minima lie close to the minima generated from the crystal structure in the respective environments.

Graphical display and analyses were carried out using QUANTA96 (Molecular Simulations Inc., San Diego, California), SQUID (Oldfield, 1992), and MOLSCRIPT (Kraulis, 1991).

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