Theoretical Studies of the Response of a Protein Structure to Cavity-Creating Mutations

Jinhyuk Lee,* Kyunghee Lee,† and Seokmin Shin*

*Department of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151–742, Korea; and †Department of Chemistry, Sejong University, Seoul 143–747, Korea

ABSTRACT We have investigated the response of a protein structure to cavity-creating mutations by molecular dynamics (MD) simulations for the wild-type and the five mutants of phage T4 lysozyme. Essential dynamics (ED) analysis and the methods for calculating different components of local interaction energies are used to examine the structural and energetic characteristics associated with the mutations. In agreement with the x-ray results, it is found that the structural changes due to the replacements of a bulky side chain such as Leu or Phe with Ala within the hydrophobic core can be characterized as slight adjustments rather than substantial reorganization of the protein. The relative stability of different mutant structures can be related with the extent of structural readjustments in response to the mutation. The destabilization of the mutant Leu→Ala proteins relative to the wild-type is closely related with the loss of van der Waals contacts due to the cavity-creating mutations.

INTRODUCTION

It is generally agreed that one chooses a site-directed mutagenesis in such a way as to minimize reorganization of the structure of the enzyme, either locally or globally (Fersht, 1999). An enzyme or enzyme complex can tolerate a cavity within it because there is just the loss of the noncovalent interaction energies. Therefore, replacements with smaller residues are preferred to mutations that increase the size of the side chain. Understanding structural and energetic changes in a protein due to cavity-creating mutations can provide valuable information for analyzing experimental studies by site-directed mutagenesis.

The hydrophobic effect is usually considered as the major factor in determining the stability of the folded structures of globular proteins (Dill, 1990; Tanford, 1980). Mutations with the creation of larger cavities may induce substantial changes in the structure of a protein. In such cases, hydrophobic effect cannot be predicted by just considering specific residues involved in the mutation. In other words, the same type of substitution has been found to give a wide range of changes in the free energy of folding for different mutant structures (Kellis et al., 1988; Matsumura et al., 1988; Sandberg and Terwilliger, 1991; Shortle et al., 1990).

Eriksson et al. studied cavity-creating mutants within the hydrophobic core of T4 lysozyme (Eriksson et al., 1992, 1993; Xu et al., 1998). Substitutions of either Leu or Phe with Ala were found to decrease the stability of the protein by different amounts. The high-resolution x-ray structures of the mutants as well as the wild-type were determined. It was found that removal of the wild-type side chain allowed some of the surrounding atoms to move toward the vacated space, but a cavity always remained. The destabilization of the mutant Leu→Ala proteins relative to the wild-type can be approximated by a constant term, which corresponds to the difference in hydrophobicity of leucine and alanine, plus a term that increases in proportion to the size of the cavity. Either the cavity volume or the cavity surface area can be used to express the size of the cavity. These experimental results provided plausible explanations for a number of conflicting reports concerning the strength of the hydrophobic effect in proteins.

T4 lysozyme (T4L) is one of the most widely studied proteins. More than 200 T4L structures crystallized in more than 25 different crystal forms are available (Zhang et al., 1995) and T4L is a rather small protein suitable for extensive molecular dynamics (MD) simulations. From the experimental and theoretical studies on a large number of x-ray conformers, it has been shown that a hinge-bending motion is an intrinsic property of T4L (Arnold and Ornstein, 1997; de Groot et al., 1998; Faber and Matthews, 1990; Matthews and Remington, 1974; McCammon et al., 1976; McHaourab et al., 1997).

In the present study, we have performed MD simulations for cavity-creating mutants of T4L. The essential dynamics (ED) analysis (van Aalten et al., 1995) was applied to MD trajectories of the wild-type in order to examine the intrinsic flexibility of the protein structure. The ED analysis was also used to study structural changes due to various mutations. The relation between the rigidity of the mutation sites and the relative stability of the mutant structures was investigated. Detailed analysis of the changes in the local environments is made by calculating different components of interaction energies for the residue involved in the mutation. Possible correlation between a specific interaction and the destabilization of the mutant protein is explored.

MODEL AND SIMULATION DETAILS

High-resolution crystal structures of a wild-type and several mutants from T4 lysozyme were used as model structures

Received for publication 26 October 1999 and in final form 10 January 2000.

Address reprint requests to Seokmin Shin, Department of Chemistry, Seoul National University, Seoul 151–742, Korea. Tel.: 82-2-880-6639; Fax: 82-2-889-1568; E-mail: sshin@plaza.snu.ac.kr.

0006-3495/00/04/1665/07 $2.00

for theoretical studies (Eriksson et al., 1992). Four of the mutants were created by replacements of Leu residues with Ala (e.g., $Leu^{46} \rightarrow Ala \equiv L46A$). One mutant with Phe→Ala substitution was also considered. The structural data were obtained from Protein Data Bank (1l63[WT*], 1l67[L46A], 1l83[L99A], 1l69[L133A], 200l[L121A], and 1l85[F153A]). The mutant L133A was created by using the gene for wild-type lysozyme as a template. All of the other mutants were constructed with the gene for a pseudo-wild-type lysozyme (C54T/C97A, or WT*), in which the two Cys residues were replaced with Thr and Ala.

MD and ED analysis

MD simulations were performed using the GROMOS program (Scott et al., 1999). Simulations including explicit solvent molecules were done in a truncated octahedral box filled with simple point charge water molecules. A cutoff distance of 8 Å was used for the nonbonded interactions and the long-range electrostatic interactions were truncated at 10 Å. The SHAKE algorithm (Ryckaert et al., 1977) was applied to constrain bond lengths. Simulations were performed at 298K with a time step of 2 fs and the equations of motion were solved using the Verlet algorithm (Allen and Tildesley, 1989).

Simulations were started from the x-ray structures of the wild-type and mutants of T4 lysozyme. Each structure was subjected to a steepest descent (SD) energy minimization to relax any possible strain in the molecule. The minimization was followed by a period of equilibration MD simulation, where the initial velocities were taken from a Maxwellian distribution at 298K. Simulations were continued for 500 ps, and their trajectories were used for analysis including ED method. The stability of a simulation was examined by monitoring energies and geometrical properties of the system.

The ED method (van Aalten et al., 1995) is based on the diagonalization of the covariance matrix built from atomic fluctuations in an MD trajectory from which overall translation and rotations have been removed:

$$C_{ij} \equiv \langle (x_i - x_{i,0})(x_j - x_{j,0}) \rangle, \quad (1)$$

where $x_{i,j}$ are separate Cartesian coordinates of the atoms with corresponding average values denoted by $x_{i,0}$. The value expressed within $\langle\ \rangle$ represents an average over the whole MD trajectory. The coordinates of the C_α atoms are used for the analysis. Diagonalization of the covariance matrix yields a set of eigenvectors and eigenvalues, which are sorted by the size of the eigenvalue. The eigenvectors indicate directions in the total configuration space, representing correlated displacements of groups of atoms in the system. The corresponding eigenvalues indicate the total mean square fluctuations, i.e., the amplitude of the correlated motions, along these directions. The basic idea of essential dynamics is that only the correlated motions represented by the eigenvectors with large corresponding eigenvalues are important in describing the overall motion of the protein, closely related with its specific function. The ED method has been found to be useful for revealing functionally significant fluctuations in various protein systems (Peters et al., 1996, 1997; van Aalten et al., 1996).

In order to investigate the changes in the dynamics of a protein due to the mutations, the difference of each mutant structure from the wild-type is used for the essential dynamics analysis. The MD trajectories of the wild-type and the mutants are redefined relative to a common reference structure. The difference in the trajectory of each mutant from that of the wild-type is used for the essential dynamics analysis. The essential dynamics analysis was carried out using the WHAT IF modeling program (Vriend, 1990).

Interaction energies

The overall interaction energy and corresponding force for each residue in a biomolecule such as a protein can be decomposed into various components. The relative importance of these components can be correlated with a specific structure or function of the protein in certain situations. Recently, a new method for calculating different components of interaction energy or force for each residue in a protein has been developed (Lee, J., S. Shin, and S.-H. Jung, submitted for publication). It is implemented as a command (INRE) for the CHARMM program (Brooks et al., 1983). For instance, van der Waals (*evdw*), electrostatic (*eelec*), and total (*etot*) interaction energies of each residue with the rest of the molecule can be obtained. Other examples include main chain (*emain*) and side chain (*eside*) components of the total interaction energies of each residue; main chain self-energies (*esm*), side chain self-energies (*ess*), and interaction energies between main chain and side chain (*ems*) for each residue. The corresponding force components are defined similarly. Starting with the x-ray crystal structures, the structures were optimized by the SD and adopted basis Newton–Raphson methods using CHARMM all-H potential with a nonbonding condition. The minimized structures were used for the evaluation of interaction energies. For each component of interaction energies, the difference between the wild-type and a mutant is calculated and compared with the thermodynamic data describing the relative stability of such a cavity-forming mutation.

RESULTS AND DISCUSSION

We have done MD simulations on the wild-type and five mutants of the T4 lysozyme as described in the previous section. The stability of the simulations was checked by computing several structural properties. Using the stable trajectories, the ED analysis was performed to examine the changes in the dynamical structures of the systems. The ED

analysis of the MD trajectories for the wild-type (WT*) showed that only a few eigenvectors are found to represent the essential motions in the protein as in other studies. Fig. 1 shows the displacements as a function of residue number averaged over the first six eigenvectors for the wild-type simulations. T4 lysozyme is known to consist of two domains: an N-terminal domain comprising residues 15 to 65 and a C-terminal domain with residues from 80 to the C-terminus. It has been suggested that a hinge-bending motion of the two domains is an intrinsic property of the protein. Residue 13 and residues 70–75 were designated as forming the locus of the hinge-bending (Faber and Matthews, 1990). The results of the ED analysis qualitatively confirm the previous findings. Relatively large displacements are occurring within the two domains while the locus of the hinge-bending shows smaller movements. The overall pattern of displacements shown in Fig. 1 is similar to the results of the previous study on the domain motions in T4 lysozyme (de Groot et al., 1998).

The main focus of the present study is to examine the structural response to cavity-creating mutations. In Fig. 1, we have also indicated the positions of the mutations. The magnitude of displacement seen in the dynamics simulation represents the flexibility of the local protein structure, which can be related with the extent of structural readjustments in response to a mutation. It is very interesting to observe that the mutation sites show relatively small displacements. This indicates that the local structures around the five mutation sites are rather rigid. One can expect that the response of the protein to cavity-creating mutations on such sites will be more like slight adjustment than major repacking. Our conclusion is consistent with the experimental findings on the same protein (Eriksson et al., 1992). The relative stability of mutations can be inferred from the way a protein responds to perturbations. Mutant proteins relax or adapt their structures to ameliorate the consequences of potentially destabilizing changes. The most destabilizing replacements tend to occur in the most rigid part of a protein structure, because in such cases it requires large energetic costs to adjust in response to the mutation. From the results of Fig. 1, the relative stability of the five mutants is expected to be the following, in order of decreasing stability: L46A > (L133A, L121A) > (L99A, F153A). The stability data obtained from the experiments showed the same trends as the above predictions (Eriksson et al., 1992). Xu et al. (1998) examined the response of T4 lysozyme to other large-to-small substitutions within the core, such as Leu→Ala, Phe→Ala, Val→Ala, and Ile→Ala mutations. We observed that most of these mutant sites are relatively rigid with small displacements in the ED analysis of the wild-type. In order to examine the correlation between the relative stability of different mutant structures and the flexibility of the mutation sites, we plotted the thermodynamic stability against the displacement of the ED analysis in Fig. 2. We note that the stability of the mutant can be qualitatively correlated with large displacement. This trend is more pronounced for the same types of mutant structures, as illustrated for the Val→Ala mutations in the inset of Fig. 2.

ED analysis of the MD trajectories for the five mutant structures showed that the overall protein motions of the mutants are similar to those of the wild-type, whereas there exist subtle differences in the displacements of local residues. We have examined the main eigenvector motions for

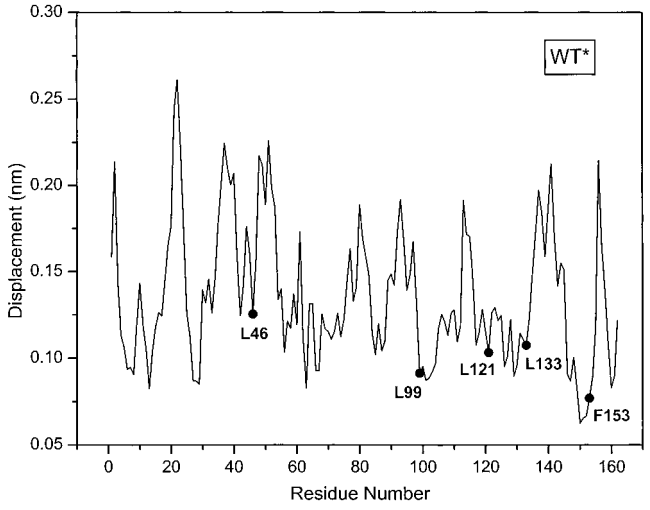

FIGURE 1 Absolute value of the displacements as a function of residue number averaged over the first six eigenvectors for the MD simulations of the wild-type of T4 lysozyme (WT*). Solid circles indicate the residues to be replaced by Ala in the five mutant structures.

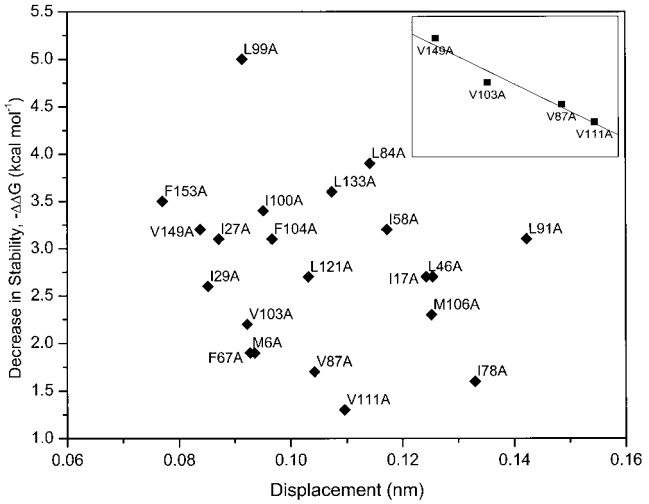

FIGURE 2 The relationship between the displacement of the mutation site from the ED analysis of the wild-type T4 lysozyme and the decrease in stability of mutant lysozyme as given by the change in the free energy of folding ($-\Delta\Delta G$). The inset shows the same plot only for the Val→Ala mutant lysozymes with the straight line representing the least-squares fit of the data. The designations of mutant structures and the thermodynamic stability data refer to the previous experimental results (Eriksson et al., 1992; Xu et al., 1998).

the trajectories of the five mutants, especially the cavity region in the vicinity of Leu^{99}, Met^{102}, Ser^{117}, Leu^{118}, Leu^{121}, Leu^{133}, and Phe^{153}. The flexibility around the cavity region is either small or moderate, depending on the type of mutations. It can be concluded that a cavity remained in the mutant structure in all cases. In order to compare different dynamical behavior of the five mutants relative to the wild-type, we have done an ED analysis based on the difference in the trajectory of each mutant from that of the wild-type. In other words, the wild-type structure was used as a common reference to determine the fluctuations in the trajectories of the mutant structures. Fig. 3 shows the eigenvalues of such ED analyses of the mutant simulations. Only a few eigenvectors contribute to the total mean square fluctuations. The magnitudes of the eigenvalues represent the extent of dynamical fluctuations of the mutant structures relative to the wild-type. They can be related with the structural changes due to the mutations. The results indicated that when Phe^{153} and Leu^{121} are replaced with Ala, the overall structural adjustments are larger than for the case of the replacement of Leu^{99} with Ala. The same observations were made in the previous experimental study (Eriksson et al., 1993, 1992). In Fig. 4, we plotted the averaged displacements of the first six eigenvectors as a function of residue number for the five mutants. The difference between

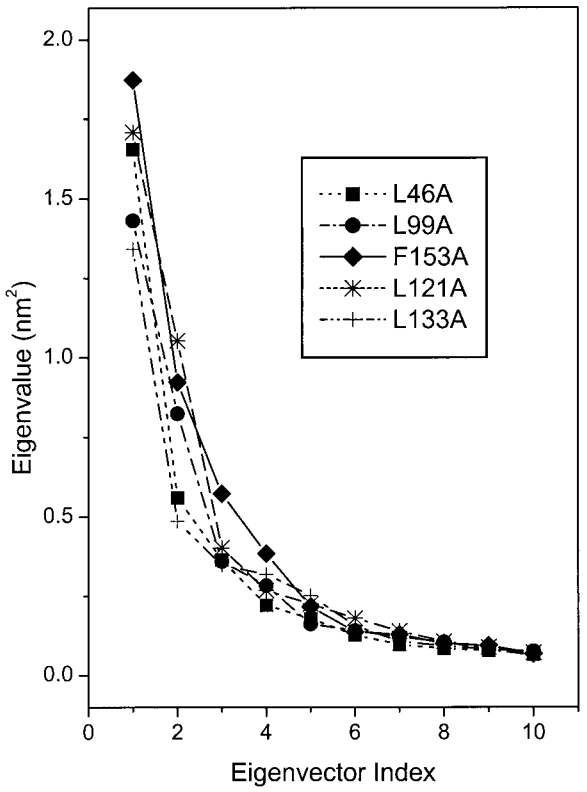

FIGURE 3 Eigenvalues obtained from the essential dynamics analyses of the MD simulations for the five cavity-creating mutant structures.

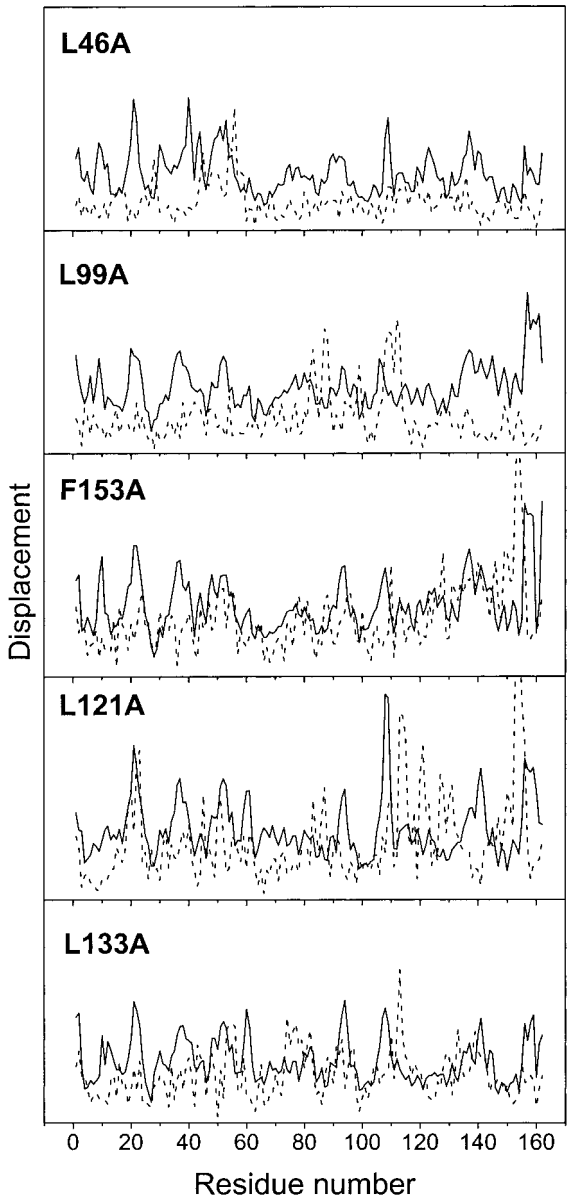

FIGURE 4 Absolute value of the averaged displacements as a function of residue number from the essential dynamics analyses of the difference trajectories of the five cavity-creating mutant structures relative to that of the wild-type (*solid line*). The range of the displacements is 0.0 to 0.75 nm for all the figures. Also shown is the difference between the backbones of the mutant structure and the wild-type (*dotted line*) with the range of 0.0 to 0.75 Å.

the backbones of the mutant structure and the wild-type is also shown in the same figure. The ED displacements and the changes in structure (mutant versus wild-type) show somewhat different pattern as a function of residue number. However, the relative sizes of the overall fluctuations for different mutants exhibit consistent results in both cases. L121A and F153A are found to show larger ED displacements and structural changes.

Cavity-creating mutations such as the replacements of Leu or Phe with Ala can be viewed as reflecting the changes in the local interaction energies around the mutation sites. We have calculated different components of interaction energies for a specific residue involved in the mutation. The main concern is to examine the possible correlation between changes in interaction energies for the mutants compared with the wild-type and the reduction in protein stability due to the mutation. Of the five mutant structures, only four cases with the same Leu→Ala replacement were considered. Experimental values for the change in the free energy of unfolding (ΔΔG) of mutant lysozymes relative to the wild-type were used to represent the reduction in protein stability.

Fig. 5 shows the relation between the decrease in protein stability and the changes in energies such as electrostatic (*eelec*), van der Waals (*evdw*), or total (*etot* = *eelec* + *evdw*) interaction energies. It is found that there exists a linear relation between increase in van der Waals energies and reduction in protein stability. Previously, decrease in stability, caused by cavity-creating mutations, was found to be correlated with increase in cavity volume or surface area (Eriksson et al., 1992). It was shown that change in the free energy of unfolding associated with a Leu→Ala replacement consists of a constant energy term of 1.9 kcal mol^{-1} plus a second energy term that depends on the size of the cavity created by the substitution. Our results are consistent with these interpretations. When a bulky residue such as leucine is replaced with a smaller one like alanine, many favorable van der Waals contacts in the original folded protein will be removed. The cavity-dependent part of the destabilization associated with cavity-creating mutants is due to the loss of such van der Waals contacts. The relative stability of different mutants will be determined by the extent of readjustments of the protein to restore part of the van der Waals interactions.

Interaction energies of a residue can be divided into contributions from the main chain and the side chain parts of the protein. The relation between the decrease in stability for the cavity-creating mutants and change in main chain (*emain*) and side chain (*eside*) components of the total interaction energies is shown in Fig. 6. The side chain component accounts for most of the increase in interaction energy and reduction in protein stability seems to be related with increase in *eside*. This makes sense because the cavity-creating mutation corresponds to the replacement of a bulky side chain with a smaller one. It is interesting to note that main chain component of interaction energy decreases in some cases. When the readjustments of a protein after the mutation are substantial, the reduction of the size of the cavity will increase the overall compactness of the protein, allowing more favorable interactions among main chain parts of the protein. It is generally known that the main chain interactions are mostly electrostatic (hydrogen-bonding) in character, whereas the side chain interactions inside a protein represent hydrophobic (van der Waals) interactions. It is interesting to note that *emain* and *eside* show behavior similar to *eelec* and *evdw*, respectively (Figs. 5 and 6).

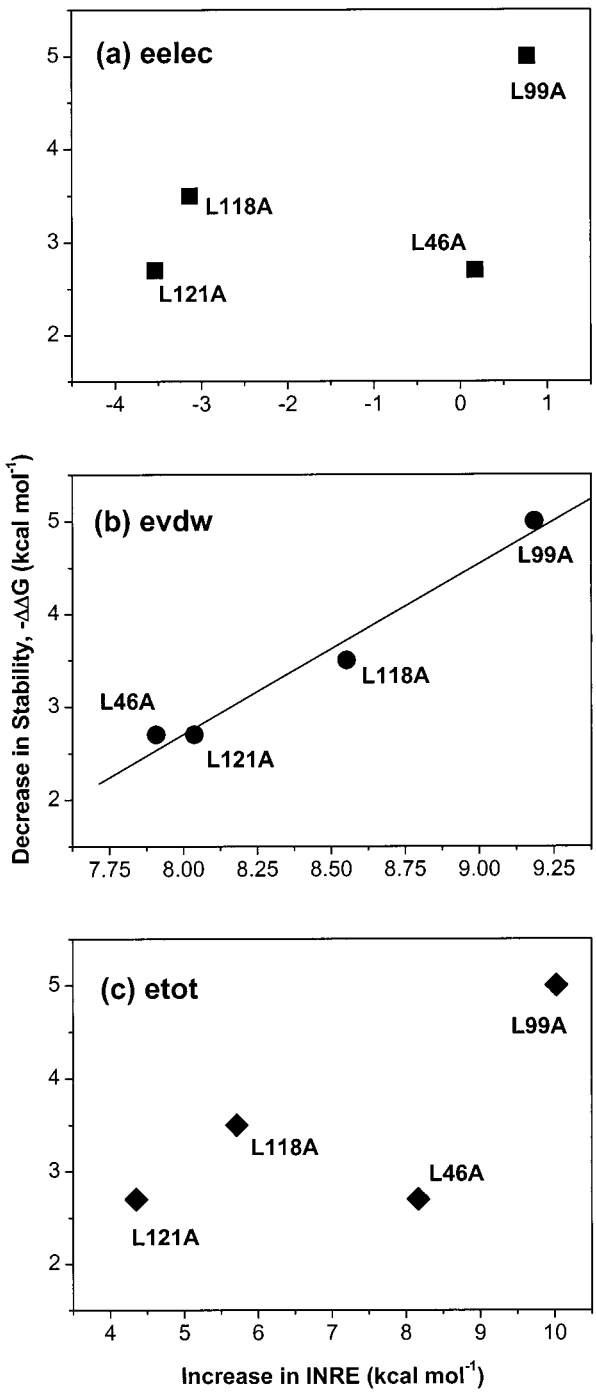

FIGURE 5 Change in the free energy of folding (−ΔΔG) of mutant lysozymes relative to wild-type plotted as a function of the component of interaction energies for the residue involved in the mutation. (*a*) van der Waals (*evdw*), (*b*) electrostatic (*eelec*), and (*c*) total (*etot*) components of the interaction energies of each residue are considered. The straight line for the *evdw* plot shows the correlation between the van der Waals interaction and the stability of the mutant structure.

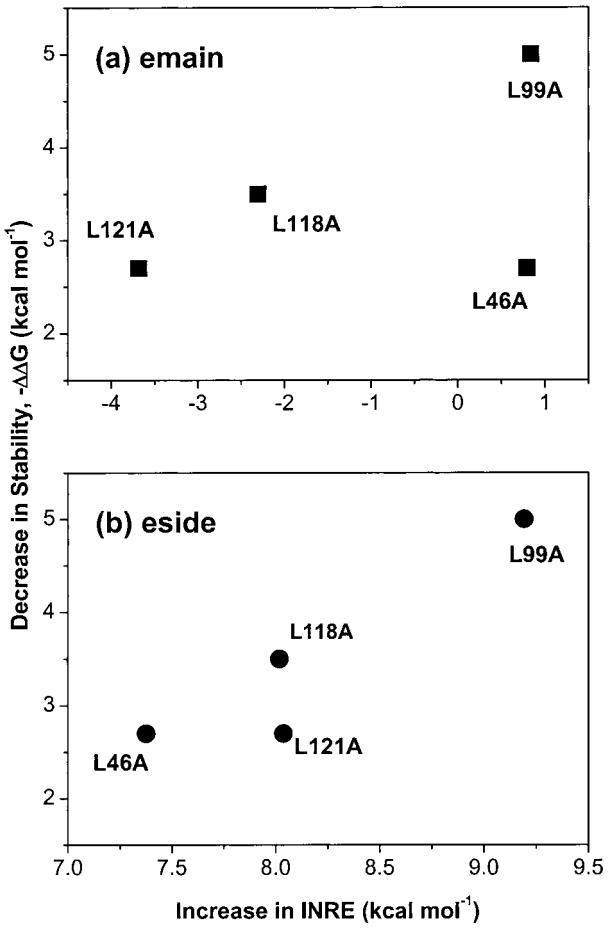

FIGURE 6 Change in the free energy of folding ($-\Delta\Delta G$) of mutant lysozymes relative to wild-type plotted as a function of the component of interaction energies for the residue involved in the mutation. (*a*) Main chain (*emain*) and (*b*) side chain (*eside*) components of the interaction energies of each residue are considered.

CONCLUSIONS

We have investigated the response of a protein structure to cavity-creating mutations. Molecular dynamics (MD) simulations and subsequent essential dynamics (ED) analysis for the wild-type and the five mutants of the T4 lysozyme have been done to examine the structural characteristics associated with the cavity-creating mutations. It is found that the structural changes due to the replacements of a bulky side chain such as Leu or Phe with Ala within the hydrophobic core can be characterized as slight adjustments rather than substantial repacking of the protein. The mutation sites located mostly around the cavity region are found to be rather rigid, which is consistent with the above findings. The amount of structural change differs from case to case and the relative stability of the mutant structures can be related to the extent of readjustments of the protein in response to the mutation.

Understanding the relative importance of different components of interaction energies in determining protein stability can provide valuable information. We have studied possible correlations between decreases in protein stability as represented by the free energy of unfolding ($\Delta\Delta G$) of mutants and changes in the different components of interaction energies due to the mutation. It is found that the destabilization of the mutant Leu→Ala proteins relative to the wild-type is closely related to the increase in the van der Waals interactions. The destabilization is caused by the loss of van der Waals contacts due to the cavity-creating mutations. The relative stability of different mutants depends on how well the protein responds to the mutation and restores a favorable folded structure. The side chain components of interaction energies account for most of the changes in van der Waals energies. It is generally agreed that the hydrophobic effect is the major factor in stabilizing the folded structures of proteins. The present study suggests that one must consider the contributions due to the structural relaxation in proteins in addition to an intrinsic hydrophobic effect associated with the substitution of a specific pair of residues. Our studies have also demonstrated the usefulness of theoretical methods such as the essential dynamics analysis and the evaluation of the interaction energy component in providing insights into understanding the structure and function of protein systems.

This work was supported by the Korean Science and Engineering Foundation (KOSEF) through the Center for Molecular Catalysis at Seoul National University. J. L. and S. S. thank Sun-Hee Jung for helpful discussions.

REFERENCES

Allen, M. P., and D. J. Tildesley. 1989. Computer Simulation of Liquids. Clarendon, Oxford.

Arnold, G. E., and R. L. Ornstein. 1997. Protein hinge bending as seen in molecular dynamics simulations of native and M6I mutant T4 lysozymes. *Biopolymers.* 41:533–544.

Brooks, B. R., R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan, and M. Karplus. 1983. CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. *J. Comp. Chem.* 4:187–217.

de Groot, B. L., S. Hayward, D. M. F. van Aalten, A. Amadei, and H. J. C. Berendsen. 1998. Domain motions in bacteriophage T4 lysozyme: a comparison between molecular dynamics and crystallographic data. *Proteins Struct. Funct. Genet.* 31:116–127.

Dill, K. A. 1990. Dominant forces in protein folding. *Biochemistry.* 29: 7133–7155.

Eriksson, A. E., W. A. Baase, and B. W. Matthews. 1993. Similar hydrophobic replacements of Leu99 and Phe153 within the core of T4-lysozyme have different structural and thermodynamic consequences. *J. Mol. Biol.* 229:747–769.

Eriksson, A. E., W. A. Baase, X. J. Zhang, D. W. Heinz, M. Blaber, E. P. Baldwin, and B. W. Matthews. 1992. Response of a protein structure to cavity-creating mutations and its relation to the hydrophobic effect. *Science.* 255:178–183.

Faber, H. R., and B. W. Matthews. 1990. T4 lysozyme displays five different crystal conformations. *Nature.* 348:263–266.

Fersht, A. 1999. Structure and Mechanism in Protein Science. Freeman, New York.

Kellis, J. T., K. Nyberg, D. Sali, and A. R. Fersht. 1988. Contribution of hydrophobic interactions to protein stability. *Nature.* 333:784–786.

Matsumura, M., W. J. Becktel, and B. W. Matthews. 1988. Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature.* 334:406–410.

Matthews, B. W., and S. J. Remington. 1974. The three-dimensional structure of the lysozyme from bacteriophage T4. *Proc. Nat. Acad. Sci. USA.* 71:4178–4182.

McCammon, J. A., B. Gelin, M. Karplus, and P. G. Wolynes. 1976. The hinge-bending motion in lysozyme. *Nature.* 262:325–326.

McHaourab, H. S., K. J. Oh, C. J. Fang, and W. L. Hubbell. 1997. Conformation of T4 lysozyme in solution: hinge-bending motion and the substrate-induced conformational transition studied by site-directed spin labeling. *Biochemistry.* 36:307–316.

Peters, G. H., S. Toxvaerd, O. H. Olsen, and A. Svendsen. 1996. Dynamics of proteins in different solvent systems: analysis of essential motion in lipases. *Biophys. J.* 71:2245–2255,.

Peters, G. H., D. M. F. van Aalten, A. Svendsen, and R. Bywater. 1997. Essential Dynamics of lipase binding sites: the effect of inhibitors of different chain length. *Protein Eng.* 10:149–158.

Ryckaert, J.-P., G. Ciccotti, and H. J. C. Berendsen. 1977. Numerical integration of the Cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J. Comp. Phys.* 23: 327–341.

Sandberg, W. S., and T. C. Terwilliger. 1991. Energetics of repacking of a protein interior. *Proc. Natl. Acad. Sci. USA.* 88:1706–1710.

Scott, W. R. P., P. H. Hunenberger, I. G. Tironi, A. E. Mark, S. R. Billeter, J. Fennen, A. E. Torda, T. Huber, P. Kruger, and W. F. van Gunsteren. 1999. The GROMOS biomolecular simulation program package. *J. Phys. Chem.* 103:3596–3607.

Shortle, D., W. E. Strites, and A. K. Meeker. 1990. Contributions of the large hydrophobic amino acids to the stability of staphylococcal nuclease. *Biochemistry.* 29:8033–8041.

Tanford, C. 1980. The Hydrophobic Effect. Wiley, New York.

van Aalten, D. M. F., A. Amadei, A. B. M. Linssen, V. G. H. Eijsink, and G. Vriend. 1995. The essential dynamics of thermolysin-conformation of the hingebending motion and comparison of simulations in vacuum and water. *Proteins Struct. Funct. Genet.* 22:45–54.

van Aalten, D. M. F., J. B. C. Findlay, A. Amadei, and H. J. C. Berendsen. 1996. Essential dynamics of the cellular retinol binding protein: evidence for ligand induced conformational changes. *Protein Eng.* 8:1129–1135.

Vriend, G. 1990. WHAT IF: a molecular modeling and drug design program. *J. Mol. Graph.* 8:52–56.

Xu, J. A., W. A. Baase, E. Baldwin, and B. W. Matthews. 1998. The response of T4 lysozyme to large-to-small substitutions within the core and its relation to the hydrophobic effect. *Protein Sci.* 7:158–177.

Zhang, X. J., J. A. Wozniak, and B. W. Matthews. 1995. Protein flexibility and adaptability seen in 25 crystal forms of T4 lysozyme. *J. Mol. Biol.* 250:527–552.