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Nonneutral evolution of volume fluctuations in lysozymes revealed by normal-mode analysis of compressibility

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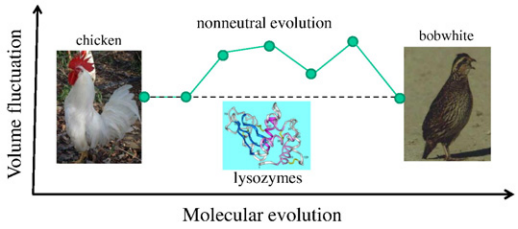
HIGHLIGHTS

- Compressibility of evolutionary lysozymes are calculated by normal mode analysis.
- Volume fluctuation of lysozymes follows a nonneutral evolutionary pathway.
- Internal cavity is an important factor regulating volume fluctuation of lysozymes.

GRAPHICAL ABSTRACT



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ABSTRACT

The evolution of structural fluctuations of proteins was examined by calculating the isothermal compressibility (β_T) values of chicken lysozyme and its six evolutionary mutants at Thr40, Ile55, and Ser91 (a ternary mutant corresponding to bobwhite lysozyme) from their X-ray structures by normal-mode analysis at 300 K. The β_T values of the two extant lysozymes from chicken and bobwhite were 1.61 and 1.59 $Mbar^{-1}$, respectively, but five other evolutionary mutants showed larger β_T values of up to 2.17 $Mbar^{-1}$. These results suggest that ancestral lysozymes exhibit larger volume fluctuations than extant ones, and hence that the molecular evolution of lysozymes has followed a nonneutral evolutionary pathway. The evolutionary mutants contained large amount of cavities, although no change was visible in the X-ray structures. There was a linear correlation between β_T and total cavity volume, predicting that the cavity volume or atomic packing is an important factor regulating volume fluctuations during the molecular evolution of this protein.

1. Introduction

There has been considerable controversy about whether protein evolution follows a neutral or nonneutral pathway [1–4], but this problem remains undefined at the molecular level because few proteins have a series of evolutionary variants. Game-bird lysozymes consist of 129 total amino-acid residues, 3 of which (residues 40, 55, and 91) are occupied either by the triplet Thr40/Ile55/Ser91 (TIS) for chicken or by the triplet Ser40/Val55/Thr91 (SVT) for bobwhite. These three residues are located together just beneath the active-site cleft in the three-dimensional structure [5,6], and they play an important role in the enzyme function. Lysozymes in game birds are encoded by a single gene [7] in which the possibility of simultaneous double or triple mutations is negligibly small. Hence, their ancestral lysozymes could be expected to evolve along the possible three-step pathways between these triplets shown in Fig. 1: TIS (chicken) → SIS (variant with Ser40/Ile55/Ser91), TIT (variant with Thr40/Ile55/Thr91), TVS (variant with Thr40/Val55/Ser91) (single mutation) → SIT (variant with Ser40/Ile55/Thr91), SVS (variant with Ser40/Val55/Ser91), and TVT (variant with Thr40/Val55/

Abbreviations: TIS, wild type with Thr40/Ile55/Ser91 (chicken); SVT, wild type with Ser40/Val55/Thr91 (bobwhite); SIS, variant with Ser40/Ile55/Ser91; TIT, variant with Thr40/Ile55/Thr91; TVS, variant with Thr40/Val55/Ser91; SIT, variant with Ser40/Ile55/Thr91; SVS, variant with Ser40/Val55/Ser91; TVT, variant with Thr40/Val55/Thr91; PDB, Protein Data Bank.

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Thr91) (double mutation) → SVT (bobwhite); however, none of these six intermediates (SIS, TIT, TVS, SIT, SVS, and TVT) are found in extant lysozymes. Malcolm et al. [5] constructed these six intermediates by site-directed mutagenesis of chicken lysozyme and found that the thermostabilities of the variants fell outside the range of the two extant lysozymes from chicken and bobwhite. This finding suggests that these mutations have followed a nonneutral evolutionary pathway. It is of interest how the structural fluctuations of these ancestral lysozymes have changed during the molecular evolution to gain functional adaptation.

The structural fluctuation of proteins has been investigated by various techniques, including X-ray crystallography (B-factor), NMR (order parameter), hydrogen-deuterium exchange, compressibility, and computer simulation [8]. Compressibility is an important measure of volume fluctuation [9] because it is sensitive to packing defects or cavities in the interior of a protein molecule, and these are easily compressed by pressure and result in internal motions or flexibility in response to thermal or mechanical forces. Directly measuring the isothermal compressibility (β_T) of a protein is generally difficult, as reflected by the small number of related reports [10,11]. This has led to most studies of protein compressibility considering the adiabatic compressibility, β_s, which can be determined more easily and accurately by sound-velocity measurements [12–21]. An important finding of these compressibility studies is that substituting a single amino acid can induce large changes in β_s, and that mutants having larger β_s values exhibit higher enzyme activity [18–21]. This means that the local structural changes associated with mutation significantly modify the overall dynamics of a protein so as to affect its function. Computer simulation of β_T is useful to obtain a detailed description of molecular movement at the atomic level, and can be implemented by the molecular dynamics and the normal-mode analysis [22–29]. The former is more common due to simulations now extending into the nanosecond range, while normal-mode analysis is advantageous for rapidly calculating β_T based on a harmonic approximation of the potential energy function around a minimum energy conformation.

To address how volume fluctuations vary during protein evolution, the present study calculated the β_T values of the above extant and evolutionary lysozymes (except for SVS, whose X-ray structure has not yet been determined) by normal-mode analysis because no experimental β_T values are known for these evolutionary lysozymes. The β_T values obtained are discussed in terms of the modified atomic packing and the molecular evolution of lysozymes.

2. Methods

2.1. Normal-mode analysis

The β_T values of lysozymes were calculated as a sum of the volume fluctuations from individual normal modes within the potential energy of the harmonic range, essentially according to the method of Yamato and colleagues developed for studying the dynamics of deoxymyoglobin and TIM-barrel protein [27,28]. The atomic coordinates of the X-ray crystal structures of lysozymes at 1.8-Å resolution were obtained from the Protein Data Bank (PDB) [14,15] and converted into dihedral-angle coordinates using the program FEDER/2 constructed by Wako et al. [30]. The following PDB IDs were used: 1HEL (chicken), 1HER (SIS), 1HEO (TVS), 1HEM (TIT), 1HEQ (SIT), 1HEN (TVT), and 1HEP (bobwhite). We defined the volume of a protein with the excluded volume calculated by the method of Higo and Go using a 1.4-Å probe for the radius of water [31].

The energy minimization of the protein structures was carried out in torsion angle space using the program FEDER/2 with the energy function ECEPP/2 and Newton's method [30]. Conformational energy function E was defined as a sum of five contributions:

$$E = E_c + E_{hyd} + E_{es} + E_{tor} + E_{ss} \quad (1)$$

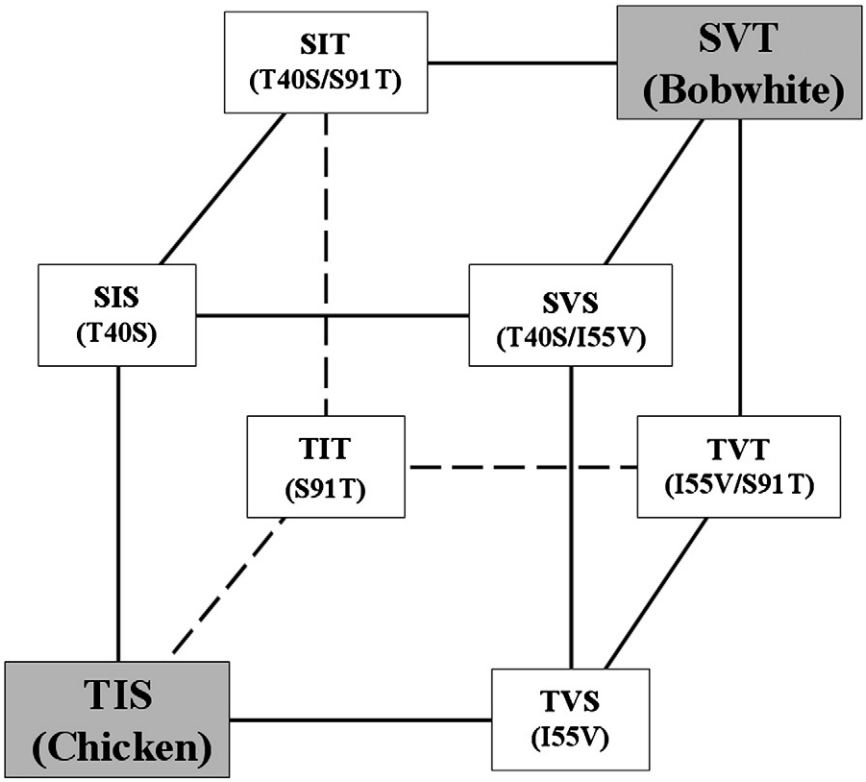

Fig. 1. Assumed evolutionary pathways between two extant lysozymes (chicken and bobwhite): TIS (chicken) → SIS, TIT, TVS (single mutation) → SIT, SVS, TVT (double mutation) → SVT (bobwhite).

where E_c is the van der Waals force (Lennard–Jones type 6–12 potential), E_{hyd} is the hydrogen-bond energy (Lennard–Jones type 10–12 potential), E_{es} is the electrostatic energy, E_{tor} is the torsional energy associated with the barriers to rotation about a rotatable bond, and E_{ss} is the disulfide-bond (loop-closing) energy. Using normal-mode variable σ_i for the i-th harmonic oscillator with angular frequency ω_i, the conformational energy (E) and the mean-square amplitude ($<\sigma_i^2>$) of the equilibrium fluctuation of each normal-mode variable are given by

$$E = (1/2)\left(\Sigma\, \omega_i{}^2 \sigma_i{}^2\right) \tag{2}$$

$$<\sigma_i{}^2> = \kappa_B T/\omega_i{}^2 \tag{3}$$

where κ_B is the Boltzmann constant and T is the absolute temperature. In addition to the harmonic approximation of the conformational energy, we assumed a linear volume change (ΔV) due to deviation from the minimum energy conformation with volume V:

$$\Delta V = \Sigma v_i \sigma_i \tag{4}$$

where v_i is the gradient of the volume with respect to the i-th normal-mode variable at the minimum energy conformation. From Eqs. (3) and (4), the mean-square volume fluctuation $<(\Delta V)^2>$ is given by

$$<(\Delta V)^2> = \kappa_B T \Sigma\left(v_i{}^2/\omega_i{}^2\right). \tag{5}$$

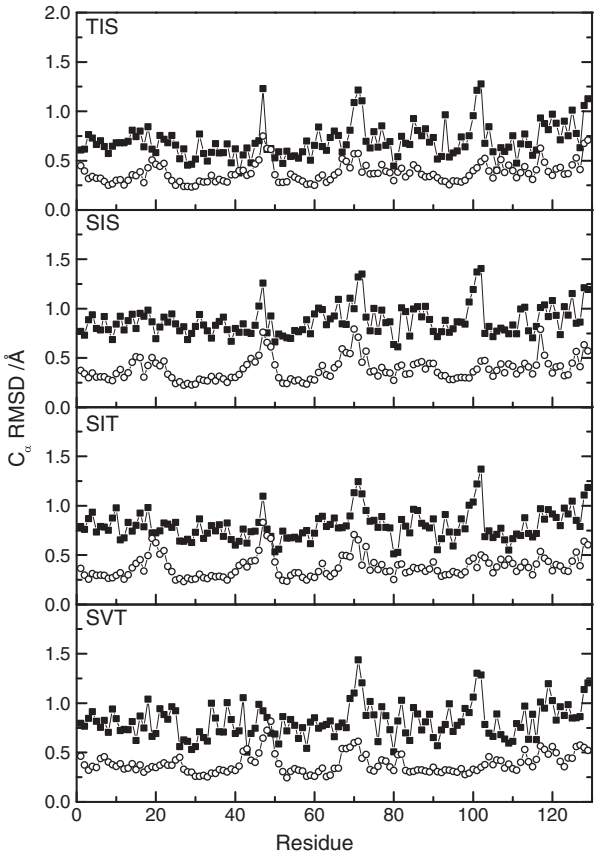

Fig. 2. Comparisons of displacements of C_α atoms of amino-acid residues in extant (TIS and SVT) and evolutionary (SIS and SIT) lysozymes estimated by normal-mode analysis (○) and from the X-ray structure (B-factor, ■).

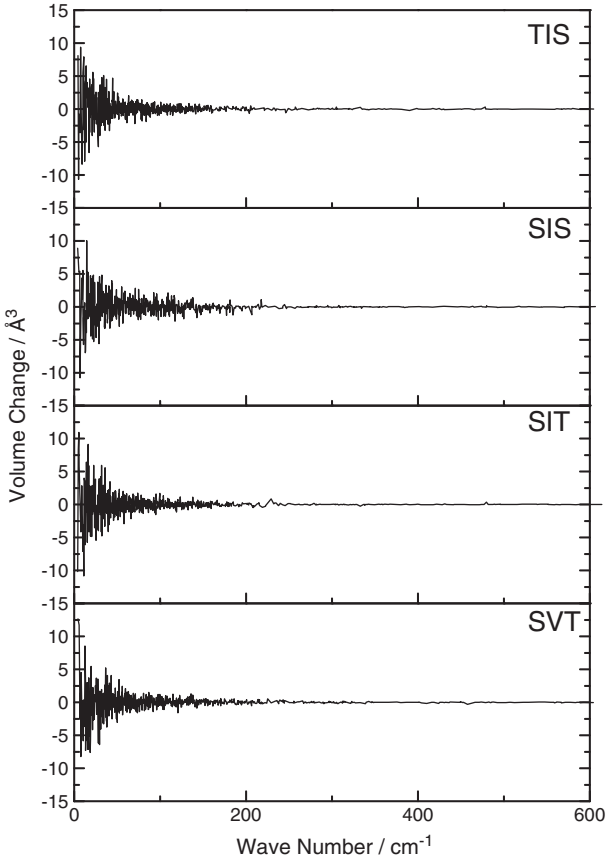

Fig. 3. Volume changes from the energy-minimum conformation of extant (TIS and SVT) and evolutionary (SIS and SIT) lysozymes as functions of the wave number for the normal mode.

The volume fluctuation is directly related to β_T from statistical thermodynamics [9]:

$$<(\Delta V)^2> = \kappa_B T\, V \beta_T. \tag{6}$$

Thus, isothermal compressibility β_T can be calculated using the following equation [27,28]:

$$\beta_T = \Sigma\left(v_i{}^2/\omega_i{}^2\right)/V. \tag{7}$$

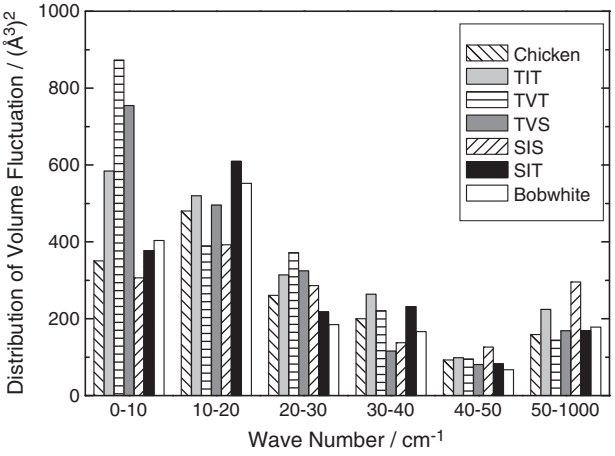

Fig. 4. Distribution of volume fluctuations of lysozymes within 10-cm^{-1} ranges below 50 cm^{-1} and within the range of 50–1000 cm^{-1}.

Table 1
Compressibilities and volume fluctuations of extant and evolutionary lysozymes.

Lysozyme	PDB ID	β_T (Mbar^{-1})	$<(\Delta V)^2>$[a] (Å^3)	V cav[b] (Å^3)	T m[c] (°C)
TIS (Chicken)	1HEL	1.61	39.3 (0.17)	65.8	73.9
SIS	1HER	1.61	39.3 (0.17)	100.8	73.0
TVS	1HEO	1.98	44.0 (0.19)	119.2	71.2
TIT	1HEM	2.08	44.8 (0.19)	211.2	77.5
SIT	1HEQ	1.75	41.1 (0.18)	143.0	75.5
TVT	1HEN	2.17	45.7 (0.20)	163.5	74.5
SVT (Bobwhite)	1HEP	1.59	39.4 (0.17)	95.0	73.4

[a] Values within parentheses represent ratios (%) of $<(\Delta V)^2>$ to the total molecular volume.
[b] Mean total cavity volumes estimated with probe radii of 1.05–1.20 Å.
[c] Taken from Ref. [5].

In the present study, β_T was calculated at 300 K.

2.2. Calculation of accessible surface area and cavity volume

The accessible surface area (A_s) and the internal cavities of lysozymes were evaluated from their X-ray structures using the program GRASP (Graphical Representation and Analysis of Structural Properties) [32]. The A_s value was calculated with a probe radius of 1.4 Å, which corresponds to the size of the water molecule. Cavities are analytically defined by the surface described by rolling a probe sphere around an internal atomic surface [33,34]. The total volume (V_{cav}) of cavities is sensitive to the probe size: a probe that is either too large or too small cannot detect small cavities. The cavity is often estimated using probes with radii (R_c) of around 1.2 Å [35,36]. In the present study, the mean values of V_{cav} estimated with four R_c values (1.05, 1.00, 1.15, and 1.20 Å) were used in correlation analysis with compressibility data. The number of internal water molecules in cavities was zero for SIS, TVS, and SIT, one for TIS, TVT, and SVT, and three for TIT. These water molecules were eliminated in the calculation of V_{cav}; however, its correlation with compressibility was not affected by the presence or absence of internal water molecules in cavities.

3. Results and discussion

3.1. Volume fluctuation and isothermal compressibility

Fig. 2 compares the displacements of C_α atoms of amino-acid residues estimated by normal-mode analysis for two extant lysozymes

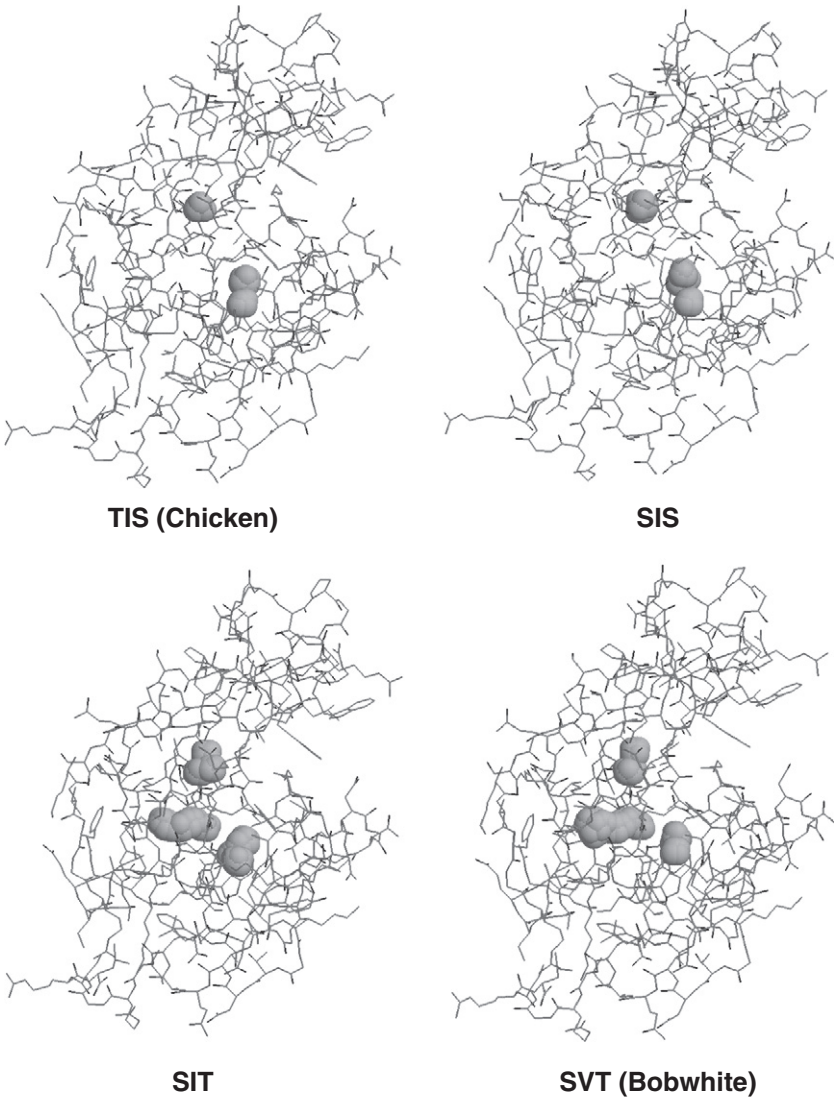

Fig. 5. Distribution of cavities in extant (TIS and SVT) and evolutionary (SIS and SIT) lysozymes. Cavities were evaluated with a probe radius of 1.15 Å and are indicated by gray balls.

(TIS and SVT) and two evolutionary intermediates (SIS and SIT) with those estimated from the B-factor of the X-ray structure. The overall features of the C_α displacements were similar in both estimations for all of the lysozymes. The C_α displacements were determined to be smaller by the normal-mode analysis than by the B-factor, which is as expected since normal-mode analysis is carried out for an energy-minimum structure whereas the X-ray structure involves the static disorder of the crystal. The RMSD (root-mean-square deviation) values of C_α displacements for all of the lysozymes was 0.12 Å, indicating that the energy-minimized structures of these intermediates were close to each other and to those of the two extant lysozymes.

Fig. 3 shows the volume changes of the protein molecule from its minimum energy conformation as a function of wave number for extant (TIS and SVT) and two evolutionary (SIS and SIT) lysozymes. Other intermediates showed similar features (data not shown). It is evident from the figure that atom movements faster than 200 cm^{-1} induce no substantial changes in volume, but that slower movements produce larger volume changes of up to 15 $Å^3$. Thus, normal modes with low frequencies play a substantial role in pressure-induced deformations or volume fluctuations of these proteins.

The distributions of volume fluctuations summed within 10-cm^{-1} ranges below 50 cm^{-1} and within the range of 50–1000 cm^{-1} are shown as histograms for all of the lysozymes in Fig. 4. Each lysozyme showed different fluctuation patterns that depended on the rates of movements: extant lysozymes (TIS and SVT) had the largest fluctuation within the range of 10–20 cm^{-1}, whereas most of the evolutionary intermediates showed enhanced fluctuations for slower movements. The following residues exhibited changes in their mean dihedral angles of over 10° in total mode: residue 129 for TIS (chicken); residues 1 and 127 for SVT (bobwhite); residue 117 for SIS; residues 17, 100, 102, 103, and 129 for SIT; residues 1, 102, 117, and 129 for TIT; residues 41, 67, 71, 102, 117, 120, 121, 126, 127, and 129 for TVT; and residues 1, 48, and 104 for TVS. These results indicate that the local structures are sensitively influenced by the substitution of a single amino acid, especially in evolutionary intermediates. The atomic movements were not analyzed in detail since the local compressibility changes fell outside the scope of the present study.

The calculated values of β_T and $<(\Delta V)^2>$ are listed in Table 1 together with V_{cav} and the thermal transition temperature (T_m) reported by Malcolm et al. [5]. The β_T values of chicken and bobwhite lysozymes were almost the same (1.61 and 1.59 $Mbar^{-1}$), but all of the evolutionary mutants showed larger β_T values of up to 2.17 $Mbar^{-1}$ although the β_T value of SIS was similar to that of the extant lysozymes, suggesting that volume fluctuations are larger for ancestral lysozymes than for extant ones. Volume fluctuations amount to only 0.17–0.20% of the excluded volume, but they induce large variations of up to 16% (39.3–45.7 $Å^3$) among the mutants (Table 1).

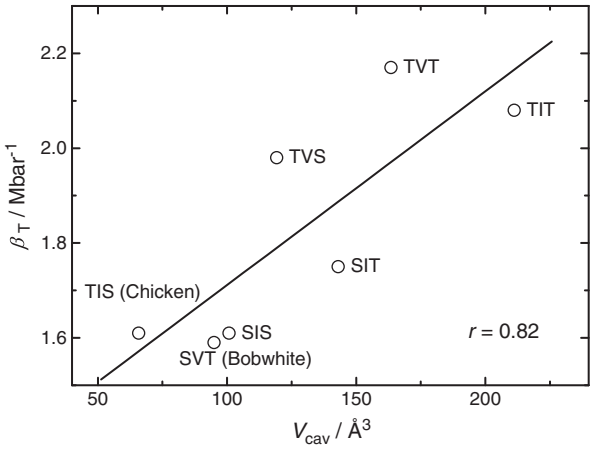

Fig. 6. Plot of β_T against V_{cav} for lysozymes. Solid line represents the least-squares linear regression. V_{cav} is the mean value estimated with four probe radii (1.05, 1.00, 1.15, and 1.20 Å).

3.2. Comparison of calculated and experimental values of β_T

The β_T value of chicken lysozyme has been experimentally estimated to be 4.7 and 9.8 $Mbar^{-1}$ from the X-ray crystal structures under high pressures [37,38], and 7.5 $Mbar^{-1}$ by a high-pressure NMR study [39]. A similar value was estimated from the partial adiabatic compressibility β_s (4.7 $Mbar^{-1}$) determined from sound-velocity measurements in solution [12]. These experimentally observed β_T and β_s values are considerably larger than the value of 1.61 $Mbar^{-1}$ obtained in the present study, although direct comparison between the calculated and experimental values of compressibility is intrinsically difficult because the experimental values are partial quantities involving complicated contributions from hydration. Such a low β_T value obtained by normal-mode analysis is not improbable since lysozyme is classified as a rigid protein in the accumulated compressibility data, and a protein should be more rigid in the energy-minimum crystal structure due to suppressed large-scale motions of the molecule [23].

This present study employed a simple computational procedure for the systematic comparison of compressibility among different mutants, but accurate predictions of β_T fell outside the scope of this work. Future studies should address two issues: (1) accurate models of protein dynamics and (2) accurate definitions of protein volumes. Regarding the first of these issues, in the normal-mode analysis in vacuo, large-scale molecular movements are associated with low-frequency normal modes. In solution, such movements would be better described by multiple hierarchical modes. For instance, Kitao et al. proposed a JAM (jumping-among-minima) model for the energy landscape of human lysozyme in solution at room temperature [40]. Regarding the second issue, the computational estimate of the compressibility depends on the definition of protein volume. Imai et al. recently proposed a method to evaluate partial volumes based on a statistical mechanics approach, which provides a powerful computational tool to calculate molecular volumes [41]. In spite of these two issues, it is noteworthy that evolutionary mutants of lysozyme have different β_T values when using the same calculation procedure, and it is likely that these differences will be magnified in solution.

3.3. Contribution of the cavity volume to compressibility

It is of interest that the evolutionary lysozymes have considerably different β_T values since only small changes are visible in their tertiary structures [6]. Such significant mutation effects have also been observed in the β_s values determined by sound-velocity measurements in solution [18–21]. Two main factors contribute to the partial compressibility of a protein in solution: cavities and hydration [12,13]. In general, cavities contribute positively and hydration contributes negatively to partial compressibility, and hence the experimentally observed partial compressibility is determined by the relative contributions of these two terms. However, the contribution of hydration is not included in normal-mode analysis, and so the observed difference in β_T values of evolutionary lysozymes could be dominantly ascribed to modifications to the cavities. Since the apparent compressibility of a cavity is about tenfold larger than that of water [12], even a small perturbation in the cavity would cause large changes in both β_T and β_s.

Fig. 5 shows typical distributions of the cavities in four lysozyme molecules, as evaluated with an R_c of 1.15 Å. The figure indicates that the number and positions of cavities vary among the mutants. The mean values of V_{cav} estimated with four R_c values (1.05, 1.00, 1.15, and 1.20 Å) are listed in Table 1. It is noteworthy that any

mutation induces a large variation in V_{cav} and that all of the evolutionary intermediates have larger V_{cav} values than the extant lysozymes.

Fig. 6 shows plots of β_T against V_{cav} for all of the lysozymes examined, from which it is evident that β_T increases with the total cavity volume (correlation coefficient $r = 0.82$). We observed a similar positive correlation between the β_s and V_{cav} values of 27 globular proteins (unpublished data). Therefore, the cavity volume or atomic packing would be an important factor determining fluctuations in the molecular evolution of lysozyme.

3.4. Relationship of volume fluctuation to evolution and function

According to the neutral theory of molecular evolution [2], the overall properties of evolutionary intermediates should fall within the range exhibited by the extant species. As indicated in Table 1, the values of β_T and $<(\Delta V)^2>$ of the evolutionary mutants fall outside the range of the two wild types, which is negligibly narrow in the present case. This constitutes evidence that volume fluctuations of these lysozymes have followed a nonneutral evolutionary pathway. Such a lack of neutrality was also found for the thermal stability [5]: the T_m values of some intermediates fall outside the range of the two wild types (Table 1). We found a linear positive correlation ($r = 0.67$) between β_T and T_m except for the data for TVS in Table 1. A similar correlation ($r = 0.64$) was previously found between the β_s and T_m values of 14 globular proteins [13]. These results suggest that highly flexible intermediates are tolerant to large thermal fluctuations in the molecular evolution of lysozyme, although the thermodynamic mechanism for this correlation is unclear since the thermal stability of a protein is determined by the change in heat capacity between the native and denatured states.

It is presently unknown how the volume fluctuations are related to the function of these evolutionary lysozymes, because their enzyme activity has not been measured. However, the differences (~16%) in the volume fluctuations among these evolutionary lysozymes would be sufficient to affect the function because volume fluctuations of chicken lysozyme reduce by 3%, 7%, and 14% when it is bound to its inhibitors: monomer, dimer, and trimer of *N*-acetylglucosamine, respectively [42]. In fact, there is a considerable amount of experimental evidence that mutation induces comparable changes in compressibility and enzyme function. Single amino-acid substitutions in aspartate aminotransferase, dihydrofolate reductase, and cyclic AMP receptor protein induce significant changes in β_s, and mutants with larger β_s values exhibit higher enzyme activity [18–20]. Evolutionally designed hyperactive dihydrofolate reductase has a very large β_s value, as predicted from the results obtained for a single mutation [21]. The β_s values of lysozyme and ovalbumin from chicken are larger than those of the respective proteins from turkey, which is more evolved [43]. These previous results together with those of the present study suggest that proteins become less compressible as they evolve. Ancestral proteins might be more flexible and functionally more active than the extant ones in order to allow them to adapt to diverse environments.

It is probably difficult to confirm the above hypothesis on the fluctuation-related stability and function experimentally because series of evolutionary mutants rarely exist in various proteins. However, the present study has demonstrated that normal-mode analysis as well as other types of computer simulation can yield useful information on the compressibility and the role of structural fluctuations in the molecular evolution of proteins when evolutionary intermediates are constructed by site-directed mutagenesis and their tertiary structures are resolved. Further computational and experimental studies on the structural fluctuation of evolutionary intermediates would provide new insight into the structure–function relationship of extant proteins.

4. Conclusions

Normal-mode analysis was applied to calculate the β_T values of chicken lysozyme and its six evolutionary mutants. The evolutionary intermediates were more compressible than the extant lysozymes and fell outside the range of the neutral evolutionary pathway. The observed linear correlation between β_T and V_{cav} indicates that the cavity volume or atomic packing is an important factor regulating volume fluctuations in the molecular evolution of proteins. Computer simulations of compressibility would be useful for studying the role of structural fluctuations in the molecular evolution of proteins.

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