

Structural Basis of Hierarchical Multiple Substates of a Protein. I: Introduction

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ABSTRACT A computer experiment of protein dynamics is carried out, which consists of two steps: (1) A Monte Carlo simulation of thermal fluctuations in the native state of a globular protein, bovine pancreatic trypsin inhibitor; and (2) a simulation of the quick freezing of fluctuating conformations into energy minima by minimization of the energy of a number of conformations sampled in the Monte Carlo simulation. From the analysis of results of the computer experiment is obtained the following picture of protein dynamics: multiple energy minima exist in the native state, and they are distributed in clusters in the conformational space. The dynamics has a hierarchical structure which has at least two levels. In the first level, dynamics is restricted within one of the clusters of minima. In the second, transitions occur among the clusters. Local parts of a protein molecule, side chains and local main chain segments, can take multiple locally stable conformations in the native state. Many minima result from combinations of these multiple local conformations. The hierarchical structure in the dynamics comes from interactions among the local parts. Protein molecules have two types of flexibility, each associated with elastic and plastic deformations, respectively.

Key words: protein conformation, dynamics, Monte Carlo simulation, conformational energy, minimization, spin glass, conformational substates, conformational heterogeneity, hierarchy in dynamics, trypsin inhibitor

INTRODUCTION

Proteins have a thermodynamically stable state, the native state, under physiological conditions, in which they take a well-defined three-dimensional structure as revealed from X-ray crystallography. The structure is, however, not a static but a dynamic one, which is fluctuating around an average conformation. Understanding the biological functions of proteins in molecular terms should therefore be based not only on static but also on dynamic structure. Many efforts have been made both in experi-

mental and theoretical studies on the dynamic structure of proteins.¹⁻³ We are, however, still far from a complete understanding.

It has now become evident that the dynamics of the protein native state have both harmonic and anharmonic aspects depending on the physical quantities by which we observe proteins. The harmonic aspect is the one in which experimentally observed physical quantities can be well reproduced (or predicted) by the normal mode analysis. This analysis is based, at least on the surface, on the assumption that the protein conformation in the thermal equilibrium is fluctuating within a range of conformational space in which the conformational energy surface can be approximated by a multidimensional parabola. In this analysis internal motions of the molecule are represented by a superposition of normal modes, and all of dynamic properties of the molecule can be calculated analytically. The normal mode analysis has been carried out for several proteins, and many of their dynamic properties have been calculated and have been shown to reproduce experimentally observed results well.⁴⁻⁸ However, proteins show at the same time also anharmonic aspects, if different physical quantities are observed. In such aspects proteins appear to exist in the conformational space in which there are many local energy minima.

The rebinding process of CO or O₂ to heme of myoglobin after their photodissociation has been observed to be nonexponential in time at temperatures below 200 K.⁹ This fact has been interpreted as follows. In the native state the molecule has many conformational substates corresponding to different energy minima, and its structure is fluctuating over them at room temperature. When the temperature is lowered below 200 K, each molecule in the sample is frozen into one of the substates. Height of the activation energy of the rebinding reaction in individual substates is different from each other, and is distributed in a certain range. The rebinding process

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of the whole sample, which is a summation of processes exponential in time but with distributed relaxation times, is observed to be nonexponential.⁹

Temperature factors of atoms in myoglobin were measured at several temperatures in X-ray crystallography^{10,11} and Mössbauer spectroscopy.¹² Magnitude of contribution from lattice disorder to temperature factors in X-ray crystallography was estimated by comparing them with temperature factors in Mössbauer spectroscopy, because the latter contains contributions only from dynamic fluctuations. From temperature dependence of mean square atomic displacements calculated from the X-ray temperature factors after removing this contribution, mean square atomic displacements at absolute zero temperature were estimated and found nonvanishing. This fact also suggests the existence of conformational substates, in one of which each molecule in the crystal is frozen at low temperatures. The conformational heterogeneity of these substates explains the nonvanishing atomic displacements.^{11,12}

In recent studies of high-resolution X-ray crystallography, refinement of crystallographic models has been carried out by allowing the possibility of multiple conformations of side chains or local main chain segments.¹³⁻¹⁵ Multiple local conformations have been actually revealed in several side chains and a main chain segment in these studies. These observations provide direct evidences of conformational substates.

Existence of conformational substates has been shown also from theoretical studies. Imagine that conformation of a protein is fluctuating at room temperature within such a region of conformational space that contains many local minima. If the temperature is lowered very quickly down to 0 K, the conformation will be trapped in one of the many local minima. This hypothetical process has been simulated by computer experiments. Thermal fluctuation of a protein at room temperature has been simulated either by molecular dynamics or by Monte Carlo methods. By starting from a number of conformations in the record of such simulations, the conformational energy of the molecule was minimized. Multiple minima were obtained, indicating the existence of conformational substates.¹⁶⁻¹⁸ The concept of conformational substates indicates experimentally observable distinct conformations with significant populations either at room or low temperatures. Minimum energy conformations are those which have locally minimum conformational energy. There may be cases in which minimum energy conformations obtained by energy minimization from thermally excited conformations are not significantly populated even in low temperatures. Except for these supposedly rare cases, minimum energy conformations correspond to conformational substates.

Now evidence for conformational substates is

strong both experimentally and theoretically. Natural questions to follow are: How are minima distributed in the conformational space? How do conformations corresponding to different minima differ? How are thermally excited conformations related to minimum energy conformations? These questions are clearly pertinent to the elucidation of the nature of protein dynamics in the native state. This and the following papers are devoted to these questions.

We have carried out a simulation of the thermal fluctuations in a protein, bovine pancreatic trypsin inhibitor (BPTI), by a Monte Carlo method,¹⁹ and that of quick freezing by the method of energy minimization. Then, multiple energy minima have been actually found. We have analyzed conformations of these minima. From the analysis has emerged a new detailed picture of thermally fluctuating protein conformation, which accounts also for the experimentally observed anharmonic aspects of protein dynamics. In this series of papers²⁰⁻²³ we describe methods of the computer simulation and of the analyses of the results thereof, report results of the analyses, and discuss the results in terms of a new picture of fluctuating protein conformation. This first paper is devoted to a brief description of the methods and the obtained new picture of fluctuating protein conformation. Detailed descriptions of the methods and results will be given in the following papers.

COMPUTER EXPERIMENT

The computer experiment we carried out here consists of two steps. The first step is the simulation of thermally fluctuating conformations of the molecule, BPTI, at temperature 300 K. The Monte Carlo simulation¹⁹ was carried out for 5×10^5 steps. The record generated by this simulation contains a sample of conformational fluctuations that would occur during a time interval in the range of 2.5 ~ 25 nsec. The molecule was treated as isolated *in vacuo*. Limitations of the analyses in this paper due to this treatment of the molecule in the artificial environment will be discussed later in this paper.

The second step is the simulation of quick freezing of the molecule into one of energy minima. One of the sampled thermally fluctuating conformations is chosen, which we call an instantaneous conformation. The molecule in the instantaneous conformation has thermally excited conformational energy. If the molecule in the instantaneous conformation is frozen quickly down to the absolute zero temperature, the conformational energy will become one of minimum values. This process can be simulated by minimization of the conformational energy of the molecule by starting from the instantaneous conformation. We choose a number of conformations from the sampled thermally fluctuating conformations, and carried out the energy minimization. The Newton's method has been used in the minimization. We

actually obtained a number of different minima. Details of this computer simulation will be described in the next paper in this series.²⁰

ANALYSES OF STRUCTURES OF ENERGY MINIMA

We focus our attention on differences of minimum energy conformations (MECs) obtained in the simulation.

At first we quantify a conformational difference between a pair of minima in terms of mean-square deviations of C α atoms. This measure defines a distance in the conformational space. Distribution of the minima in this metric space is studied. Details of this study is described in the second paper in this series.²⁰

In the next step we study details of conformational differences among minima. MECs are analyzed from the following points of view:

1. Differences in side chain conformations and in main chain local conformations.
2. Differences in atomic arrangements in interfaces between residues which are in contact with each other in the folded structure.
3. Relative displacements between centers of gravity of individual residues.

The first and second points focus attention on local conformations of the molecule. The third aims to detect nonlocal changes in the whole molecule in conformational changes among minima.

Are conformations of a pair of energy minima different in most parts of the molecule, or mainly in some parts of it? Localization of conformational differences and heterogeneity of local conformations are studied by analyzing conformations of energy minima from the point of view of (1). Complete descriptions of this study are given in the third paper in this series.²¹

What is the origin of an energy barrier between a pair of MECs? In the native state of a protein molecule atoms are packed densely. It is naturally expected that each local minimum is a minimum point within an area in the conformational space where conformations have the same topology of atom packing. Here we define a topology of atom packing by a set of atom pairs which are covalently nonbonded but in contact in space in the three-dimensional structure. The topology of atom packing is expected to vary from one to another minimum. Such changes will be observed in interfaces between residues in contact in the folded structure. These are questions addressed by the analysis from the point of view of (2). Study of this part is presented in the forth paper in this series.²²

How do changes in local conformations affect the whole structure of the molecule? This is studied by the analysis from the point of view of (3). In the fifth

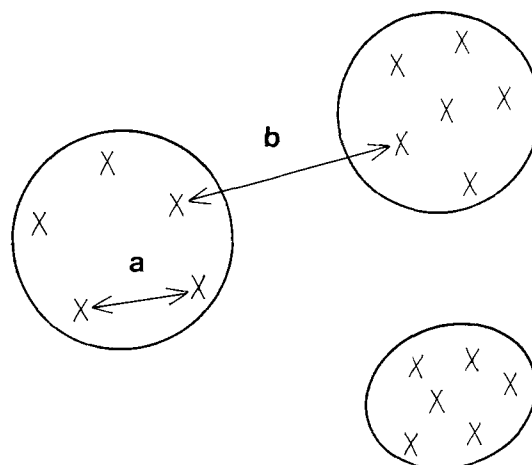


Fig. 1. A schematic illustration of the distribution of energy minima in the conformational space. X, an energy minimum; each of the clusters of minima is encircled. a: A transition between minima within one cluster. b: A transition between clusters.

paper in this series this study is described in detail.²³

PICTURE OF THERMAL FLUCTUATIONS

The following somewhat idealized picture of thermal fluctuations of the protein molecule has emerged from the analyses of the results of the computer experiment. The raw results are sometimes a bit more complicated than in the following picture, which is presented here as an essence of the results.

Multiple Energy Minima Exist in the Native States and They Are Distributed in Clusters in the Conformational Space

The conformational energy surface has many thermally accessible minima in the region of the native state. These minima are distributed in clusters in the conformational space. Dynamics in the time range between 10^{-14} and 10^{-10} seconds can be described as a superposition of vibrations within each energy minimum and transitions among minima within each cluster. In the time range of 10^{-9} seconds or longer dynamics is characterized by transitions between clusters of minima (Fig. 1). The protein dynamics has such a hierarchical structure consisting of at least two levels. We will call the fluctuations occurring in the shorter and longer time ranges those in the first and second levels, respectively. The hierarchical dynamics in the two levels is thus observed in the simulation covering the time range of 2.5 ~ 25 nsec. The number of levels may increase with the time range of dynamics. Elucidation of dynamic structure in the longer time ranges remains to be done in the future.

Local Parts of a Protein Molecule Can Take Multiple Conformations in the Native State, and Many Minima Result from Combination of These Multiple Local Conformations

A few multiple stable local conformations exist in most of side chains and local segments of a main chain of the molecule. By quick reduction of temperature each of the parts is frozen into one of its multiple conformations. Minimum energy conformations can thus be described in terms of combinations of these multiple local conformations. Though interaction among the parts will restrict the possible combinations, a large number of energy minima exist in the native state. These conclusions are drawn from the observation that, when we compare conformations of a pair of energy minima differences between them occur as combinations of locally different conformations in several side chains and a few local main chain segments.

The Hierarchical Dynamic Structure Originates from Interactions among Side Chains and Local Main Chain Segments Which Have Multiple Conformations

Atoms are packed densely in a protein molecule. Multiple conformations of each part are stabilized in respective atom packing. A transition of a side chain from one to another of its multiple conformations changes the shape of an interface between it and a surrounding side chain. We call a transition of a side chain or a local segment of a main chain among its multiple conformations *switching* of the local conformation. Also we say that such a part has a *switching degree of freedom*. If the surrounding side chain also has multiple conformations and atomic interactions are unfavorable in the newly changed interface, switching may again occur simultaneously in such surrounding side chains so as to realize a suitable interface. In other words a switching degree of freedom may interact very strongly with neighboring switching degrees of freedom. Local conformational switching of one part may entail by such a mechanism some neighboring parts to switch their local conformations. In other words *collective switching* occurs within a certain region. Near the surface of the molecule it propagates over a region consisting of less than a few residues in most of cases. Atom packing in interfaces among parts within a region and in interfaces between parts in the region and the remaining part of the molecule is rearranged. The remaining part of the molecule is deformed slightly without rearrangement of atom packing within itself (Fig. 2a). Magnitude of deformation in such remaining part of the molecule is within the range of thermal vibrations around a single energy minimum. Such collective conformational switching discussed here occurs in various regions near the surface. Various combinations of collectively

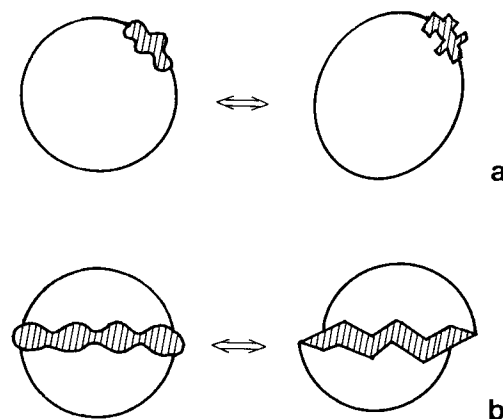


Fig. 2. Schematic illustrations of collective conformational switching and subsequent deformation of the shape of the whole molecule. The collective conformational switching occurs in the shaded parts. **a:** The collective switching occurs in a region consisting of a few residues near the surface of the molecule, and slight and elastic deformation is observed in the remaining part of the molecule. **b:** The collective switching occurs in an extended region involving the core of the molecule, and the remaining two parts of the molecule move relatively to each other.

switched conformations in such regions bring forth a number of minima, which belong to the same cluster.

When local conformational switching occurs in the core of the molecule, the switching may propagate across the molecule. In such an event switching occurs collectively in a wide region consisting of more than 10 residues. The topology of atom packing changes within such a region and in interfaces between the region and the remaining parts. Remaining parts are deformed slightly without rearrangement of atom packing within itself. In one particular case observed in the simulation carried out in this study a region of collective conformational switching extends across the whole molecule in such a way that it divides the remaining part into two (Fig. 2b). In this case the relative location and orientation between the two divided parts changed very much due to the extended collective conformational switching in the central region. The shape of the whole molecule subsequently deformed much. Its magnitude is beyond the range of thermal vibrations around a single minimum. Conformational changes of this type correspond to transitions from one minima in one cluster to another in another cluster.

Thus, conformational changes both in the first and in the second levels occur as a result of interactions between switching degrees of freedom. In the first level, collective switching occurs only in scattered surface regions. In the second level, collective switching of a large scale, going across the molecule, occurs.

As mentioned in the first point a transition between minima within the same cluster occurs in the time range of less than 10^{-10} seconds, and that be-

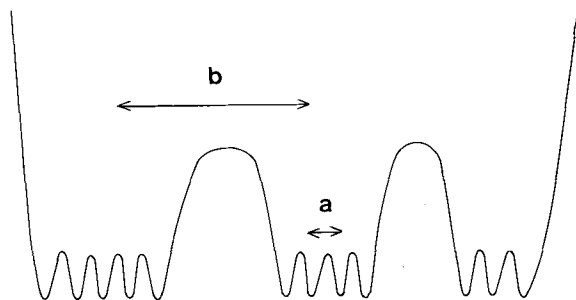


Fig. 3. A schematic illustration of the structure of energy surface and the hierarchical structure of the protein dynamics. **a**: A transition among minima within one of clusters of them, which is observed in fluctuations of the first level. **b**: A transition between the clusters of minima.

tween cluster of minima occurs in the range of 10^{-9} seconds or longer. Therefore, the activation energy of the latter transition should be higher than that of the former. It is naturally expected that collective conformational switching in a few residues near the surface of the molecule occurs more easily than those involving more than 10 residues in the core of the molecule. Thus, the conformational energy surface may look like Figure 3.

DISCUSSION

Two Types of Flexibility in a Protein Molecule

Implicit in the description of the above point (3) is that conformational changes involving switching degrees of freedom are active causes and changes in the remaining parts, in which topology of atom packing is maintained, are passive results. These two types of changes correspond to two types of dynamic flexibility in the protein. This point will be discussed here.

Let us first define *elastic deformation* in a protein as a conformational change involving no change of topology of atom packing and similarly *plastic deformation* as that involving a change of topology. We will use these terms also for changes in a part of the protein molecule with an obvious meaning. Use of these terms is reasonable, if we imagine placing the protein molecule in absolute zero temperature. In the case of an elastic deformation, the molecule recovers its original conformation when some applied external force is removed. When the molecule is deformed plastically, it keeps the deformed conformation even after the removal of the external force.

In an elastic deformation displacement vectors of atoms close in space are similar to each other. Relative displacements among them are small. In other words, in elastic deformations changes in each small region is small. However, they may accumulate to cause large deformations of the whole molecule as in a well-known example of very-low-frequency normal modes.⁴

A plastic deformation localized in a small region is associated with large relative atomic displacement vectors. Therefore deformation in the small region is large. Concerning deformations of local parts plastic deformations are more flexible than elastic ones.

As mentioned in point (3) in the preceding section plastic deformation in conformational changes in the first level is restricted within scattered small surface regions, and the remaining part is deformed elastically. In this case the global deformation of the whole shape of the molecule is determined mainly by the elastic properties (Fig. 2a). On the other hand it is determined mainly by the plastic flexibility in conformational changes in the second level (Fig. 2b).

Is Simulation *In Vacuo* Meaningful

We treat the protein molecule as isolated *in vacuo*, considering solvent effects partially by using the dielectric constant, 4.0, in electrostatic interactions. The average of the simulated conformation will deviate from that in solution. Its root-mean-square deviation of C^α atoms from those in the X-ray structure is about 1 Å. Though more work is clearly necessary to elucidate effects of solvent on protein dynamics, the simulated conformational fluctuations around the shifted structure in the artificial environment are expected to have many relevant characteristics of thermal fluctuations in the real protein molecule. Therefore, we think that the picture of the protein dynamics obtained in this study, especially as to the structural basis of the hierarchical multiple substates, is mostly true in the dynamics in solution. We should, however, discuss the following point. For structures of the surface of the protein molecule, hydrogen bonds with water molecules and atom packing of them are essential. Multiple conformations of the local parts of the protein surface should be determined with those of water molecules around them. Though the conformations of surface side chains or local main chain segments in the energy minima studied in our work may, in some instances, not be very realistic, the multiplicity of them will truly exist even in solution.

The glass temperature of the protein observed experimentally in low temperatures, i.e., the temperature above which protein conformations are released from trapped local energy minima, is found slaved by the viscosity of solvent, i.e., the solvent determines the glass temperature.²⁴ Dynamics of the local conformations of the protein surface in the room temperature must also be influenced by that of the water molecules. A recent molecular dynamics study of water revealed that change in atom packing involving change in hydrogen-bond network occurs in the time range of 10^{-12} to 10^{-11} seconds.²⁵ In our picture, collective conformational switching in small surface regions occurs in fluctuations in the first level. They occur in the time range of 10^{-14} to 10^{-10} seconds. Because the dynamics of water takes place

roughly in the same time range, it is unlikely that the characteristic time of the fluctuations in the first level changes drastically in orders by the presence of the water. For a complete understanding of these problems, a simulation of dynamics of a system consisting of the protein and water molecules should be carried out.

Comparison with Experimental Studies

Parak et al. analyzed the Mössbauer spectrum of the iron atom in myoglobin and reported that motion of the atom is characterized by that in three time ranges of shorter than 10^{-9} seconds, 3×10^{-9} seconds, and 10^{-7} seconds or longer at room temperature.^{12,26,27} The iron atom is in the interior of the molecule. The motion in the second or third ranges may occur by the same mechanism as that of fluctuations of the second level in the picture of protein dynamics proposed here.

Frauenfelder and co-workers demonstrated a hierarchical structure of protein dynamics from their intensive studies of the relaxation process from carbon monoxide myoglobin to deoxymyoglobin.²⁸ They classified fluctuations over conformational substates by heights of activation energy barriers. More work, however, is necessary to discuss the relation between their experimental and our simulation results.

Analogy with Spin Glass

Stein proposed a spin glass model for the protein conformational substates.²⁹ In that model each residue is assumed to have two local metastable conformations in the native state and the protein conformational substates are described in terms of combinations of the two local metastable states of each residue. Each residue is represented by a spin which has two states, (-1) and $(+1)$ corresponding to the two local metastable states. The conformational energy of the protein is described in the same way as in the spin glass hamiltonian. Complicated interactions between a pair of residues, which are likely to cause energetic frustrations in the protein conformational substates, are represented by the random interactions between a pair of spins. In the present study we have found that each of most of the side chains and local main chain segments has more than two multiple metastable conformations. The spin glass model, adopted by Stein, is clearly an oversimplified one. However, the existence of a large number of energy minima and the hierarchical structure in the distribution of them, which were observed in the protein, are observed also in spin glasses and glasses. We think studies of these phenomena common to proteins and spin glasses will provide more insights into protein dynamics.

It should be noted that the native state of a protein molecule is not in the spin glass phase at the room temperature. The molecule is frozen into one of

energy minima and bounded there at low temperatures, but transitions among minima is thermally excited at the room temperature. In this respect, the native state of the protein at room temperature is clearly not solid-like, but rather, fluid-like. However, it does not flow. It is a new state, not yet clearly characterized.

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