Theory of cooperative transitions in protein molecules. II. Phase diagram for protein molecule in solution

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Theory of Cooperative Transitions in Protein Molecules. I. Why Denaturation of Globular Protein is a First-Order Phase Transition

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Synopsis

A theory of equilibrium denaturation of proteins is suggested. According to this theory, a cornerstone of protein denaturation is disruption of tight packing of side chains in protein core. Investigation of this disruption is the object of this paper. It is shown that this disruption is an "all-or-none" transition (independent of how compact is the denatured state of a protein and independent of the protein–solvent interactions) because expansion of a globule must exceed some threshold to release rotational isomerization of side chains. Smaller expansion cannot produce entropy compensation of nonbonded energy loss; this is the origin of a free-energy barrier (transition state) between the native and denatured states. The density of the transition state is so high that the solvent cannot penetrate into protein in this state. The results obtained in this paper make it possible to present in the following paper a general phase diagram of protein molecule in solution.

INTRODUCTION

Denaturation and renaturation of protein globules have been intensively studied for many years (see Refs. 1–7 and references therein). The interest in these phenomena is due to their universality and to their importance for understanding the mystery of the protein self-organization.

It is now known that denaturation of small (one-domain) proteins is a cooperative "all-or-none" transition from the native to denatured state[5] with a drastic alteration of many (although sometimes not all—see Refs. 2, 4, and 6) features of the molecule. This transition is accompanied by a large heat effect (about 0.5–1 kcal/mole of residues).

The most astonishing fact for a physicist is that denaturation of a native protein, this irregular heterogeneous system where every atom has its unique position in space, is a regular first-order phase transition. One would rather think that the breakdown of such a complex structure might result from gradual destruction of the "fusible" regions (as in DNA[8]).

The question why denaturation is an all-or-none transition seems even more puzzling if we recollect that protein is a heteropolymer: very often heterogeneity smoothes and destroys phase transitions.[9]

Theories of homopolymers[10] and statistical heteropolymers[11,12] show that unfolding of globular chains is usually rather smooth: coil arises after a pronounced swelling of globule.

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Thus one could expect that the globule–coil transition would differ drastically from melting or sublimation of a solid. But in fact native proteins are solid "aperiodical crystals" and their denaturation resembles just a disruption of solids.

A sharp transition resembling all-or-none unfolding has been observed in computer experiments using a "protein chain" with a unique set of nonbonded interactions.[13,14] In this model, which neglects motion of side chains, denaturation leads unavoidably to a large-scale rearrangement of backbone conformation.

We suggest an alternative treatment of denaturation that takes into account liberation of side chains in denatured protein. We show that in this case disruption of tight packing is always an all-or-none transition independent of the extent of rearrangement of the backbone. This resembles disruption of crystals, which may lead to liquid as well as to vapor.

MODEL OF PROTEIN GLOBULE

A reasonable model of the protein globule (domain) can be imagined as a stack of a few large rigid blocks (α-helices and β-sheets) covered by a large number of side chains [Fig. 1(a)]. These blocks are fixed by hydrogen bonds and they are stable at least while the globule is dense enough and water does not penetrate inside it.

A hydrophobic core is surrounded by these segments. The core is tightly packed in native protein, and side chains that form this core are fixed[15] (Fig. 2).

We shall concentrate on the disruption of tight packing, which provides us with the possibility of neglecting (1) the rearrangement of the backbone and (2) the water environment. The first point is considered in more detail in the discussion section. The solvent effects are crucial for the state of denatured protein only; the forthcoming paper[16] is devoted to this problem.

Thus we now consider a protein globule in vacuum with a fixed backbone fold.

The interactions of each group depend on the chemical nature of the neighbor (in space) residues and on their positions. If the neighbors remain the same, the local density determines both the energy of the interactions and the entropy of the movements of a residue.

The mutual positions of residues and the local densities are strongly correlated by rigid backbone: the core residues belong to stiff blocks (such as α-helices and β-sheets) that stretch from one side of the globule to the other. Thus, displacement of a segment governs a simultaneous change of many side-chain interactions.

We shall show (and this is the main point of this paper) that there exists some region of densities ("barrier") at which protein globule is absolutely unstable. To this end it is enough to consider the uniform displacement of the structural segments, taking the volume of globule as the only essential macroscopic parameter that determines stability (or instability).

To estimate the stability, the "mean-field" ("molecular field") approximation is used. This method is common to the theories of liquids, crystals, solid solutions, polymeric globules, etc. Under this approximation all the compli-

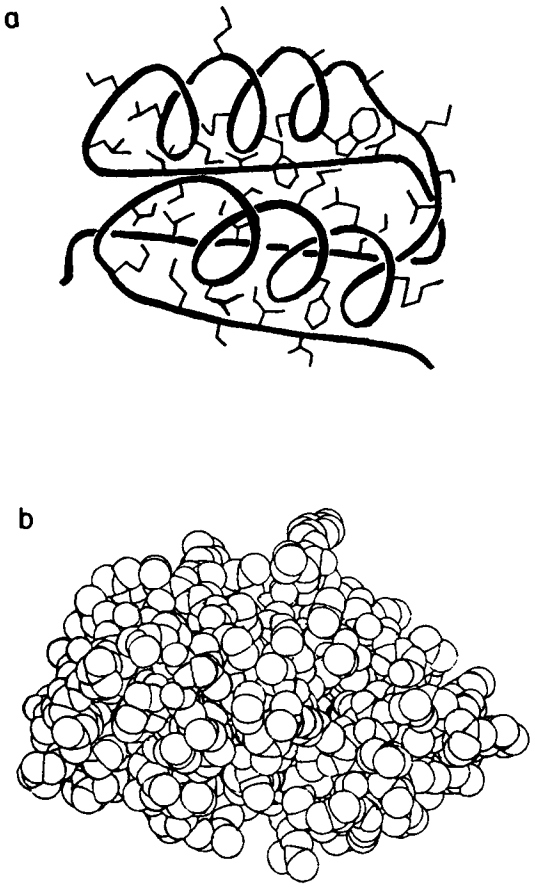

Fig. 1. Protein globule: The scheme (a) shows the backbone (solid line) forming secondary structure segments (here: two α-helices and a β-sheet of three strands) connected by loops; the backbone is covered by the numerous side chains. The space-filling model (b) shows the compactness and tight packing of a protein globule.

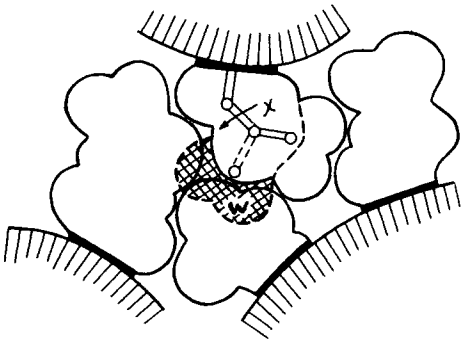

Fig. 2. Scheme of side-chain packing. Only a small part of the core is shown, including several side chains attached to the structural segments. The shaded region ω stands for the alternative rotamer of the central group (χ is the torsional angle). This rotamer is forbidden by the tight packing.

cated multiparticle interactions are replaced by the sum of the single-particle ones: each particle moves in a "field" produced by other particles and determined by the macroscopic state of the total system (e.g., by the volume, crystal lattice type, etc.).

It is well known (see, e.g., Ref. 17) that a molecular field approximation is always valid except for the vicinity of a critical (a second-order phase transition) point. No critical events are observed in thermodynamical experiments with proteins[5]; we shall not encounter such events in this theory either.

It is necessary to stress here the peculiarity of protein (as compared with such "simple" substances as liquids, ordered crystals, etc.): in the native protein each atom constantly has its own (in other words, unique) surrounding. This makes the molecular field tremendously heterogeneous: each residue is under the action of its "own" force field. It bears similarity with spin glasses where the molecular field approximation is quite well justified.[18,19] However, the problem of disruption of tight packing is even simplier as the *change* of all these fields is a function of a single macroscopic parameter (volume of the globule).

We should like to stress that the mean field approximation does not assume that one in the same force field acts at all particles. It assumes only that the force field acting at a given particle from the side of other ones is nearly constant in time and thus it may be represented as some "mean" field.

INTERACTIONS IN THE PROTEIN MOLECULE

The main contributions to the Helmholtz free energy F in our model are the following:

1. Free energy of intramolecular hydrogen bonds, F_H, which is large but constant if secondary structure is preserved, as it is assumed above.

2. Energy of nonbonded interactions; the most important for us is the energy $\tilde{E}_w$ of the van der Waals interactions in the core of the globule. This is the interaction of side chains with one another and with structural segments. (Here and below the tilde denotes that the value corresponds to the hydrophobic core.)

3. Free energy of torsional motions, $\tilde{F}_t$, which is determined first by the entropy of side-chain movements and then by the entropy of backbone motions in loops.

The nonbonded energy $\tilde{E}_w$ goes up in the usual way (see $\tilde{E}_w$ in Fig. 3) if the density of the globule increases.

Torsional movements become pronounced if the globule expands. First they go up in the usual way ($\tilde{F}_{ar}$ and $\tilde{F}_{al}$ drop down in Fig. 3); then, however, the amplitudes of aliphatic side chains remain nearly constant, restricted by the torsional potential (see Fig. 4 below) and then, again, these amplitudes increase (see $\tilde{F}_{al}$ in Fig. 3) when expansion exceeds such a threshold that structural restrictions on rotational isomerizations disappear.

As a result, there exists a range of densities where entropy of movements is still low while energy of nonbonded interactions is yet high. Thus there exists the free-energy barrier corresponding to such densities and segregating tightly packed (native) and all kinds of friable (denatured) states of a protein. It should be emphasized that this barrier is due to the peculiar behavior of

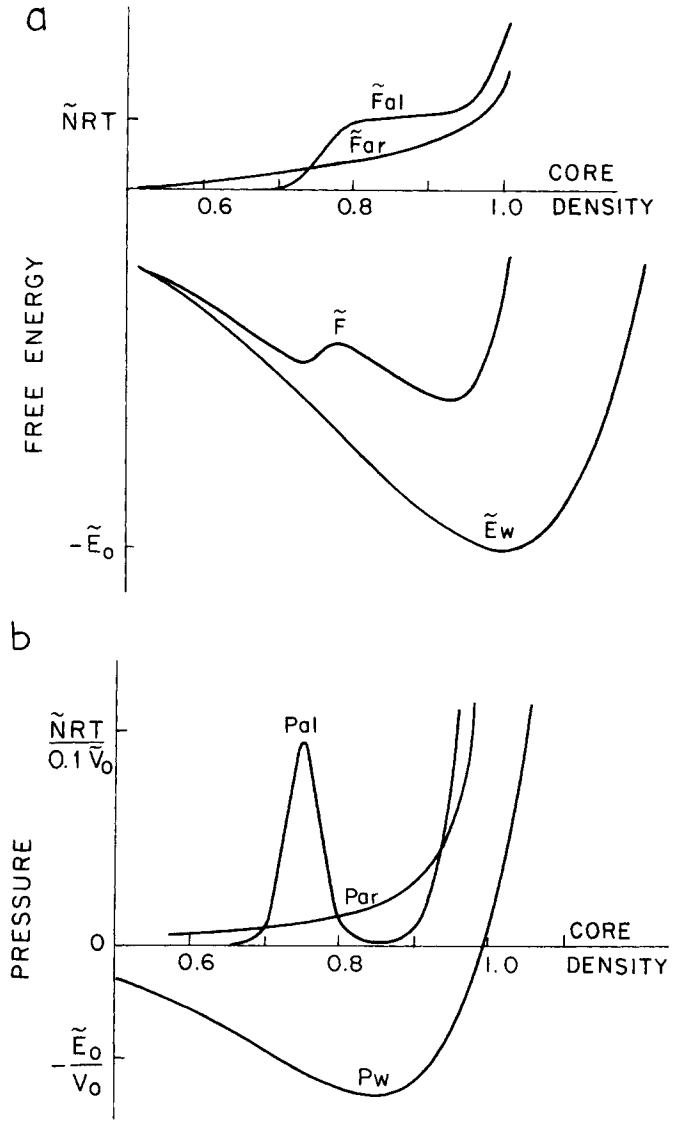

Fig. 3. The plot of free energy (a) and the main components of the internal pressure (b) vs the relative density $\tilde{V}_0/\tilde{V}$ of the hydrophobic core. The total free energy $\tilde{F}$ consists of the van der Waals energy $\tilde{E}_w$ and the free energy of torsional motions ($\tilde{F}_{al}$ for the aliphatic and $\tilde{F}_{ar}$ for the aromatic degrees of freedom). For the evaluation we have assumed that $\tilde{N} = 50$ side chains of the core have $\tilde{M}_{al} = 80$ aliphatic and $\tilde{M}_{ar} = 20$ aromatic degrees of freedom; for the other parameters, see the text.

torsional motions, which are restricted (for aliphatic side chains) by two factors: steric hindrances and torsional potentials.

The reader who is not interested in theoretical details and numerical estimations may skip the remaining part of this section and all the next one.

Energy of van der Waals Interactions

The energy of van der Waals interactions can be taken in the usual form,

$$\tilde{E}_w = \frac{1}{2}\sum_{i\neq j}\left[\frac{A_{i,j}}{(r_{i,j})^{12}} - 2\frac{B_{i,j}}{(r_{i,j})^6}\right] \tag{1}$$

where $A_{i,j}$ and $B_{i,j}$ are the Lennard–Jones parameters for interaction of atoms i and j, and $r_{i,j}$ is the distance between these atoms. As the intrasegment interactions are not practically changed in the course of globule expansion, the summing [Eq. (1)] has to take into account only atoms from the residues that belong to different segments.

If expansion is uniform, the change of distances between such residues is proportional to the change of distances between segments, i.e., to the change of the core diameter, $\tilde{V}^{1/3}$. In a rough approximation, it can be assumed that the interatomic distances change in the same way:

$$r_{i,j} = r^0_{i,j}\tilde{V}^{1/3} \tag{2}$$

From Eqs. (1) and (2) it follows that

$$\tilde{E}_w(\tilde{V}) = \tilde{E}_0(q^{12} - 2q^6) \tag{3}$$

where

$$q = (\tilde{V}_0/\tilde{V})^{1/3} \tag{4}$$

while

$$\tilde{E}_0 = \frac{1}{2\tilde{V}_0^2}\sum_{i\neq j}\frac{B_{i,j}}{(r^0_{i,j})^6} \tag{5}$$

$$\tilde{V}_0 = \left[\sum_{i\neq j}\left[\frac{A_{i,j}}{(r^0_{i,j})^{12}}\right] \Big/ \sum_{i\neq j}\left[\frac{B_{i,j}}{(r^0_{i,j})^6}\right]\right]^{1/2}$$

stand for minimal van der Waals energy and the core volume corresponding to this minimum.

The values $\tilde{V}_0$ and $\tilde{E}_0$ can be easily estimated because a native protein is as tightly packed as a molecular crystal.[15] This means that the "optimum" for the van der Waals energy volume $\tilde{V}_0$ of the protein core is close to the sum of the side-chain volumes: $V_{cryst} = \Sigma v_i^{cryst}$. An "average" protein domain (of $N \simeq 100$ residues) contains $\tilde{N} \simeq 50$ side chains in a core; $\tilde{N}$ includes both hydrophobic groups and nonpolar parts of hydrophilic ones. The average

molecular weight of a side chain is about 50 daltons and its average volume is about 100 Å^3. So $\tilde{V}_0 = \tilde{N} \cdot 100$ Å^3 while the total volume of a native globule $V_N = N \cdot 150$ Å^3. If we assume that just one half of the side-chain interactions corresponds to the intersegmental ones, $\tilde{E}_0 = \tilde{E}_w(\tilde{V}_0) = 4 \cdot \tilde{N}$ kcal/mol (see, e.g., Ref. 20: sublimation of a 50-dalton molecules "costs" about 7–8 kcal/mol).

It is worthwhile to note that the greatest contribution to energy is given by the nearest neighbor atoms. The distance between such atoms is about $(\tilde{V}/\tilde{N})^{1/3} - d$, provided the distance between the centers of neighbor side chains is $(\tilde{V}/\tilde{N})^{1/3}$ and $d \simeq 2$Å is the average atom-to-atom distance within the side chain. The improved value

$$q = \left[(\tilde{V}_0/\tilde{N})^{1/3} - d\right] \Big/ \left[(\tilde{V}/\tilde{N})^{1/3} - d\right] \tag{4a}$$

introduced into Eq. (3), gives a more precise value of $\tilde{E}_w(\tilde{V})$.

Figure 3 shows the van der Waals energy E_w, and the corresponding pressure

$$P_w(\tilde{V}) = -\frac{dE_w}{d\tilde{V}} = \frac{4\tilde{E}_0}{\tilde{V}_0}(q^{12} - q^6) \Big/ \left[1 - d(\tilde{N}/\tilde{V})^{1/3}\right] \tag{6}$$

as functions of the relative hydrophobic core density $\tilde{V}_0/\tilde{V}$.

The important parameter $\tilde{E}_0/\tilde{V}_0 \simeq 0.04$ kcal/(mol Å^3) $\simeq 3$ kbar determines the density of the van der Waals energy, i.e., the characteristic pressure of the van der Waals forces; $\tilde{E}_0/\tilde{V}_0$ is somewhat higher for a core rich in aromatic (more "dense") groups than for a core rich in aliphatic ones.

Torsional Motions

The main effect of decay of a "solid" protein structure is the liberation of motion of the numerous side chains involved in the hydrophobic core.

Each side-chain movement has its own torsional potential $U_i(\chi_i)$. The most typical are two types of torsional potentials (cf. Ref. 21). The first are for "aliphatic" degrees of freedom (i.e., the angle χ_i corresponds to rotation around the $-CH_2-CH_2-$ covalent bond); the high ($U_0 \simeq 3$ kcal/mol $\gg RT$; R is the gas constant and T is the absolute temperature; at $T \simeq 350$ K, $RT = 0.7$ kcal/mol) torsional barriers (Fig. 4) hinder these rotations. The second potentials have low ($< RT$) torsional barriers and correspond to the rotation of aromatic rings. Therefore we may take

$$U_{al}(\chi) = \tfrac{1}{2}U_0(1 + \cos 3\chi)$$

for aliphatic and

$$U_{ar}(\chi) = 0$$

for aromatic degrees of freedom.

Besides, each rotation is limited in some range,

$$\chi_i^0 < \chi_i < \chi_i^1$$

by collision of the side chain with its own backbone.

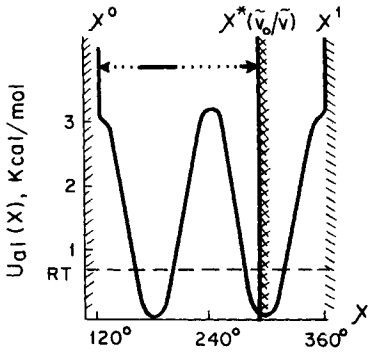

Fig. 4. The internal torsional potential U as a function of the torsional angle χ for aliphatic degrees of freedom. The shaded regions are forbidden due to collision of the side chain with its own backbone. The sterically allowed (at a given $\tilde{V}_0/\tilde{V}$) region of χ is shown by the arrow; the dotted parts of the arrow correspond to the regions that are practically forbidden by the high torsional potential.

And, most important, side-chain rotations are restricted by collisions with neighbor segments up to total fixation in the tightly packed core (Fig. 2). We may assume that each rotation is restricted, from one side, by collision with its own backbone, and from the other side, by collision with the neighbors:

$$\chi_i^0 < \chi_i < \chi_i^*\left(\tilde{V}_0/\tilde{V}\right)$$

Here, according to the accepted "molecular field" approximation, the amplitude of each motion depends on a single macroscopic parameter, the average core density $\tilde{V}_0/\tilde{V}$. Thus the total free energy of the $\tilde{M} = 2\tilde{N}$ motions of the $\tilde{N}$ side chain is

$$\tilde{F}_t^s(\tilde{V}) = \sum_{i=1}^{\tilde{M}} F_i^t\left(\tilde{V}_0/\tilde{V}\right) = -RT\sum_{i=1}^{\tilde{M}} \ln\frac{z_i(V_0/V)}{z_i^0} \tag{7}$$

where

$$z_i^0 = \int_{\chi_i^0}^{\chi_i^1} \exp\left[-U_i(\chi)/RT\right] d\chi \tag{8}$$

is the partition function of "free" motion and

$$z_i\left(\tilde{V}_0/\tilde{V}\right) = \int_{\chi_i^0}^{\chi_i^*\left(\tilde{V}_0/\tilde{V}\right)} \exp\left[-U_i(\chi)/RT\right] d\chi \tag{9}$$

corresponds to the motion restricted by collisions of the side chain with neighbor segments.

We can estimate $\chi_i^*(\tilde{V}_0/\tilde{V})$ by taking into account that free rotation (i.e., rotation at about 180°) needs a free volume of about ω_i, where ω_i is the

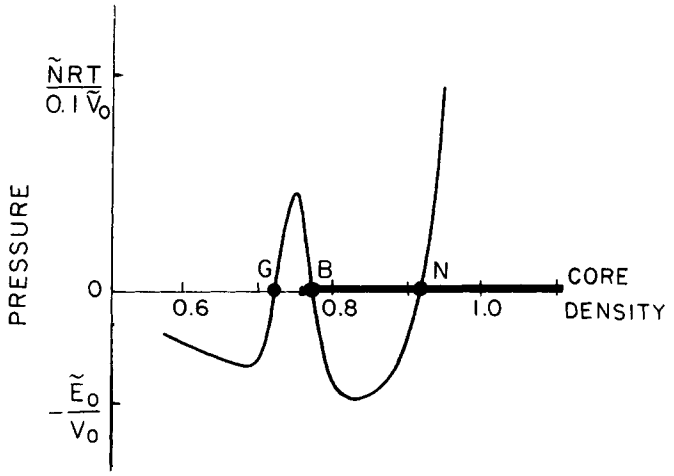

Fig. 5. The internal pressure in the protein globule in vacuum. The spots stand for the roots of the equation of state (18) at $P_{out} = 0$. They correspond to two stable states, the native (N) and denatured (G) ones, and to the unstable transition ("barrier") state B. The solid part of the abscissa axis marks the densities at which water molecules cannot penetrate the protein. In this region the vacuum model is also valid for proteins in solution.

volume of the asymmetric part of the side chain (see Fig. 2). Thus

$$\chi_i^*(\tilde{V}_0/\tilde{V}) - \chi_i^*(1) = \frac{\pi}{\omega_i}\left(\frac{\tilde{V} - \tilde{V}_0}{\tilde{M}}\right) \tag{10}$$

The volume ω_i can be evaluated as $\omega_{al} = 30$ Å^3 for aliphatic degrees of freedom (the volume of the $-CH_3-$ group) and $\omega_{ar} = 50$ Å^3 (the volume of $-CH=CH-$) for the aromatic ones.

The region $\chi_i^0 < \chi_i < \chi_i^*(1)$ stands for vibration in the most dense protein. It seems that $\chi_i^*(1)$ is close to χ_i^0 or, better expressed, to the minimum of the torsional potential which is the nearest to χ_i^0 (Fig. 5) because side chains in native proteins are fixed, as a rule, in the vicinity of such minima.[22]

Figures 3 and 4 represent the free energy of side-chain torsional motions and the corresponding "entropic" pressure,

$$P_{ar,al}(V) = -dF_{ar,al}/dV \tag{11}$$

as the functions of hydrophobic core density, $\tilde{V}_0/\tilde{V}$.

The part of the pressure due to $\tilde{M}_{ar}$ barrier-free rotations (i.e., vibrations) of aromatic groups is represented very simply:

$$P_{ar} = RT\sum_i^{\tilde{M}_{ar}} \frac{1}{\tilde{V} - \tilde{V}_0 + \tilde{M}\omega_i(\chi_i^*(1) - \chi_i^0)/\pi} \tag{12}$$

To calculate pressure precisely, one must take into account all the amplitudes, $\chi_i^*(1) - \chi_i^0$, in the most dense protein. According to Ref. 23, the range of amplitudes of side-chain vibrations in the hydrophobic core is $\simeq 0.2$ Å, i.e.,

small enough, and we may neglect them if

$$\tilde{V} - \tilde{V}_0 \gg \frac{\chi_i^*(1) - \chi_i^0}{\pi} \tilde{M}\omega_i \tag{13}$$

i.e., if $\tilde{V} - \tilde{V}_0 > 0.1\tilde{V}_0$.

This approximation improves when the protein core is less dense. This is true both for aromatic and aliphatic groups.

One can see (Fig. 3) that all the peculiarities are connected with the torsional pressure of aliphatic side chains. These peculiarities are due to the pronounced torsional barrier that separates their rotational isomers. The volume at which $\chi_{al}^* - \chi_{al}^0 = 2\pi/3$,

$$\tilde{V}_G \approx \tilde{V}_0 + \tfrac{2}{3}\tilde{M}\omega_{al} \approx 1.4\tilde{V}_0 \tag{14}$$

is needed for the intensive rotational isomerization of aliphatic groups (usually these groups include the majority of all side-chain degrees of freedom). This volume corresponds to the peak of the pressure P_{al} in Fig. 3(b).

The pressure due to *backbone motions* can be estimated in a very similar way. The only difference is that every backbone motion shifts several residues; thus the backbone "asymmetry parameter" ω_b is comparatively very large. Therefore backbone motions (M_b degrees of freedom) resemble vibrations [see Eq. (12)] that produce additional pressure:

$$P_t^b \approx RTM_b/(\tilde{V} - \tilde{V}_0) \tag{12a}$$

The total torsional pressure is

$$P_t = P_t^s + P_t^b = RT(\tilde{M}_{ar} + M_b)/(\tilde{V} - \tilde{V}_0) + P_{al} \tag{15}$$

In conclusion, we stress that the characteristic behavior of torsional pressure is due to the following qualitative features of side chains and protein architecture:

1. Aliphatic chains have two types of motion: vibrations (in local minimum) and rotational isomerization (jumps from one minimum to another). These jumps are comparatively large, because the narrow "allowed" regions [where $U(\chi) < RT$] are separated by the broad "forbidden" ones [where $U(\chi) \gg RT$].

2. If a protein is dense (i.e., the side chains move in small "cages"), only vibrations are possible. The cage must exceed some minimal volume (which corresponds to rotation at about $\pi/2$) and only then rotational isomerization can start. So, the volume

$$\tilde{V}_B \simeq \tilde{V}_0 + \tfrac{1}{2}\tilde{M}\omega_{al} \simeq 1.3\tilde{V}_0 \tag{16}$$

corresponds to the free energy barrier (energy is already lost as compared to the tightly packed state, while entropy is still not yet gained).

The collective side-chain movements (which are so important for protein dynamics) cannot alter the obtained thermodynamic results. This is because

the coherent rotation of many groups (specially examined in[24]) can make only minor contribution to the entropy.

EQUATION OF STATE FOR THE PROTEIN GLOBULE AND THE NATURE OF PHASE TRANSITION

Figure 5 shows the resulting pressure within the protein globule as a function of its core density. It corresponds to the sum of the plots of P_w and $P_t = P_{al} + P_{ar}$ shown in Fig. 3.

In equilibrium, the internal pressure is just counterbalanced by the outer one and it is the same at all points of a molecule. Thus one can find the stable states of the globule as the roots of the equation of state,

$$P_{in} = -dF/dV = -P_{out}$$

Here

$$F(V) = \tilde{F}(\tilde{V}) + F_H = \tilde{E}_w(\tilde{V}) + \tilde{F}_t(\tilde{V}) + F_H \tag{17}$$

thus

$$P_w(\tilde{V}) + P_t(\tilde{V}) = -P_{out} \tag{18}$$

The outer pressure P_{out} is the pressure of the solvent interacting with the protein. The protein–solvent interaction will be considered in the second paper of this study[16]; here it is neglected (i.e., we assume $P_{out} = 0$).

The graphic analysis of the equation of state (see Fig. 5 and also Fig. 3) gives three solutions:

N—native protein: stable state, free energy minimum;

B—barrier: unstable state, free energy maximum; and

G—denatured protein, stable state, free energy minimum.

The stable states N and G are segregated by B, a free energy barrier. The transition N ⇔ G occurs when the free energies of the N and G states become equal. This is a regular first-order phase transition.

The numerical estimations according to expressions (3), (4a), and (7–10) make it possible to obtain the thermodynamic parameters of protein melting (in vacuum):

energy change: $\Delta E \simeq E_w(V_N) - E_w(V_G) \simeq E_0/2 \simeq 100$ kcal/mol

entropy change: $\Delta S \simeq R\tilde{M} \simeq 200$ cal/(mol · deg)

temperature of melting: $T_m \simeq \Delta E/\Delta S \simeq 500$ K

The energy and entropy estimations (for protein of about 100 residues) seem reasonable (cf. Refs. 2–7), but the denaturation temperature is too high. However, it is necessary to note that all the solvent effects are neglected (i.e., $P_{out} = 0$) here; they are considered in the next paper.[16]

The denatured protein is globular in the vacuum model, which neglected the solvent environment. It is trivial. The vacuum model is valid if the solvent does not penetrate the globule and exerts only some surface pressure (cf. Ref. 25). But if the solvent penetrates the hydrophobic core in the course of denaturation it can change, in principle, the nature of the transition itself. Some expansion of the native globule is necessary for penetration of the

solvent. How large should this expansion be? It must be at least as large as to open some pores, which can include a solvent molecule. The volume of a water molecule is very close to the volume of a methyl group, ω_{al}. So, in principle, the solvent can penetrate the protein core only when rotational isomerization starts (see Fig. 5).

Therefore, interaction with the solvent virtually does not influence the native state N and the transition (barrier) state B; the solvent does not change the nature of the phase transition. But it determines, of course, the state of denatured molecule (it may make the denatured globule more friable or even convert it to coil; see Ref. 16).

The predicted barrier seems to be an energetic one (determined mainly by $\tilde{E}_w$; see Fig. 3), if it is passed from the native state. This barrier is *independent of the solvent*, which starts to penetrate the globule only after the barrier has been overcome. In contrast, this barrier seems to be entropic (determined mainly by $\tilde{F}_{al}$; see Fig. 3) if it is passed from the denatured state. Such a difference has indeed been observed,[26,27] and the measured activation energy of denaturation (50 kcal/mol for lysozyme) is in accord with our theoretical estimation [$\tilde{E}_w(\tilde{V}_B) - \tilde{E}_w(\tilde{V}_N) = 60$ kcal/mol; see Fig. 3).

DISCUSSION

The main result of this paper is that expansion of a native (tightly packed) state encounters a free energy barrier at a rather high protein density (Fig. 6). That is why the decay of tight packing occurs as an "all-or-none" two-state reaction *independent* of the state of a denatured molecule.

This result has been obtained under two important assumptions, which we are going to discuss now:

1. Expansion of the globule is uniform.
2. The chain fold remains the same.

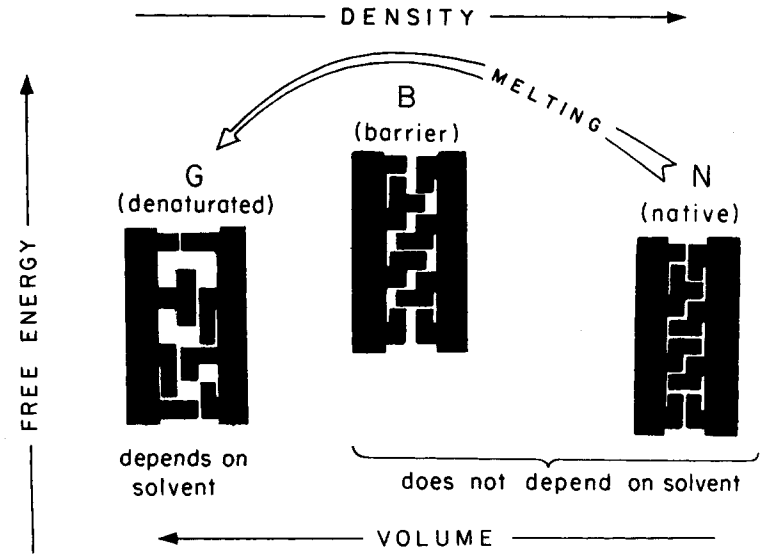

Fig. 6. Scheme of melting of a tightly packed protein core. The right part of this scheme is also valid for proteins in solution. The compactness of the denatured state depends on solvent.

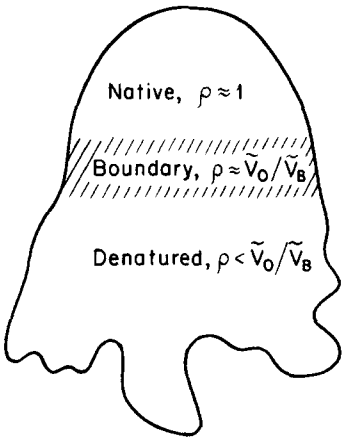

Fig. 7. Distribution of density ρ in a half-molten protein. The density at the boundary corresponds to the free-energy maximum.

A nonuniform expansion of the globule forms a boundary between the native and the denatured phases. The density at the boundary is "intermediate" and just corresponds to the barrier B. The surface tension at this boundary makes such "half-molten" states unstable (Fig. 7).

The "half-denatured" states may be stable only in extremely heterogeneous proteins. If a protein consists of two parts with the melting temperatures T_1 and T_2 ($T_1 > T_2$), it cannot be half-denatured when the free energy of the boundary

$$n\gamma > \frac{\Delta E}{2} \frac{T_1 - T_2}{T_1 + T_2} \tag{19}$$

Here $\Delta E \simeq \tilde{N} \cdot 2$ kcal/mol is the energy of protein denaturation [see Fig. 3(a)], n is the number of residues at the boundary, and γ is the surface free energy per one residue ($\simeq 0.5$ kcal/mol; see Fig. 3).

If Eq. (19) is satisfied (for the compact core where $n \sim \tilde{N}^{2/3} \simeq 10$, this means that 50 K $> T_1 - T_2$), the half-molten states *cannot* serve as the *stable* intermediates in de- and renaturation (but they can be the unstable *transition* states in the kinetics of these processes).

A nonuniform denaturation may occur when a contact between parts of a protein is weak, i.e., it is favored by multidomain or a one-dimensional structure of a protein.

The assumption of uniqueness of a protein fold made it possible to use density as a single macroscopic parameter relevant for protein denaturation. Under this assumption, we calculated the density range that corresponds to a free-energy barrier. But this density range itself does not depend on a particular chain fold. Thus we see that the uniqueness of a chain fold is not obligatory for all-or-none protein denaturation. The only necessary condition is the absence of such rearrangements, which segregate the refractory regions from the fusible ones [see Eq. (19)].

The uniqueness of protein structures (regardless of some small deformations) has been demonstrated in numerous x-ray and nmr studies. The reason

for this general fact is not clear yet. One may hope to find (or to create, using protein engineering) some drastic transformations of the native ("solid") structures resembling the polymorphous transitions in crystals. However, even in this case the decay of the tight packing must be a first-order phase transition.

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