*Physics – Uspekhi* **45** (11) 1131 – 1151 (2002) 

*REVIEWS OF TOPICAL PROBLEMS*

PACS numbers:: **87.10. + e**, 87.15.By, 87.15.He, **87.80. – y**

# Dynamical properties and energy landscape of simple globular proteins

Yu F Krupyanskiĭ, V I Goldanskiĭ

DOI: 10.1070/PU2002v045n11ABEH001145

## Contents



**Abstract. Analysis of dynamic properties of a simple globular protein, myoglobin, has demonstrated that it possesses a hierarchically organized energy landscape. It shows two types of specific protein motions, besides vibrations: 1) individual motions of small atomic groups — transitions between conformational substates (CS) of the lower tier 2, and 2) cooperative motions of secondary structure elements (α-helices) — transitions between CS of the upper tier 1. The profile of macromolecule dynamic properties is highly heterogeneous. Only vibrations occur near the active center. The number of CS grows towards the periphery where specific type 1 and 2 motions become predominant. Such a picture is consistent with the concept of a protein as 'a random copolymer slightly edited in the vicinity of the active center'.**

**Yu F Krupyanskiĭ, V I Gol'danskiĭ** N N Semenov Institute of Chemical Physics, Russian Academy of Sciences
ul. Kosygina 4, 117977 Moscow, Russian Federation
Tel. (7-095) 939 73 00
Fax (7-095) 137 83 18
E-mail: yufk@chph.ras.ru

Received 20 November 2001, revised 11 March 2002
*Uspekhi Fizicheskikh Nauk* **172** (11) 1247 – 1269 (2002)
Translated by Yu V Morozov; edited by M S Aksent'eva

*"... periodic crystals ... constitute one of the most fascinating and complex material structures by which inanimate nature puzzles his wits. Yet, compared with the aperiodic crystal, they are rather plain and dull. The difference ... is of the same kind as that between an ordinary wallpaper... and ... a Raphael tapestry, which shows no dull repetition, but an elaborate, coherent, meaningful design traced by the great master."*

E Schrödinger [1]

## 1. Introduction

Practically all cellular functions depend on proteins. Together with DNA and RNA molecules, protein molecules are the most complex of all others known to scientists. Enzymatic proteins catalyze biochemical reactions; elucidation of the mechanisms of these reactions is a central problem of biological physics [1 – 9]. After detailed data on the human genome structure was published in 2001, mankind all of a sudden found itself living in a new, 'postgenomic' era. The description of the genome gave powerful impetus to the development of a new science concerned with the behavior of protein pools in the cell at a given time and biochemical interactions among proteins (the so-called 'proteomics').

The objective of the rapidly developing structural genomics is to elucidate and reliably predict the three-dimensional structure of a large number of proteins that came to be known

as a result of deciphering human and many bacterial genomes, etc. In the pursuance of this goal, new large biochemical centers are being built (mostly in the United States) to carry out multidisciplinary investigations into the three-dimensional structure of proteins using X-ray analysis, NMR-spectroscopy, and theoretical prediction of spatial configuration from the known amino acid sequences of these molecules. Russian scientists have made and continue to make an important contribution to the prognostication of protein structure [10 – 12].

Let us now return back to the 'genomic' era. Enzymatic proteins show very high catalytic activity [2 – 4], and numerous studies were undertaken to interpret it. In chemistry, there is a well-known logical connection between the object's structure (rigid molecule) and functional properties. In biological physics, the functional activity of macromolecules also depends on their structure. However, this relationship is much more complicated than in the chemistry of rigid molecules. Investigations into the fundamental principles of protein functioning lead to the conclusion that not only the structure but also dynamic properties of these macromolecules need to be known if their activity is to be properly understood and described in quantitative terms. Hence, the necessity to explore the energy landscape of macromolecules upon which their dynamic properties depend. The energy landscapes are formed in the course of the self-organization of the protein primary structure into a spatial one. Naturally, it should be expected that, in the new 'postgenomic' era, elucidation of the three-dimensional structure of numerous new proteins will be followed by an in-depth study of their dynamic properties and energy landscapes.

Comparison of a nanocrystal particle and a protein of a roughly similar size shows that the two undergo energy, entropy, and volume fluctuations of the same order of magnitude. However, both their reactive ability and dynamic properties are dramatically different because their structural and dynamic organizations differ. Specific structural and dynamic organization of enzymatic proteins ensures their high reactive (catalytic) ability. Also, the same structural organization is responsible for the highly specific dynamic properties of these proteins that make them so much unlike ordinary solids.

Much experimental data obtained during the last 20 – 25 years indicates that the physical properties of proteins are far more complicated than they were deemed to be in the preceding period. Results of X-ray analysis (with the resolution extended to the 1 Å level) [13] taken together with high packing density [14], cooperativity of denaturation process [15], and other factors [13, 16, 17] confirm that proteins are regular 'crystal-like' systems or 'aperiodic crystals' [1, 3]. At the same time, authors of many experimental studies on protein dynamic properties, using the neutron scattering (NS) technique [18], Mössbauer spectroscopy (MS) [19 – 22], and Rayleigh scattering of Mössbauer radiation (RSMR) [22 – 26] consider specific (intraglobular) motions of proteins in the framework of bounded jump or continuous diffusion models, that is as liquid-like at small times. In such experiments, proteins have nothing in common with 'crystal-like' systems. No wonder these studies tend to expose quite different and seemingly discrepant properties of protein macromolecules.

Proteins are known to be capable of self-organization. Physics of protein self-organization provides a forum of lively debates concerning two current hypotheses of globule self-organization, post-translational and co-translational. Advocates of the former hypothesis are in turn divided into those who support the thermodynamic hypothesis and those giving preference to the kinetic hypothesis [27 – 30]. The proof of the one or the other hypothesis would immediately give answers to many questions pertinent to protein dynamics. If the thermodynamic hypothesis is correct, the native state of proteins corresponds to the global energy minimum, which means that the protein is an ordinary ideal nanocrystal, where only harmonic or quasi-harmonic vibrations are possible.[1] In this case, however, any specific protein or liquid-like (at small times) motions are out of the question. If the kinetic or co-translational hypothesis is true, protein dynamic properties should be much more diverse due to the fact that the native state is consistent with one of the local energy minima. Specific protein motions (other than the solid-like ones) resembling bounded diffusion may take place, besides harmonic vibrations inherent in solids.

Protein dynamic properties depend on the globule energy landscape being formed in the course of folding. Therefore, protein dynamics should be closely related to the mechanism of self-organization of protein globules. Investigation into dynamic properties of proteins may be conducive to understanding mechanisms of self-organization. In connection with this, the on-going discussion on dynamic properties of proteins should just as well concern the physical aspects of their self-organization. Unfortunately, only recent reviews [7, 8] and some reports at the last national and international conferences [32 – 34] appear to trace the relationship between these basic areas of protein physics.

It is universally accepted that electron-conformational interactions and structural changes in functioning proteins play a key role in enzymatic catalysis [2 – 8]. Conformational relaxation is a rate-limiting stage of the reaction [2]. Therefore, knowledge of the energy landscape and dynamic properties of macromolecules is of primary importance for the quantitative description of protein functioning. Further progress in the understanding of the basic principles of protein functioning is impossible without a deeper insight into spatio-temporal characteristics of specific protein motions.

The present review deals with the experimental data and theoretical considerations pertaining to the dynamic properties and energy landscape of myoglobin the simplest but best studied of all globular proteins. In addition, relevant data on another well-known protein, lysozyme, is presented.

## 2. Structural organization of functioning proteins

Living cells are built up of macromolecules, such as nucleic acids (DNA and RNA), proteins, polysacharides, and lipids [35]. Nucleic acids are long chains of nucleotides, while proteins are composed of amino acids. There are four types of nucleotide bases and 20 types of amino acid residues making up protein molecules.

At a higher organizational level, the cell's macromolecules are assembled in supramolecular complexes of nucleic acids and proteins, e.g. ribosomes.

The supramolecular complexes in their turn give rise to organelles exemplified by the cell nucleus. In organisms

[1] Naturally, short-lived fluctuations of the structure may occur too, so far as they are possible in small particles at all [31].

having chromosomes as components of their cell nuclei, DNA molecules are wound around protein molecules (histones) for compact storage of information and ready access to it. Without such compaction, the enormous 3 m length of a DNA molecule containing one billion basepairs would not be able to fit within the 5 micron nucleus.

The language of life, called the genetic code is based on a four-letter alphabet or a sequence of four types of nucleotides assembled in a DNA molecule. Three-letter words formed by adjacent nucleotides constitute units known as codons. Collectively, all the codons make up the genetic code. A sequence of codons in a strand of DNA forms a gene which serves as a template on which an RNA molecule is built.

The information for the construction of a particular protein is read from the DNA and transcribed onto a RNA molecule. The RNA molecule is then transported to a ribosome where the protein assembly takes place. The RNA instructs the ribosome in which order the amino acids must be assembled to form the primary structure of the protein. When the primary sequence emerges from the ribosome, it folds into the functionally active three-dimensional structure.

The protein structure has a hierarchical organization. Primary, secondary, and tertiary (sometimes quaternary) structures are distinguished.

The primary structure or the linear amino acid sequence is an immediate product of protein biosynthesis on a ribosome. It persists in the course of further thermal motion until degeneration.

Secondary structures are $\alpha$-helices or $\beta$-sheets in which elementary units consist of a few (up to ten) chain links each.

The tertiary structure refers to the folding of the protein chain back upon itself or a mode of spatial location of secondary structure elements. Formation of the tertiary structure may bring together any portions of the chain, even those infinitely far apart in the amino acid sequence. The majority of active cellular proteins exist in the form of compact aggregates, with practically all solvent molecules being forced outside the chain. Proteins having such structural organization are called globular. After the human genome's structure was deciphered, it turned out that the old 'one gene — one protein' formula is valid only for selected genes whereas many (probably most) others encode each for a family of related but essentially different proteins. In other word, the principle of 'one gene — many proteins' appears to be more appropriate.

### 2.1 Models of protein as a physical body

Macromolecular enzyme proteins are of a size in the nanometre range. Such notions of macrophysics as temperature, permittivity, etc. are applicable to bodies of this size containing thousands of simple molecules or atoms [5]. In other words, a protein macromolecule may be regarded as a macroscopic body in the physical sense of the word. A few words about the currently popular models of proteins are in order. In fact, the very names of these models indicate which analogies with the simplest aggregate states are used in them. The model of the protein as a reinforced droplet has been discussed at length in many works published by K V Shaitan and A B Rubin (see, for example [4, 6]).

Long ago, Schrödinger noted that biological systems are characterized by an astonishingly high degree of ordering which explains why the behavior of a living organism resembles a mechanical rather then a thermodynamic one [1]. D S Chernavskiĭ and co-workers suggested that mechanics adds to statistics even at the level of individual biopolymer macromolecules (proteins) [36]. Protein molecules are known to carry information contained within their amino acid sequence which directs both their spatial structure and functional activity. In other words, a protein is assembled under a certain 'plan' [5]. D S Chernavskiĭ maintains that analogs of an enzyme protein are only other objects built according to a plan, i.e. constructions. This argument makes the essence of the 'protein-machine' concept [5].

It was mentioned in the previous paragraph that biological systems are distinctive for the high degree of ordering. However, it is difficult to think of a living cell organized more regularly than a water droplet of comparable size. L A Blyumenfel'd [2] undertook a quantitative evaluation of biological ordering and arrived at the conclusion that ordering of the biological organization of the human body does not exceed 300 entropy units (e.u.) [2]. A major contribution to this value is made by the regular distribution of amino acid which residue in protein molecules. For this reason (in agreement with physical criteria), any biological system is ordered no more than a piece of rock of the same weight [2]. The impression of the high ordering of biological systems is produced by the fact that this ordering (or information, to be precise) has sense [37] or value [38].

A glass-like dynamical model of protein macromolecules was proposed by the authors of this review some 20 years ago [39, 24]. By that time, a concept of conformational substates (CS) in a protein macromolecule had been formulated based on the results of kinetic and dynamic studies [40 – 42]. Also, it had been shown that glasses possess some specific properties or anomalies manifest at low temperatures, such as the presence of a linear term in the low-temperature heat capacity at $T < 1$ K.

It turned out that this anomaly directly ensued from the complexity of the energy landscape in glasses, that is, the presence of local energy minima or CS, with tunneling of atoms or small atomic groups between them being responsible for the appearance of the linear term [43, 44]. This analogy was so obvious that we immediately suggested that protein macromolecules must show similar 'glassy' anomalies. Subsequent experiments confirmed this suggestion. The concept of 'protein-machine' lays emphasis on those characteristics of proteins that make them related to 'animate' nature. In contrast, the glass-like dynamical model takes into consideration only those properties of the protein macromolecules that are inherent in objects of 'inanimate' nature.

It will be shown in Section 5 that protein macromolecules retain many features of 'inanimate' nature. The assertion that protein macromolecules possess anomalies intrinsic in glass is sometimes interpreted as the identification of proteins with this material (see, for example, [5]). It is certainly not the case. A review of current studies developing ideas of this model is presented in Sections 5 and 6.

### 2.2 About phase transitions in macromolecules

In statistical physics, phase transitions and different phases are strictly determined only for systems with an infinite number of particles (in fact, for the number of particles on the order of Avogadro's number $N = 6 \times 10^{23}$). The largest (longest) protein chains contain 100 – 1000 links, i.e. their number is much smaller than Avogadro's number. For this

[2] 1 entropy unit = 1 e.u. = 1 calorie per degree$^{-1}$ [2].

reason, the states of a given finite biological macromolecule are not always possible to relate to a definite phase. Near phase transition, there must be a transitional temperature zone of finite width ($\Delta T$) between regions reliably referred to as different phases.

This problem should be discussed bearing in mind the generally accepted classification of phase transitions. According to this classification, a first-order phase transition occurs when the first derivatives of thermodynamic potentials (entropy, density, magnetization, etc.) undergo abrupt changes at the transition point. In second-order phase transitions, the second derivatives (heat capacity, susceptibility, etc.) undergo similar leaps. This classification is inapplicable to a finite system in which no abrupt jumps occur (see [45] for details). Unlike systems with an infinite number of particles, proteins must have a transitional temperature zone of finite width $\Delta T$ relative to a certain critical temperature $T_c$ that separates regions reliably referred to as different phases. This line of reasoning fits equally well glass transition.

### 2.3 Major requirements for the structural organization of functioning proteins

It is well known that enzymatic catalysis and performance of many other protein functions demand definitiveness (ordering, unambiguity) of mutual arrangement and orientation of amino acid residues in the active center. This suggests a mechanism maintaining the ordering of the active center structure of the active center and protecting the said mutual arrangement and protecting the said mutual arrangement and orientation of amino acids from destruction under effect of thermal fluctuations.

The most likely mechanism for the purpose is the integration of the active center into a cooperative system (structure) subject to destruction only as a whole, i.e. in the course of intraglobular phase transition after the pattern of first-order transition. In this case, free energy that stabilizes the native structure of the active center is close to the free energy stabilizing the entire three-dimensional protein structure. In other words, it is rather high. Such a mechanism does exist in globular proteins. Indeed, the melting of a native three-dimensional globule is (to the accuracy of modern experiment) a first-order transition.

O B Ptitsyn and M V Vol'kenstein have formulated the following general requirements for functioning globular proteins [46]:

1) the presence of the active center (definitely arranged atomic groups directly involved in protein functioning); 2) integration of the active center into an expanded structure whose frame is formed by $\alpha$-helices and/or $\beta$-sheets with closely-packed side-groups; 3) stability of the three-dimensional protein structure in a functioning molecule.

Efficiency of the enzyme performance is evaluated from the turnover rate, i.e. the number of catalytic actions of a single protein molecule per unit time. For certain enzymes, the turnover rate is as high as hundreds of thousands per second. The energy of ligand binding is close to denaturation energy. Therefore, the stability of the structure or its ability to fully recover after a single catalytic act is one of the main requirements for the spatial structure of a protein globule. Because only crystals, of all other simple substances, have a stable structure, it was natural to suggest that proteins are 'crystal-like', as was done in a few fundamental works [1, 3].

## 3. Principal properties of glasses and supercooled liquids

In the general case, any solid having no periodic structure and characterized by the absence of long-range (crystal) order is referred to as a noncrystalline or amorphous one. The conventional definition of 'glass' as an amorphous solid obtained by rapid cooling of the melt is no longer deemed sufficient. Under the glassy state or simply glass (not to be confused with glass as a building and household material) is meant an amorphous solid undergoing calorimetric transition to a glass-like substance near a certain characteristic temperature $T_g$ [47, 48]. Then, the solid state of matter at $T < T_g$ is termed glass (having viscosity higher than $10^{13}$ – $10^{14}$ poises) while a glass-forming system at $T_m > T > T_g$ is called metastable long-lived supercooled liquid. Here, $T_m$ is the melting point. The crystalline state is the ground and equilibrium state of such system.

Slow cooling of a liquid (melt) to the melting point $T_m$ (Fig. 1a) leads to crystallization (route *a*). Rapid cooling of a sufficiently viscous melt may result in its overcooling without crystallization (route *b*). Let us introduce a cooling rate $R = -\mathrm{d}T/\mathrm{d}t$. Each substance is characterized by such $R_{min}$ that it turns to a supercooled liquid at $R > R_{min}$. At $R < R_{min}$, crystallization occurs. The higher the viscosity at the melting point the higher the likelihood of vitrification. Because the viscosity ($\eta$) of metals is much lower than

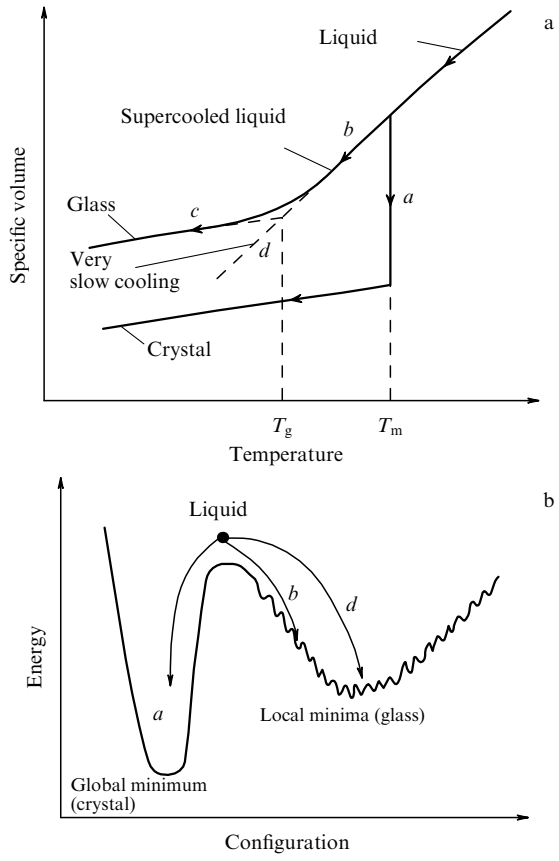


**Figure 1.** (a) Temperature dependence of specific volume illustrating peculiarities of liquid-to-glass transition. (b) Diagram showing glass or crystal formation from a liquid (represented in energy-configuration coordinates). The ground (crystalline) state of the system corresponds to the global minimum, local minima correspond to the glassy state. See the text for details.

that of silicates (e.g. $\eta(T_m) = 0.07$ poises for Fe and $\eta(T_m) = 10^7$ poises for $SiO_2$), the former are cooled down by path *a* while the latter by path *b*.

In dielectric glasses, $R_{min}$ is usually of the order of $10^{-5}$–1 K $s^{-1}$. Metallic glasses have very high $R_{min}$ values: $R_{min} \sim 10^6 - 10^{10}$ K $s^{-1}$ [48, 49]. Computer modeling gives even higher values $R_{min} \sim 10^{11} - 10^{13}$ K $s^{-1}$ for small systems composed of $10^3$ atoms. Thus, glass transition occurs even in simple monoatomic fluids like liquid argon [49]. Assuming that computer modeling reflects processes in real substances, any substance can be supposed to have such $R_{min}$ at which rapid cooling leads to its vitrification. In other words, glass transition is an universal natural phenomenon.

The crystalline state of a substance is known to be thermodynamically stable. Supercooled liquids remain in the metastable state during rather a long (although finite) lifetime $\tau_l$. In most cases, this period is almost as long as freezing time $\tau_f$ which, in turn, depends on the mean cooling rate $R$:

$$\tau_l \sim \tau_f \approx \frac{T_m - T_g}{R} .$$

For example, if $R \sim 10^{-5}$ K $s^{-1}$ and $T_m - T_g = 100$ K, then $\tau_f \approx 3.5$ years. Glasses are also metastable , but for them $\tau_l \gg \tau_f$. It is known that simple window glasses remain stable for many centuries.

The glass structure is in disequilibrium in two respects: 1) relative to the equilibrium crystalline structure and 2) relative to the metaequilibrium (at a given temperature) structure of glassy matter. Upon glass transition the substance retains the structure of liquid (melt) corresponding to $T \geqslant T_g$. Below $T_g$, the structure is equilibrated so slowly that it does not relax to its metaequilibrium state during the period of observation. For this reason, the structure and properties of matter in a glassy state depend on their thermal history.

Annealing (heating a glass, holding for a certain time at a given temperature, and then cooling) may result either in crystallization or in approximation to the metaequilibrium state (if high viscosity prevents crystallization). This process is referred to as glass stabilization. Annealing normally leads to structural renewal. This indicates the absence of memory in glasses.

If a substance 'chooses' route *a*, i.e. undergoes crystallization, further cooling of the system can not induce the transformation of the crystalline state to the glassy one.

Metastability of glass-forming systems is also manifested as the dependence of vitrification point $T_g$ and other properties on the cooling rate $R$. For example, if $R' > R$, $T_g(R') > T_g(R)$. Therefore, it can be assumed that $T_m > T_g \geqslant T_g(R_{min})$. Usually, $T_g \approx 2/3 T_m$.

Simple glass-forming liquids are normally characterized by an ordered low-temperature (crystalline) phase with translational symmetry. This phase, that can be avoided (as above) by fast cooling, is actually the ground state of a system described by the global minimum of energy. A supercooled liquid or glass has a complicated energy landscape with many local energy minima or conformational substates (CS) (Fig. 1b). At high temperatures, the system performs a random walk across the energy landscape including those with high conformational energy. In the case of slow cooling, the system can, in all probability, fall into a low-energy state (route *d*). Rapid cooling can just as well drive the system into areas with enhanced conformational energy (route *b* in Fig. 1b). Thus, Fig. 1b is a good illustration of glass disequilibrium with respect to the metaequilibrium state and the ground one (i.e. global energy minimum).

Heating above a vitrification point $T_g$ results in the conversion of glass into a supercooled liquid. This transition is reminiscent of a second-order transition. According to a current concept, however, glass transition can not be reduced to usual second-order thermodynamic phase transitions just because $T_g$ depends on the cooling rate [47].

At low temperature, glasses exhibit a number of specific properties called anomalies because neither ideal crystals nor 'nonglassy' amorphous substances normally possess them. In the context of this paper, a most important anomaly is the presence of a linear term in the low-temperature heat capacity at $T < 1$ K. This anomaly results directly from the complexity of the energy landscape in glasses, that is the presence of local energy minima or CS. Tunneling of atoms or small atomic groups between them is responsible for the appearance of the linear term [43, 44].

The response of an ordinary liquid (in the high-temperature limit) to mechanical or electrical perturbations is described by a conventional Debye relaxation model and is exponential in time [50]:

$$F(t) = \exp\left(-\frac{t}{\tau}\right) .$$

Here, $\tau$ is relaxation time.

The corresponding frequency response is determined by dynamic susceptibility:

$$\chi(\omega) = \frac{\chi_0}{1 + i\omega\tau} .$$

A fundamental property of supercooled liquids is a slow relaxation process with macroscopically large relaxation time. This process is usually referred to as primary or $\alpha$-relaxation. In this temperature range, the response of a supercooled liquid to mechanical or electrical perturbations is largely determined by $\alpha$-relaxation and shows non-exponential time dependence. The relaxation function is frequently described by the Kohlrausch–Williams–Watts expression [51]

$$F(t) = \exp\left[-\left(\frac{t}{\tau}\right)^{\beta}\right] . \qquad (3.1)$$

Dynamic susceptibility of supercooled liquids can not be described by a simple Debye model. Therefore, a relaxation time spectrum $G(\tau)$ is introduced to describe experimental data

$$\chi(\omega) = \chi_0 \int d\tau \frac{G(\tau)}{1 + i\omega\tau} .$$

Davidson and Cole [52] introduced the following empirical expression for susceptibility that fairly well describes numerous experimental findings:

$$\chi(\omega) = \frac{\chi_0}{(1 + i\omega\tau)^{\beta}} . \qquad (3.2)$$

Notwithstanding different functional form of expressions (3.1) and (3.2), they yield similar results in the numerical case [53].

## 4. Self-organization in proteins

Conversion of a polypeptide chain into a native protein is a key process in the translation of genetic information into biological activity [54]. Extensive attempts to understand this process were made in the last 5 – 10 years. In the cell, functionally active proteins are formed in the course of two processes, synthesis of the primary amino acid sequence on a ribosome (formation of chemical structure) and its folding (self-organization) into the functionally active three-dimensional protein molecule. These two processes are frequently regarded as independent and successive (post-translational folding).

Over 30 years ago, C Anfinsen [27] demonstrated that a previously denaturated protein renaturates *in vitro*, provided certain conditions are satisfied (low-rate process, small concentration, etc). In other words, the tertiary (native) protein structure can spontaneously recover. While the primary structure of proteins is synthesized on specific subcellular particles called ribosomes, no special instruments are needed to produce the tertiary structure of at least small, simple proteins. Experiments by C Anfinsen showed that the tertiary protein structure is capable of self-organization, i.e. transformation of one-dimensional information to a three-dimensional structure. It is this ability that ensures protein functioning.

It is known that the natural (evolutionary) selection of proteins occurs by means of preserving and reproducing their spatial structures. This implies an unambiguous or degenerate relationship between the genetically determined primary structure and spatial organization of a given protein [3]. In this Section we consider, in brief, current concepts of self-organization in proteins. Readers wishing to obtain a deeper insight into the problem are referred to numerous excellent reviews and original papers [54 – 62].

There is a long-lasting discussion in the literature in an attempt to clarify whether the native protein structure is consistent with the absolute (global) minimum of free energy (the so-called thermodynamic theory of protein folding [27]) or corresponds only to its local minimum (kinetic hypothesis of protein folding [28, 29]). The latter hypothesis suggests that a protein chain is unable to reach the most stable energy state (global minimum) within a few minutes (real time of protein self-assembly in the cell). The native conformation of a self-assembled protein chain is consistent with rather a deep but local energy minimum that makes the protein structure resistant to potential perturbations. In other words, the protein structure is in quasi-equilibrium or, strictly speaking, in the metastable state with a very long lifetime. It means that the self-assembly of a protein chain results in a state separated from the absolute equilibrium (global) minimum by a high barrier of free activation energy $\Delta G$ which it is unable to penetrate. Conformation corresponding to the global energy minimum is kinetically unattainable by a native protein.

The choice between the thermodynamic and kinetic hypotheses of protein folding is crucial for the purposes of this review. Dynamic properties of proteins depend on the energy landscape of the globule being formed in the course of self-organization. The proof of the thermodynamic hypothesis would immediately answer all questions concerning protein dynamics. In the context of this hypothesis, the native protein state corresponds to the global energy minimum with a very simple (harmonic or quasi-harmonic) potential-energy surface, while the protein itself has properties of an ideal nanocrystal in which only harmonic or quasi-harmonic vibrations may occur. In this case, any specific protein motions are out of the question. If the kinetic hypothesis is valid, the native protein state is consistent with rather a deep but local energy minimum. Then, the ground state is energetically degenerate, and the protein can be in any of the large number of conformational substates, i.e. have energy landscape. This hypothesis predicts a broader spectrum of dynamic properties. For example, specific protein motions like bounded diffusion may appear, besides vibrations normally inherent in solids. One can speak about liquid-like (at small times) motions.

It is usually argued that the thermodynamic hypothesis of protein folding is confirmed and proved by the classical experiments of C Anfinsen. Indeed, results of these renaturation experiments suggest reproducibility of mutual arrangement of secondary structure elements, but they do not provide evidence of reproducibility of the position of each atom in the protein macromolecule and hence do not give a direct answer to the question which energy minimum, local or global, is attributable to it [29].

It is still unclear how proteins of different size acquire 'their own' structure choosing it among a huge number of possible variants. If a native structure is chosen as the most stable one, how does the protein chain come to recognize it as such for a few minutes that self-organization lasts? Indeed, it has been shown that cosmological time would be taken to search through all $10^{100}$ possible protein structural combinations (where 100 is the number of amino acid residues, each having around 10 conformations) — the so-called Levinthal's protein-folding paradox [28]. An alternative suggested by Levinthal [28] is that the native structure is not the most stable one but takes the shortest time to be formed by folding.

The sense of Levinthal's famous paradox can be formulated taking into consideration the work of A V Finkelstein [63]. Folding of the protein chain is accompanied by a decrease of its entropy $S$ (due to enhanced chain ordering) and energy $E$ (as contacts are being formed between chain links that come closer to one another). It should be recalled that the expression for free energy has the form

$$G = E - TS\,. \tag{4.1}$$

The decrease of entropy enhances while that of energy lowers the chain's free energy. If, in the course of the folding the chain all but acquires its final structure before stabilizing contacts begin to form (i.e. the chain looses almost all its entropy before it starts gaining energy), a rise in the free energy at the first stage is high and the folding is very slow [3]. It is this situation (complete loss of entropy prior to the energy gain) that underlies the famous Levinthal's paradox [28] that a protein chain can never acquire the most stable structure during the lifetime of the Universe. Alternatively, the folding takes little time if it occurs so that a fall of entropy is almost immediately made up for by a decrease of energy [55, 64].

This line of reasoning has led researchers to postulate mechanisms of successive self-assembly of proteins that might be instrumental in surmounting Levinthal's paradox [55, 64].

Due to the active cooperation between theorists and experimenters in the last few years, considerable progress

[3] In agreement with chemical kinetics [2, 3], the time of a process exponentially depends on the maximally attainable rise in free energy.

has been made in understanding detailed mechanisms of self-organization (see [54–61]).

During the same time, views concerning proteins have changed dramatically. Some 20–25 years ago, each protein was considered to be a unique and indispensable product of nature. Naturally, this opinion was opposed since it was unclear how such a unique sequence could have come into being during the lifetime of the Universe.

Furthermore, the first computer experiments designed to simulate protein self-organization brought researchers to the extreme of assuming, for simplicity, a random sequence of amino acid residues in a protein chains (primary structure). In other words, random heteropolymers were considered as protein models. However, indepth studies demonstrated that random sequences can not be reasonably used as protein models. It turned out that they do not usually fold into a 'native' structure nor can such folding, if any, be rapid and reliable. It is small wonder because proteins could not but change in some way or other in the course of evolution. This fact is taken into account in the concept of a protein as 'a slightly edited random copolymer' suggested by M V Vol'kenstein and O B Ptitsyn in which 'editing' largely affects the vicinity of the active center [46]. In our opinion, this concept is of great help for the understanding of both protein folding processes and current views of dynamic properties of simple globular proteins.

Theoretical studies of protein self-organization were, from the very beginning, oriented towards computer simulation even though several analytical works were also performed. Early authors tried to undertake a numerical analysis of real proteins and maximally take into consideration minor details of real interactions between their constituent elements. However, it soon became clear that such detailed calculations could not be made in the near future. Therefore, a different approach was used to overcome this difficulty based on the simplest of models [58] that 1) bore a certain degree of resemblance to proteins, 2) were sufficiently sophisticated to solve nontrivial physical problems, and 3) reproduced certain essential aspects of protein self-assembly. The authors of these works warned that the results obtained with these simple models should be interpreted with great caution. It must be borne in mind that these models are none other than rough approximations, adequate only on spatio-temporal scales much greater than microscopic ones (sizes of more than 10 Å and times over 1 ms respectively [58]). In other words, lattice models fail to adequately describe small-scale structural and chemical details of protein molecules, even the localization and size distribution of secondary structure elements. 'Monomers' used in these models are actually renormalized 'quasimonomers'; therefore, small-scale details are totally lacking from the models [62]. In this context, the conformation of a lattice polymer should be regarded as a rough representation of localization of secondary structure elements. From this standpoint, a 27-mer and even 18-mer are adequate to a single-domain protein that normally consists of 50 to 200 residues [62].

New theoretical concepts, other than successive self-assembly models [55, 64] supplemented by new computer techniques provided, a basis for further progress in the development of experimental methods [57, 61]. Two groups of studies carried out in parallel are worth mentioning. One originates from the known work of Bryngelson and Wolynes [65]; the results are reported in a series of papers referred to in reviews [59, 60]. The authors emphasize the importance of the knowledge of the self-assembly energy surface for a more comprehensive understanding of its mechanism. In a rough approximation, the energy surface of self-assembly is reminiscent of a funnel with traps where a protein is retained for some time (Fig. 2). There is no unique pathway leading to the native state; instead, there are many convergent routes. Bryngelson and Wolynes conjectured [65] that proteins, unlike random heteropolymers, meet the 'principle of minimal frustration'[4]. In other words, a protein, unlike an ordinary random heteropolymer, has an amino acid sequence for which a configuration with the smallest possible energy has such a property that almost all numerous interactions are interrelated, and there are practically neither unrelated nor unrealized (frustrated) interactions. It was suggested that the results of evolution reflect the 'minimal frustration' and 'harmony' principles [65, 66].

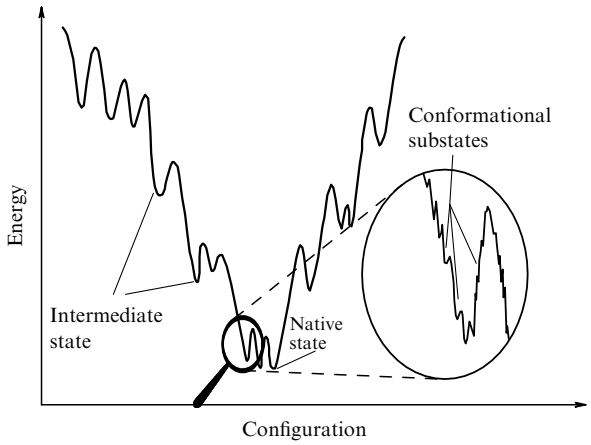


**Figure 2.** Energy surface of protein self-assembly (funnel with traps) as described in [59].

Because only 20 rather than an infinite number of different kinds of amino acids occur in nature, the authors of the above concept believe that a certain amount of frustration (hence, complicated energy landscape as in spin glasses) is inevitable [7, 59, 65].

It was always implied in consecutive self-assembly models [55, 64] that folding leads to a unique three-dimensional native structure at the atomic level that corresponds to the global minimum of free energy.

In a series of works based on the 'principle of minimal frustration' and in our original studies [36, 24, 42], the authors proceeded on a significantly different view of the unique native structure. It was believed that each native protein in an ensemble of folded proteins has identical conformation (or coarse structure, fold), but the local arrangement of molecules within a given conformation may be different. In other words, a protein of a given conformation is in one of the conformational substates (see Fig. 2). The energy landscape of a protein meeting the 'principle of minimal frustration' has several low-lying energy levels with many very similar structural characteristics. For real proteins, averaging over these configurations gives a structure detected by X-ray structural analysis. A deviation of this structure from the unique one in X-ray analysis depends on the Debye–Waller factor or B-factor.

Another line of research originating from the known works of E Shachnovich and coworkers [67, 68] and further

[4] The concept of frustration is borrowed from physics of spin glasses; see, for instance, [7] for its popular exposition.

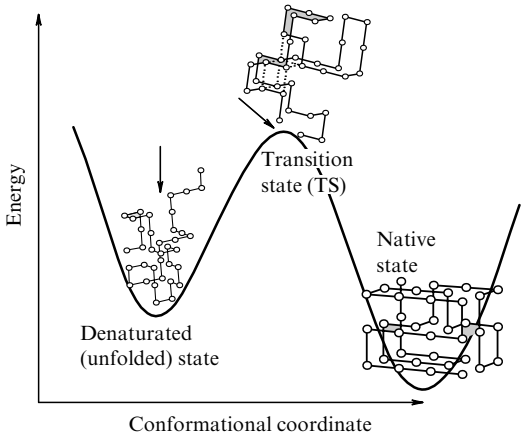


**Figure 3.** Protein self-assembly energy surface as presented in [58].

developed by the group of M Karplus and E Shachnovich and A Fersht's group is perfectly expounded in reviews [58, 69]. These works are concerned with the self-organization mechanisms in the simplest proteins. The results of experiments with small proteins containing no disulfide bridges indicate that self-assembly may be a two-step process [58]. Therefore, the free energy landscape as shown in Fig. 3 was chosen for such (small) proteins.

The two-level free energy profile suggests that the process of self-assembly is determined by two characteristic times: 1) the relaxation time of polypeptide chain motions in the potential, consistent with the free energy minimum in the denaturated (unfolded) state and 2) the time of overcoming the barrier between the denaturated and native states.

The overcoming time is assumed to be identical with the experimentally observed self-assembly time. Rapid self-organization is feasible when there is a large energy gap between the native conformation and the one energetically closest but structurally unrelated [58, 63]. The order of phase transition determines its kinetics. Cooperativity of folding implies that kinetically the mechanism of nucleation should occur as a first order transition.

In agreement with the nucleation mechanism in physical kinetics, a system fluctuates in the 'old' phase (denaturated state) until an islet of the 'new phase' (native state) is formed to be sufficiently large to grow further with decreasing free energy.

The simplest example of such a mechanism is vapor condensation preceded by the formation of nuclei of liquids [70]. If a nucleus is larger than the critical size, it continues to grow giving rise to the liquid phase. In case of proteins, it should be expected that a partly assembled fragment of the native structure identifiable in the transition state (TS) may serve as the nucleus for self-assembly.

Such kinetic behavior was actually documented in an experiment upon thorough analysis of the self-assembly mechanism of a small protein (CI 2) by the protein engineering technique [57]. In the modern 'nucleation-condensation' mechanism [57], nuclei of a new protein phase must contain at least a few non-local contacts responsible for the formation of long loops.

Evidently, the 'principle of minimal frustration' is an alternative to the 'nucleation-condensation' mechanism.

It is worthwhile to note that the authors of the 'nucleation-condensation' model easily explain how Levinthal's paradox is surmounted in nature. It is not surprising because any first-order phase transition is accompanied by a marked decrease of entropy. Therefore, there is no more of Levinthal's paradox associated with this protein-folding mechanism than with vapor condensation or any other first-order phase transition.

Until now, we have discussed the self-organization of isolated proteins. However, there are no isolated proteins in a living cell where self-organization of each macromolecule occurs in close cooperation with the surrounding ones [71]. Therefore, the patterns of self-organization under real conditions may be quite different. In the course of evolution, nature 'invented' a number of mechanisms to help avoid the effects of intermolecular interactions. The cell contains a family of 'molecular chaperones' for this purpose (see [72] for details). A S Spirin and some other authors [73, 74] maintain that in eucaryotic cells co-translational folding prevails at the expense of post-translational one considered thus far. The folding of a polypeptide chain as it grows on the ribosome starting from the N-terminal amino acid toward the C-terminus (co-translational folding) is an alternative which certain authors believe to be more realistic both biochemically and physically. Also, there are experimental findings in support of co-translational folding [73, 74].

As a matter of fact, which mechanism of protein folding, post-translational or co-translational, actually works remains a matter of considerable debate. The same is true of thermodynamic and kinetic hypotheses suggested to explain the post-translational mechanism.

We believe that the kinetic hypothesis and co-translational mechanism may have some features in common. At an early stage of folding, it may result in an energetically disadvantageous but kinetically more practicable conformation whose further packing will be governed by the previously formed 'nucleus'. The global free energy minimum of the total polypeptide chain corresponds to quite a different conformation of the 'nucleus'. However, transition of the macromolecule to a conformation consistent with the global minimum may be kinetically infeasible. Therefore, the mechanism of co-translational folding is responsible, in line with the kinetic hypothesis, for the protein in the native state remaining in the local rather than global energy minimum.

Because the precision of modern protein folding experiments is insufficient to describe minute details of the protein structure [62], experimental studies of dynamic and kinetic properties of native globular proteins are of paramount importance for providing valuable information about the energy landscape of a macromolecule and giving evidence in support of one or another mechanism of protein self-organization.

## 5. Dynamic and kinetic properties of proteins. Results of experimental studies

### 5.1 General design and conditions of experimental studies

Experimental results expounded below have been obtained in conditions that may seem rather unusual for classical biologists and biochemists. Dynamic and functional properties of proteins (best-known for myoglobin) were examined in a wide range of temperatures (from 2 K to 330 K) [40, 75], times (from femtoseconds to $5 \times 10^4$ s) [76], pressure (0.1 – 2 kbars) [77], viscosity ($10^{-2} - 10^{-7}$ poises) [78], and hydration [79, 80].

B Chance was the first to use (in the early 1960s) low temperatures to study a simple biochemical reaction with the electron transfer from cytochrome C to oxidized chlorophyll [81]. Other simple (but molecular) low-temperature reactions involving myoglobin were first investigated by H Frauenfelder in the early 1970s [40].

What was the purpose of these studies bearing in mind that proteins evolved to work at physiological (near-room) temperatures? Reactions at physiological temperatures are fast, and their elementary stages are intermixed. The entire process needs to be slowed down and separated into constituent components if the elementary stages are to be separated. These operations are possible at a low temperature. The works referred to in the preceding paragraph actually employed a physical approach (as in condensed state physics). In other words, properties of a substance (protein) were investigated at limiting (but not denaturating) ambient parameters.

### 5.2 Myoglobin — the hydrogen atom in biology

Proteins considered below were selected to be simple enough, both operationally (to enable the use of an experimental physical approach) and functionally (for a better understanding of the principles of their functioning). Yet, the protein function had to be sufficiently complicated in order to have certain biological sense. The so-called heme proteins, in the first place myoglobin, were deemed most suitable for the purpose.

Myoglobin (Mb) was used in the majority of studies reviewed in this Section. It is a well-studied simple protein involved in a simple process of binding and accumulating oxygen in the muscular tissue especially in water mammals (e.g. sperm whales). It facilitates oxygen diffusion in the cell:

$$\mathrm{Mb} + \mathrm{O_2} \rightarrow \mathrm{MbO_2}\,. \tag{5.1}$$

One of the competing reactions is carbon monoxide (CO) binding to myoglobin

$$\mathrm{Mb} + \mathrm{CO} \rightarrow \mathrm{MbCO}\,. \tag{5.2}$$

$O_2$ and CO molecules are ligands for myoglobin. These reversible reactions, (5.1) and (5.2), were (and are) used in theoretical studies of protein chemistry, functioning, and dynamic properties.

Myoglobin is an α-helical protein. Its primary sequence consists of 153 amino acid residues. The secondary structure is formed by eight α-helices (Fig. 4a). These and the side-chains, fold into a tertiary structure with an approximate dimension of $2 \times 3 \times 4$ nm$^3$ as sketched in Fig. 4b. The structure encloses a heme group (protoporphyrin IX) or heme with an iron atom at the center (Fig. 4c). This atom forms bonds with ligands (Fig. 4c). α-helical proteins normally display rough symmetry with a polyhedral arrangement of α-helices into the backbone [82]. What is known of proteins at large is also known about myoglobin. It was the first protein whose structure was deciphered by Kendrew [83]. In chemistry, the hydrogen atom is fairly well studied. Much experimental data on myoglobin obtained by different methods gives reason to conclude that in biology this protein is an analog of the hydrogen atom in chemistry. More is known about myoglobin than of other well-studied proteins considered below, such as lysozyme and some others.

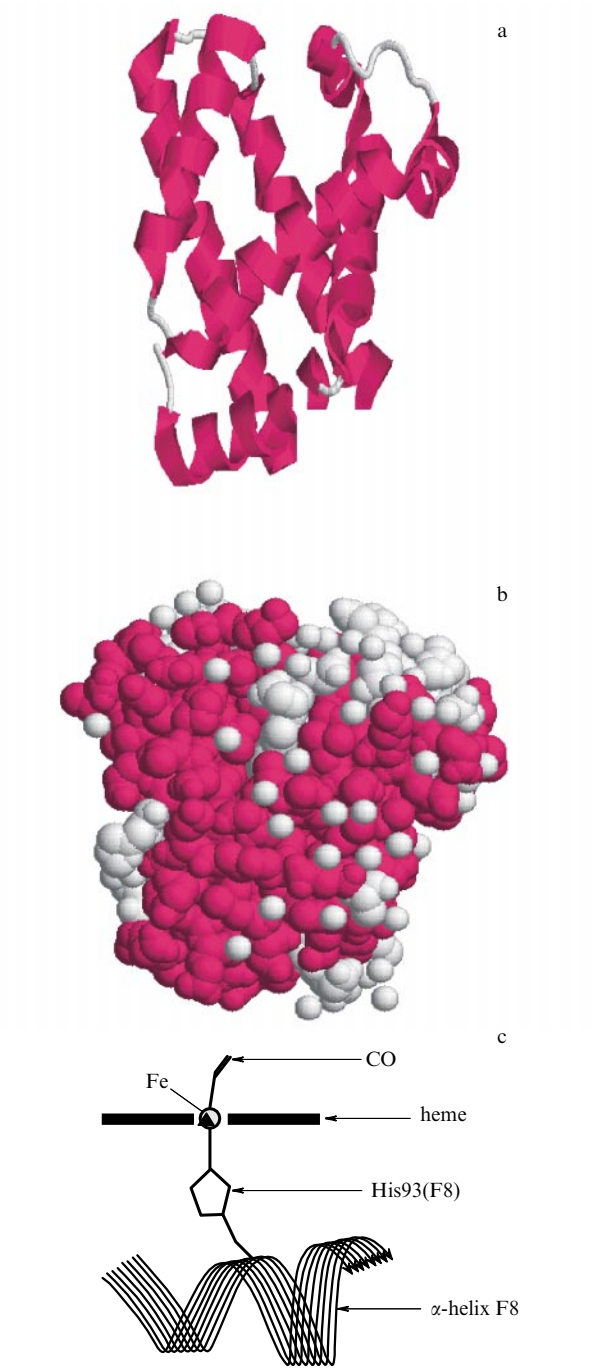


**Figure 4.** (a) Secondary structure of myoglobin (eight α-helices); (b) spatial structure of myoglobin; (c) schematic representation of the CO-bound heme and the nearest α-helix (F8).

### 5.3 Cold degeneration of proteins

Many biochemists were sceptical about the first results of low-temperature protein studies in the belief that cooling water-protein mixtures should lead to complete or partial denaturation of the proteinic component. Special studies on cold denaturation were undertaken (see, for instance, [84, 85]). Specifically, it was reported [85] that in a range of pH 3.5 to 3.9 metmyoglobin underwent denaturation-like structural transition not only upon heating but also upon cooling the system to $-5 - 10\,^{\circ}$C. It was concluded that partial denaturation of proteins occurred upon their freezing in aqueous solutions. However, the addition of the so-called cryoprotectors (e.g. glycerol or sacharose) prevented partial denatura-

tion, to say nothing about the complete one [85]. Because the studies considered below were carried out using cryoprotectors (water-glycerol mixture), potential protein denaturation (either partial or complete) can be ignored. The same refers to protein specimens in a crystalline state where the absence of denaturation has been proved by direct X-ray analysis.

### 5.4 Concept of conformational substates (CS) and energy landscape of protein macromolecule

At room temperature, Mb behaves like a very simple, 'dull' protein. However, the very first kinetic studies carried out by H Frauenfelder and his group at low temperatures demonstrated a great variety of previously unknown and very interesting phenomena. These experiments were designed to explore kinetics of CO rebinding to myoglobin after flash photolysis of MbCO [40, 75]. In the end, they have substantially advanced the formulation of the current concept of an energy landscape in protein macromolecules.

The most important experimental findings that provided a basis for the concept of conformational substates (CS) in protein macromolecules, a new one for protein physics, are listed in Table 1. According to this concept, all macromolecules in an ensemble of identical proteins have a similar coarse structure (conformation), but individual molecules somewhat differ from one another in terms of local structure (rotations around σ-bonds, hydrogen bridge shifts, fluctuative ruptures and restorations of hydrogen bonds resulting in small displacements of large molecular groups or fragments with respect to other parts of the globule) [39 – 42]). These minor structural differences account for each macromolecule having a specific barrier $H$ for binding CO (see Table 1, point A1).

The concept of CS in protein macromolecules being valid, it means that the ground state of a native protein is energetically degenerate. In other words, such macromolecule is in the local rather than global energy minimum. Hence, it is characterized by an energy landscape instead of energy levels as in atoms or simple molecules. The concept of an energy landscape in protein macromolecules leads to the following inferences: 1) the kinetic or co-translational hypothesis of protein folding is more relevant than the thermodynamic one and 2) an explicit analogy between energy landscapes in protein globules and glasses suggests that low-temperature heat capacity must, in either case, contain a linear term $C_1T$ [39]. All subsequent experiments confirmed this view (see Table 1, point A2).

### 5.5 Myoglobin energy landscape at low temperatures ($T \leqslant 20$ K)

The resonance frequency of a given chromophore optical transition depends on protein conformation; therefore, it may serve as an indicator of the protein structure at a specified moment of time. Structural changes in a chromophore-containing protein are responsible for fluctuations of transition frequencies. These fluctuations are termed spectral diffusion [100]. Methods for measuring spectral diffusion include laser-assisted spectral hole burning and photon echo which are complementary (see [100] for the detailed description of these methods and relevant experiments). This work reports a study of spectral diffusion of the myoglobin heme group that spanned the temperature range from 100 mK to 20 K for periods from nanoseconds to a few days. A combination of spectral hole burning and photon echo techniques allowed the structure of the myoglobin energy landscape, in the above temperature range, to be established in detail. Figure 12 shows its schematic representation in a range of up to 10 kJ $mol^{-1}$. This study confirmed the presence of conformational substates in myoglobin and revealed hierarchical organization of its energy landscape. It turned out that the number of CS at the lowest hierarchical level does not exceed three or four. This result is in excellent agreement with those obtained by low-temperature protein calorimetry.

### 5.6 Protein properties at $T > 220$ K. Equilibrium fluctuations

Studies of conformational substates (CS) were greatly promoted by X-ray dynamic analysis (XRDA), neutron scattering technique (NS), Mössbauer spectroscopy (MS), and Rayleigh scattering of Mössbauer radiation (RSMR), besides methods and results cited in Table 1. These techniques share the common principle that structural and dynamic properties of the substance being studied can be described by means of Van Hove's correlation functions borrowed from physics of solids [101]. These functions contain all spatial and temporal information about the substance (in our case, protein).

The above methods differ in terms of energy resolution, hence in the range of correlation times of specific protein motions. XRDA has energy resolution of 1 eV; therefore, this method yields no information on correlation times whatever and can not be used to distinguish between real motions and static disorder [102]. At the same time, XRDA gives a unique opportunity to obtain the general profile of dynamic properties of a given macromolecule by measuring individual mean-square displacements ($\langle x^2 \rangle$) of C, O, and N atoms.

The best neutron spectrometers have resolving powers of not more than $10^{-6}$ eV. Therefore, the neutron scattering technique yields data on protein dynamics within time intervals of less than 100 ps [102]. The information derived from neutron scattering experiments is averaged over all hydrogen atoms of a given macromolecule.

Mössbauer spectroscopy (MS) [102, 22] attains a resolution on the order of $10^{-9}$ eV; accordingly, it is sensitive to the motions of the Mössbauer nuclei (normally, these are nuclei of $^{57}$Fe present in the active center of the globule) in time intervals of less than 100 ns.

The authors of the present review have made an appreciable contribution to the development of a method based on Rayleigh scattering of Mössbauer radiation (RSMR) that is extensively used in on-going physical studies [102, 22, 25, 26, 103 – 105]. RSMR is a combination of X-ray structural analysis and Mössbauer spectroscopy with high (record) energy resolution equalling that of Mössbauer spectroscopy alone. Protein dynamics is normally investigated using two different experimental variants of the method. One is designed for the use under mild collimation conditions (the width the angular resolution function is 8 degrees). We employed this variant to measure a fraction of elastic scattering for different proteins and RSMR spectra [23, 25, 103]. This fraction known, the 'generalized' mean-square amplitude of protein motion displacements $\langle x^2 \rangle_R$ can be estimated while measurement of RSMR spectra allows us to determine correlation times of protein motions over a range from $10^{-7}$ to $10^{-9}$ s. The second variant of the method is used in rigorous collimation conditions (the width of the angular resolution function does not exceed 2 degrees). With the help of this approach, it is possible to have an idea of the size and shape of segments moving inside the globule based on angular dependences of inelastic scattering intensities [26, 104, 105].

**Table 1.** Experimentally observed 'crystal-like' and 'glass-like' properties of proteins at different temperatures.

| 'Crystal-like' properties of proteins | Glass-like properties of proteins | | |
|---|---|---|---|
| 1. Ability to restore the structure (stability).<br><br>2. The existence of definite stucture (conformation or fold) revealed by X-ray analysis [13].<br><br>3. High packing density [14] especially near the active centre.<br><br>4. Cooperativity of the denaturation process [15].<br><br>5. Mechanical properties of proteins (Young's modulus) resemble those of solids, such as molecular crystals and glassy polymers [16]. | The presence of energy landscape. The energy landscape changes depending on temperature and other external factors | | |
| | A. Temperature range $T < 160$ K | Transitional temperature zone ($160 < T < 220$ K) | C. $T > 220$ K |
| | 1. Non-exponential (power-law) CO-Mb rebinding kinetics at temperatures below 160 K (Fig. 5a). The non-exponential time-dependence arises because the height of the barrier to be overcome by CO in order to bind with Mb is different for each protein macromolecules. Quantity $g(H)\,dH$ determines the probability of finding a barrier in the $H + dH$ interval. Distributions $g(H)$ are first reported in this experiment (Fig. 5b) [40, 41].<br><br>2. Experiments on low-temperature heat capacity of proteins reveal a linear term $C_1 T$ in all biopolymers examined thus far [39, 86 – 89]. Specific heat of a crystalline metmyoglobin sample is illustrated by Fig. 6. $C_1$ decreases with increasing hydration level [89] (see Fig. 7).<br><br>3. Laser hole burning. Fig. 8 shows how a narrow line (or hole) can be burnt with a laser into a broadened spectral band of myoglobin [90]. | 1. Structural relaxation in proteins is non-exponential in time and non-Arrhenius in temperature [77, 91].<br><br>2. Metastable state of proteins. Metastability of a system implies that its state below the glass transition temperature depends on its history. Ref. [77] reports freezing (F) of a sample from the initial state (I) followed by pressurizing (FP). On the other pathway, the sample was first pressurized (P) and then frozen (PF). In equilibrium, the system's properties do not depend on the path (i.e. history); therefore, FP and PF should yield identical properties of the water-protein mixture. The authors measured the CO absorbtion band of a swMbCO sample imbedded in a 75% glycerol – 25% water mixture at the initial and final temperature $T_i = 225$ K and $T_f = 100$ K and pressure $P_i = 0.1$ MPa and $P_f = 200$ MPa respectively. The IR spectra obtained at two points (FP) and (PF) differed considerably [77]. This proves metastability of the protein below a certain critical temperature $T_c \approx 200$ K.<br><br>3. Glass transition in water-protein systems.<br>Phase transition near certain critical temperature $T_c \approx 200$ K in water-protein systems has been documented in calorimetric experiments [92 – 97]. At first glance, it is reminiscent of the glass-to-supercooled liquid transition in ordinary glass-forming liquids or glassy polymers. For this reason, $T_c$ is frequently referred to in the literature as the glass transition point $T_g$ (Fig. 10a). A thorough study of hydrated lysozyme [92] at a low heating rate around 0.05 K $min^{-1}$ has shown (Fig. 10b) that glass softens within a temperature range from 160 to 180 K and undergoes partial crystallization between 180 and 210 K. Notwithstanding an obvious analogy between 'vitrification' in water-protein systems and simple glasses, there is at least one essential difference due to the fact that part of the bulk water in a water-protein system behaves in a highlyunusual manner for glass transition. Namely, it first turns into a liquid but then undergoes crystallization during the course of transition as temperature increases. A further rise in temperature leads to cluster melting of this crystal water [25, 92]. | 1. Hydration dependence of $T_g$, $T_g = T_g(h)$. A few experiments reported thus far clearly indicate that glass transition temperature is a function of the macromolecule hydration level $T_g = T_g(h)$. It increases with decreasing hydration of crystalline lysozyme (Fig. 11) [96]. A similar dependence was documented for legumin [98], collagen and cytochrome C (see [99] for details). When hydration $h < 0.05 - 0.1$, the glass transition temperature is higher than room temperature (300 K); it rises to almost 400 K in case of complete dehydration [99]. |

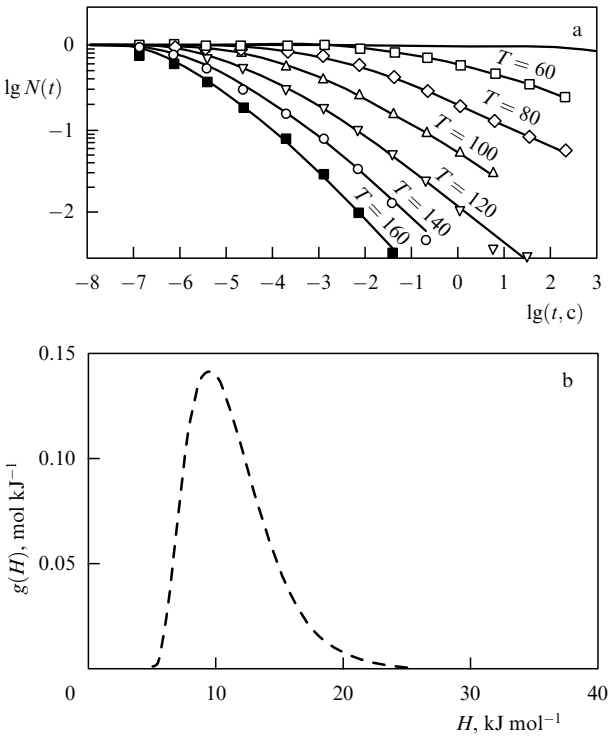


**Figure 5.** (a) CO-Mg rebinding kinetics after flash photolysis over a temperature interval from 60 to 160 K; (b) distribution function of activation enthalpy.

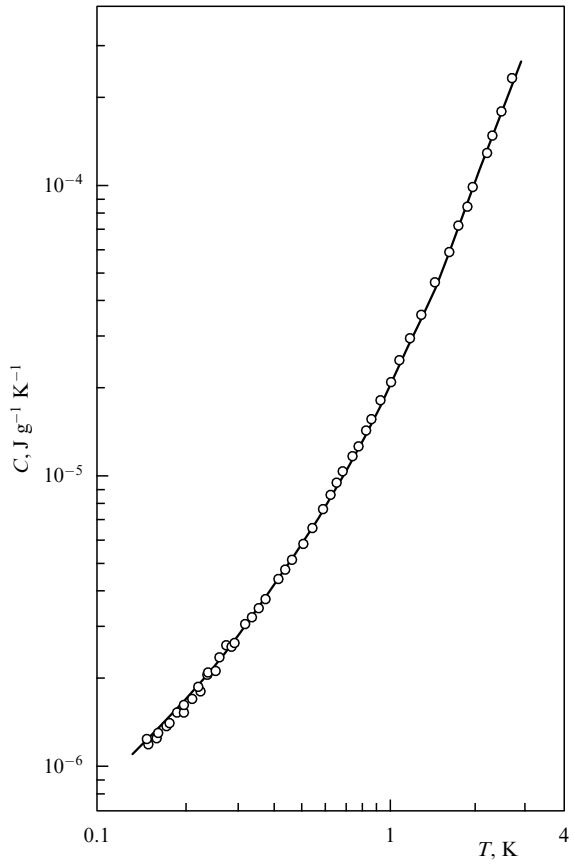


**Figure 6.** Temperature dependence of the specific heat of crystalline myoglobin.

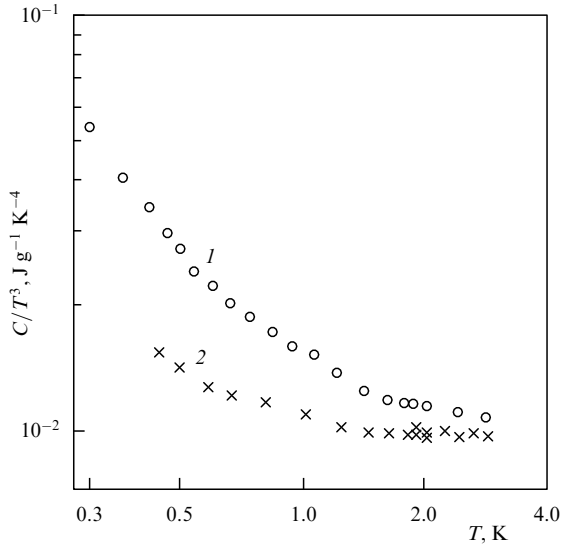


**Figure 7.** Specific heat for myoglobin samples containing either *1* — 47 % $H_2O$, *2* — 89 % $H_2O$.

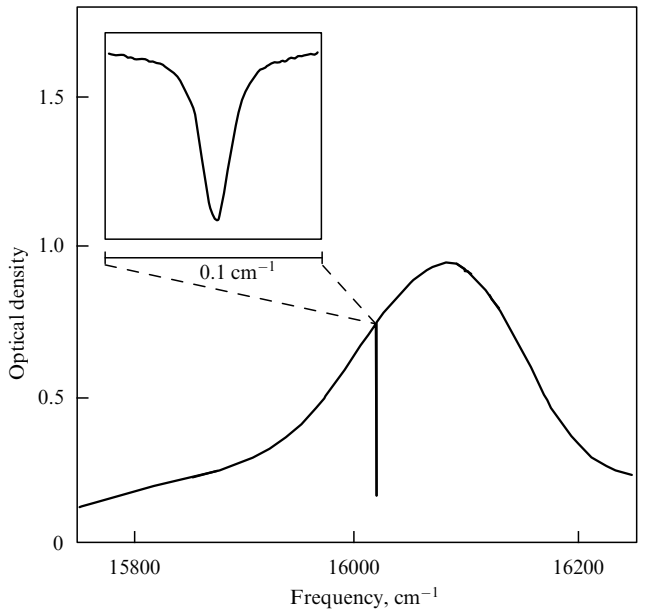


**Figure 8.** Laser burning of a narrow line (hole) into the broadened myoglobin spectral band.

**5.6.1 Results obtained by Mössbauer spectroscopy and Rayleigh scattering of Mössbauer radiation.** Mössbauer spectroscopy and Rayleigh scattering of Mössbauer radiation played an important role in the study of equilibrium fluctuations of macromolecular proteins. Here are the main experimental facts obtained with the use of MS and RSMR techniques under mild collimation conditions that await explanation [19–26].

The amplitude of mean-square displacements within the low-temperature range $\langle x^2 \rangle_{Fe}$ only slightly changes in an interval from 4.2 to 25 K [106, 107] but thereafter grows linearly (as determined by the fixed window method) with a temperature rise of up to 200 K [106, 108]. This temperature range is referred to as 'normal' because both the shape of MS and PSMR spectral lines and the behavior of $\langle x^2 \rangle_{Fe}$ and $\langle x^2 \rangle_{R}$

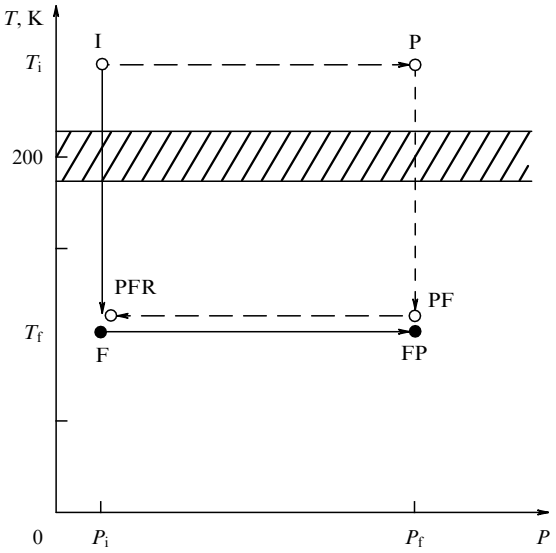


**Figure 9.** Schematic representation of an experiment to study history dependence of the system's properties. Reproduced from [77]. See Table 1 for details.

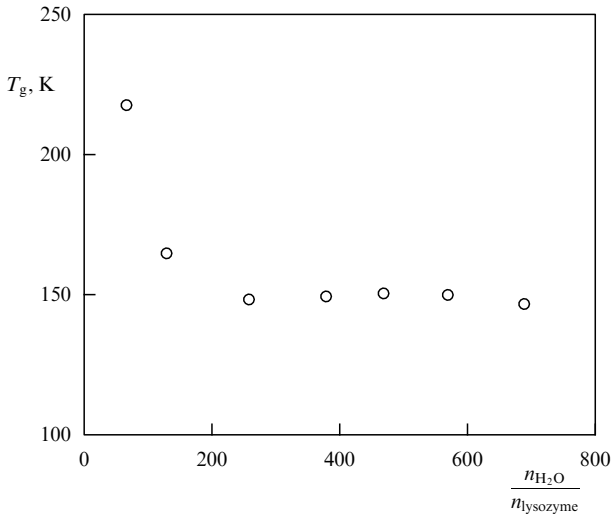


**Figure 11.** Hydration dependence of glass transition in lysozyme crystals [96].

in this range are analogous to variations of the same parameters for conventional solid bodies.

At a certain critical temperature $T_c > 200$ K, the Mössbauer effect $f'$ and elastic scattering fraction $f_R$ (in a fixed measurement window) sharply decrease for almost all studied proteins[5] (Fig. 13a).

Temperature $T_c \approx 200$ K at which a sharp rise in $\langle x^2 \rangle_{Fe}$ and $\langle x^2 \rangle_R$ begins is usually associated with glass transition temperature in water-protein systems $T_g$. A drop of $f'$ and $f_R$ is accompanied by a very peculiar modification of the spectral line; namely the so-called 'broad' component [19–25, 103, 108, 109] appears along with the 'narrow' one which results in the readily observable non-Lorentzian lineshape (Fig. 13b).

[5] Accordingly, $\langle x^2 \rangle_{Fe}$ and $\langle x^2 \rangle_R$ increase considerably and become significantly higher at room temperature than the vibration amplitude of atoms or atomic groups in dehydrated proteins or ordinary solids.

It is maintained that in this temperature range specific (i.e. other than usual harmonic vibrations) protein motions occur, and that they are responsible for the non-trivial behavior of $f'$ and $f_R$ and peculiar evolution of the lineshape. Initially, such specific behavior of $f'$ and $f_R$ as well as of Mössbauer and RSMR spectra was described with the help of the simplest models of phase transition and Brownian overdamped oscillator [20–22]. A comprehensive review of experimental results and models describing specific protein motions can be found in Refs [19–21, 110, 22, 25, 26].

Analysis undertaken in [20–26] based on different theoretical models has demonstrated at least two types of specific protein motions: 1) individual motions of small atomic groups (transitions between CS) with correlation times $\tau_c$ on the order of $10^{-9}-10^{-11}$ s and 2) complex cooperative motions of large atomic groups or parts of macromolecules with correlation times $\tau_c$ of the order of $10^{-7}-10^{-8}$ s. Both types occur as bounded diffusion and are 'liquid-like' at small times $t \ll \tau_c$ [22–26].

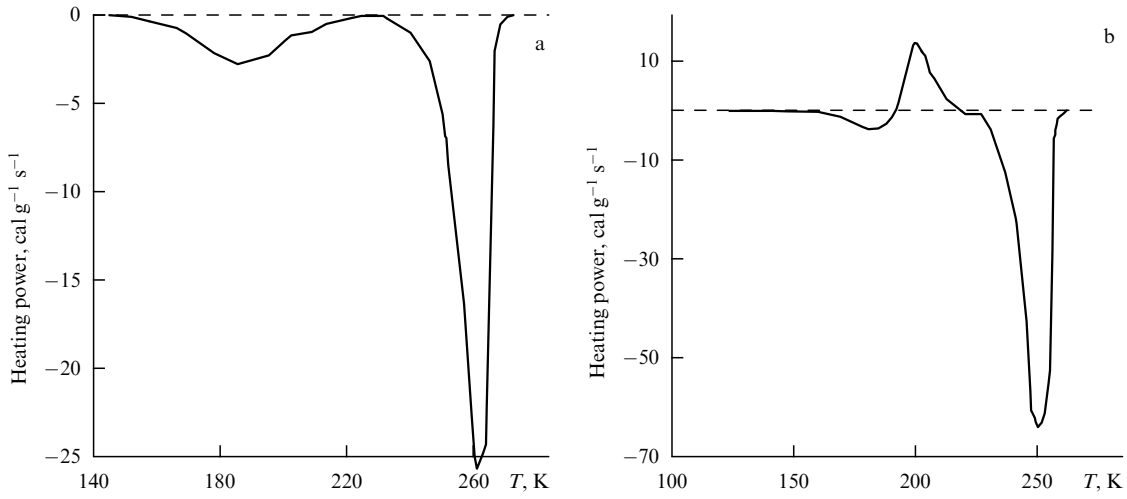


**Figure 10.** Calorimetric curve showing defreezing kinetics of hydrated lysozyme. Cooling rate of up to 77 K = 200 K min$^{-1}$. Defreezing rate: (a) 0.2 K min$^{-1}$, (b) 0.05 K min$^{-1}$. Reproduced from [92].

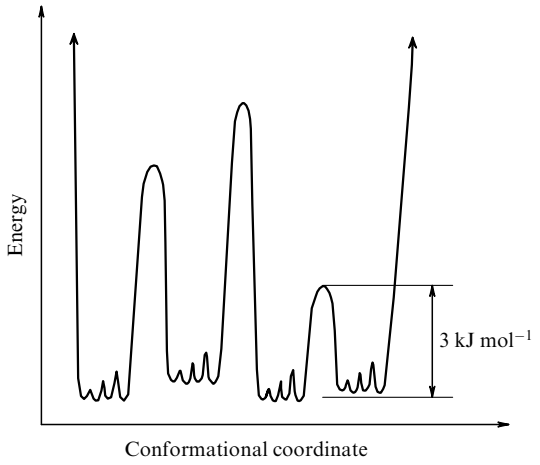


**Figure 12.** Schematic representation of myoglobin energy landscape over a range of up to 10 kJ mol$^{-1}$. The data are taken from [100].

The hierarchical organization of a protein structure is reflected in the hierarchy of amplitudes and correlation times of intramolecular movements. Evidently, the involvement of different atomic groups in intramolecular motions must be apparent as a wide spectrum of correlation times of intramolecular mobility $G(\tau)$ [20–22]. Indeed, lineshape evolution was described in later, more precise studies using a hierarchical Brownian oscillator model and taking into account correlation time spectrum $G(\tau)$ [21].

A relatively recent analysis of specific protein motions in myoglobin (by MS) and human serum albumin (by RSMR) was made using a jump diffusion model with the distributed waiting time for jump $\tau$ $G(\tau)$ [109]. The well-known Cole–Davidson, Cole–Cole, and Negami–Havriliak distribution functions [50] (or spectra of correlation times $\tau$) were chosen as distribution functions of the waiting time for jump $\tau$. Studies of angular dependences of inelastic scattering intensity for myoglobin and lysozyme [104, 105] by the RSMR technique under rigorous collimation conditions showed that complex cooperative motions are actually motions of secondary structure elements, i.e. alpha-helices in myoglobin, alpha-helices and beta-sheets in lysozyme [104, 105].

**5.6.2. Neutron scattering (NS).** Let us now turn to neutron scattering (NS) studies. Many recent reports [18, 111, 112] were devoted to 'generalized' protein dynamics. NS technique is, in certain respects, superior to the methods considered in previous Sections, e.g. RSMR. Specifically, it takes advantage of the fact that the cross section of the scattering from hydrogen is significantly larger than from deuterium. Due to this and in contrast to RSMR, if a protein is hydrated in $D_2O$, the experiment yields 'generalized' (averaged over the globule) information about the amplitude of mean-square displacements of hydrogen atoms of the protein alone, $\langle x^2\rangle_H$, rather than of the entire water-protein system. In Refs [18, 111], incoherent neutron scattering (to the energy resolution of $10^{-2}$ eV) was used to study $D_2O$-hydrated myoglobin ($h = 0.38$) within a temperature range from 4 to 350 K. An excellent resolution of elastic and inelastic scattering intensities was achieved at $T > 200$ K. These measurements were used to deduce temperature dependence $\langle x^2\rangle_H$. The behavior of $\langle x^2\rangle_H$ proved similar to that of $\langle x^2\rangle_{Fe}$ obtained by

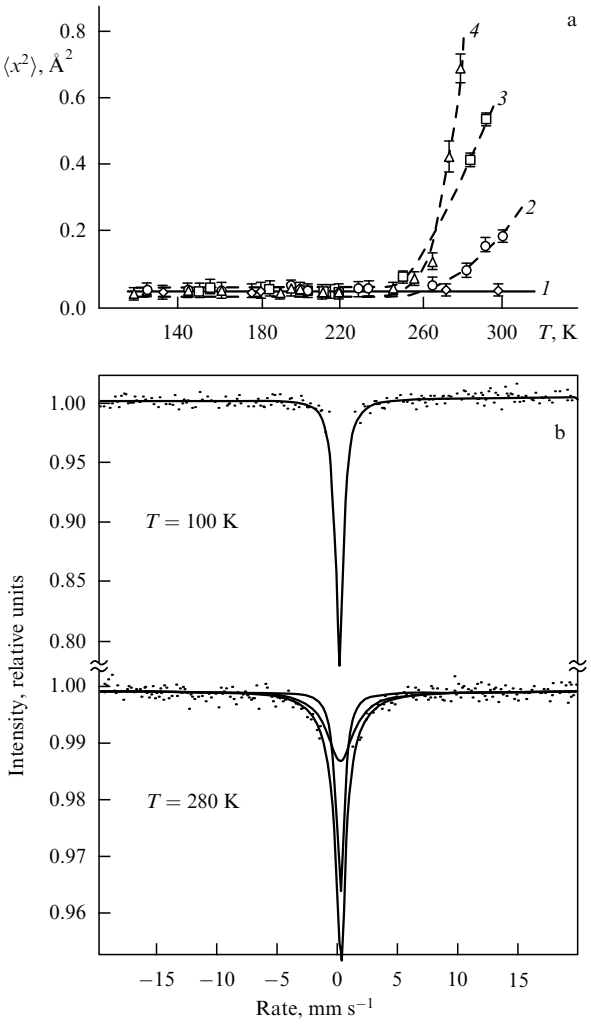


**Figure 13.** (a) Overall mean-square displacement vs temperature for different metmyoglobin samples: *1* — sample with hydration $h = 0.05$, *2* — polycrystal, *3* — $h = 0.5$, *4* — $h = 2.3$ [25]; (b) RSMR spectra for metmyoglobin with $h = 0.7$ at $T = 100$ K and $T = 280$ K [25].

Mössbauer spectroscopy and of $\langle x^2\rangle_R$ in RSMR. Over a range from 4 to 180 K, the behavior of elastic scattering intensity was analogous to that in a solid. The critical temperature $T_c$ or glass transition temperature in a protein macromolecule $T_g$ lay in the same temperature range as in RSMR and MS studies, that is between 180 and 200 K. The quasielastic scattering acquired importance at $T > 200$ K. The presence of the quasielastic component in NS spectra, as in Mössbauer and RSMR spectra, suggested specific protein motions that are usually simulated by diffusive-type motions. In the above studies [18, 111], incoherent NS was employed for the study of protein specific motions, their theoretical description using 'glass-like' models, and elucidation of the nature of transitions in proteins near 200 K.

The authors interpreted this data on the assumption of perfect analogy between dynamic properties of proteins and glasses (supercooled liquids) [18, 111]. They regarded critical temperature $T_c$ as the glass transition temperature $T_g$ and analysed the nature of transition near 200 K in the framework of a mode-coupling theory (MCT) suggested for the description of the glass-to-liquid phase transitions in simple glass-forming liquids [113]. The shape of the inelastic scattering

intensity line at $T > 180$ K was analysed either in terms of the aforementioned mode-coupling theory (MCT) or using the jump diffusion model with the distributed waiting time of a jump according to Cole and Davidson. It turned out that the mode-coupling theory fairly well describes NS data using only two adjustable parameters. Results of neutron scattering experiments are equally well described by the jump diffusion model with the distributed waiting time for jump $\tau$ and the Cole – Davidson function as the distribution function.

A recent study [112] provided information on the dynamic properties of distinguished parts of the bacteriorhodopsin (BR) molecule. The authors substituted deuterium for hydrogen in several spatial locations of BR. It was shown (see [112] and comments on this work in [114]) that $T_g$ averaged over the BR molecule equalled $T_g \approx 150$ K while $T_g$ near the active center was significantly higher, $T_g \approx 220$ K. This finding confirms our earlier inference that dynamic properties of the protein globule must be highly heterogeneous. In the case under consideration, the heterogeneity is manifest in that different parts of the protein molecule have different $T_g$ [39].

**5.6.3 X-ray dynamic analysis (XRDA).** There are already many reports on X-ray dynamic analysis of proteins in the current literature. Most of them used metmyoglobin as a model [41, 115]. The fundamental study of Frauenfelder and coworkers [41] was carried out in the temperature range from 220 to 300 K. In other studies, this interval was significantly extended to cover temperatures down to 40 K [115, 116]. The work of Frauenfelder at el. [41] was designed to obtain direct experimental evidence of conformational substates. Figure 14 borrowed from Ref. [114], shows mean-square deviations $\langle x^2 \rangle$ plotted as a function of the amino acid number in myoglobin (Mb) derived from the Debye – Waller factor for 80 K. The displacements are averaged over the $-N-C-C-$ backbone atoms of each amino acid. This picture is rather difficult to interpret in the harmonic approximation. Therefore, either the CS concept or structural distributions are used for the purpose [41, 116].

Mb macromolecules in different CS have somewhat different structures. Due to this, mean-square displacements $\langle x^2 \rangle$ obtained in the experiment for macromolecular ensembles of myoglobin are significantly greater than if they were due to atomic vibrations alone. The results of XRDA indicate that the number of CS is large in the loops and small along the α-helices of the Mb globule. Also, it shows strong temperature dependence. The following picture of protein macromolecule dynamics emerges from XRDA studies of met-Mb at room temperature: there is a 'solid' core (region with small $\langle x^2 \rangle \leqslant 0.04$ Å$^2$, where very few or no CS are observable) and 'liquid-like' outer shell $\langle x^2 \rangle \geqslant 0.04$ Å$^2$ in which the number of CS increases towards periphery of the globule. At room temperature, the 'liquid-like' shell is much bigger than the solid core. The size of the nucleus increases with decreasing temperature.

The CS concept implies that, at the transition temperature $T_g = T_c$ in the protein globule, each protein must be 'frozen' in its CS. At temperatures higher than $T_g$, atomic groups can transit from one CS to another. XRDA at $T < T_g$ reveals the 'frozen' distribution of atomic groups in different CS which accounts for $\langle x^2 \rangle \geqslant 0.04$ Å$^2$ of certain groups even at low temperatures (see Fig. 14). It should be recalled that enzymatic catalysis and performance of many other protein functions demand well-defined (ordered) mutual arrangement and orientation of amino acid residues in an active center [46]. Results of XRDA studies strongly confirm the presence of such active center. Arrows in Fig. 14 indicate five conserved amino acid residues in the Mb macromolecule (Leu-29, Phe-43, His-64, Val-68, and Ile-107) surrounding the oxygen molecule and His-93 bound to the iron atom on the proximal side of the heme group. These conserved residues are characterized by small $\langle x^2 \rangle$ (i.e. have no CS) and form, or rather belong to, the aperiodic solid core or active center of the macromolecule. The majority of the remaining amino acid residues on the proximal side of the heme group or far from it are not conserved; they are 'liquid-like' and have large $\langle x^2 \rangle$ (see Fig. 14).

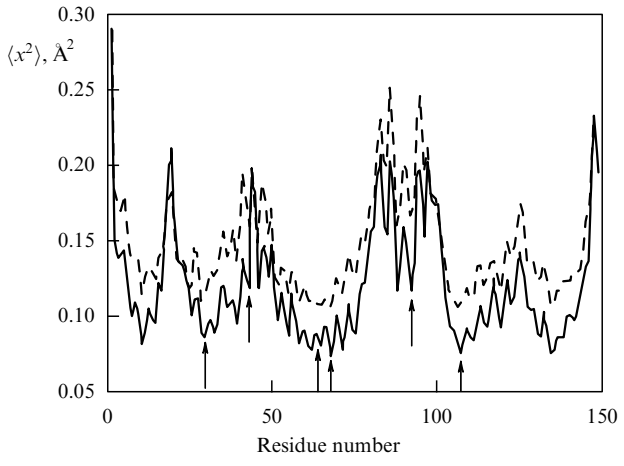


**Figure 14.** Mean-square deviations $\langle x^2 \rangle$ plotted as a function of the number of oxygen-bound (solid line) and unbound (dashed line) residues in myoglobin at 80 K. The displacements are averaged over the backbone atoms $-N-C-C-$ of each amino acid. Arrows indicate five distal conserved amino acid residues surrounding the heme pocket [114].

**5.6.4. How useful are simple harmonic models?** Experimental results summarized in Table 1 and discussed in Sections 5.6.1 – 5.6.3 give weighty arguments in support of conformational substates (CS) in macromolecules. In other words, they suggest an energetically degenerate ground state of native protein macromolecules. At the same time, there were many attempts to give an alternative explanation of this experimental data. Specifically, it was suggested that the macromolecule is in the global energy minimum and has a harmonic or near-harmonic potential energy surface. The authors used the quasi-harmonic approximation [117 – 122], normal mode approximation [120 – 124], and principal mode approximation [123] for their explanation. These approaches [117 – 124] are actually helpful in the treatment of some experimental findings, but great difficulties are encountered even in accounting for the totality of data obtained at temperatures above the critical value ($T > T_c = 200$ K) [125, 126].

All these works contain many criticisms of XRDA data for disregarding the contribution by the motion of the whole globule. It is supposed that, with this contribution given proper consideration, the absolute values of all mean-square displacements $\langle x^2 \rangle$ responsible for intramolecular mobility become much smaller and approach values characteristic of ordinary vibrations.

In our works [104, 105], RSMR, under rigorous collimation conditions, was used to study the angular dependence of inelastic scattering for myoglobin with the hydration degree

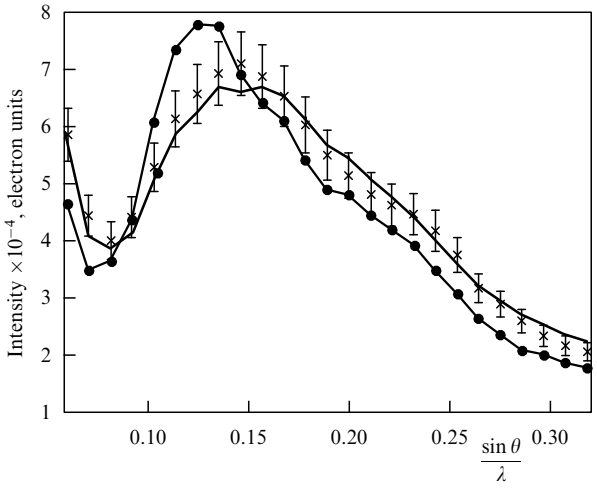


**Figure 15.** Angular dependence of the intensity of inelastic Rayleigh scattering of Mossbauer radiation for myoglobin hydrated to $h = 0.7$ (values expressed as measuring errors): solid line (best fit) - α-helices and individual motions are regarded as moving segments; – • – — motions of the macromolecule as a whole, $2\theta$ - scattering angle [104, 105].

$h = 0.7$ (Fig. 15). The calculated curve that best described experimental results contained contributions from the motions of α-helices, individual atoms, their small groups, and free water molecules, while the contribution by the motions of the macromolecule as a whole was much less important. We specially made one more attempt to describe the experimental curve on the assumption that the main type of motion was the motion of the whole globule. The result is presented in Fig. 15. The comparison of these two attempts indicates that the large contribution from intraglobular mobility of α-helices and individual motions of atoms and their small groups together with a smaller contribution by the motions of the macromolecule as a whole, much better describes the experimental data than the situation in which the most important contribution is made by the motions of the macromolecule as a whole. The smallness of the contribution from the motion of the whole globule to the RSMR data is also demonstrated in [127]. It is worthwhile to note that this conclusion does not hold for the results of X-ray analysis because RSMR reveals only motions with correlation times smaller than $10^{-7}$ s whereas X-ray data include even the contribution from the static disorder.

At low temperatures ($T < T_c$), the 'liquid-like' properties of protein macromolecules are much more prominent (see Table 1). Indeed, they are documented in a large number of studies, but it is practically impossible to explain the totality of experimental facts presented in Section 5 with the aid of models using different quasi-harmonic approximations [117 – 126].

**5.6.5. Spatio-temporal characteristics of specific protein motions.** MS, RSMS, NS, and XRDA techniques greatly promoted the development of the concept of conformational substates (CS) and complex energy landscape. XRDA allows the overall profile of macromolecule dynamic properties to be obtained from individual mean-square displacements $\langle x^2 \rangle$ of C, O, and N atoms. Accordingly, this method demonstrates the presence (and size) of the 'solid' core of the macromolecule (a region of small $\langle x^2 \rangle \leqslant 0.04$ Å$^2$ with very few or no CS containing the active center) and 'liquid-like' areas ($\langle x^2 \rangle \geqslant 0.04$ Å$^2$) with a large number of CS that further increases towards the outside of the globule.

'Vitrification' in the vicinity of $T_g$ results in defreezing a small fraction of conformational mobility of the macromolecule. However, RSMR studies indicate that the principal driving force of the defreezing process at $T > 230 - 240$ K is cluster melting of interprotein water [22, 25]. This explains why practically all analogies between proteins and glasses (except the presence of the energy landscape) vanish at $T \geqslant 230$ K.

Interpretation of the results of MS and RSMR studies, under mild collimation conditions indicates that at least two types (or rather the whole spectrum $G(\tau)$) of specific protein motions (besides ordinary ones intrinsic in solids) need to be introduced if the entire set of the available data is to be described. Individual motions of small atomic groups and cooperative motions of large ones can be distinguished in this spectrum. The individual motions are actually transitions between 'principally accessible CS' [20 – 22] and serve as 'lubricants' for large-scale collective motions of large atomic groups. Specific protein motions are akin to restricted diffusion and 'liquid-like' at small times $t \ll \tau_c$.

Investigations into angular dependences of inelastic scattering intensity for myoglobin and lysozyme using RSMR under rigorous collimation conditions [104, 105] have demonstrated that complex cooperative motions are actually motions of secondary structure elements, that is alpha-helices in case of myoglobin and alpha-helices and beta-sheets in case of lysozyme [104, 105]. Spatial characteristics of these motions and those of the whole globule are summarized in Table 2. The Table contains the following parameters: $A = N_s/N$, $B = N_i/N$, where $N_s$ is the number of atoms involved in segmental motions, $N_i$ the number of atoms involved in individual motions, $N$ — the total number of atoms in the macromolecule, $N_b$ — the number of bound water molecules, and $N_f$ — the number of unbound water molecules. For the purpose of data treatment, it was assumed that mean-square amplitudes of segmental and individual displacements equal $\langle x^2 \rangle_s = \langle x^2 \rangle_i$; $\langle x^2 \rangle_g$ is the mean-square

**Table 2.** Parameter values that best fit angular dependence curves for inelastic scattering of lysozyme and myoglobin.

| Parameters<br>Protein | $h$ | $A$ | $B$ | $\langle x^2 \rangle_g$, (Å$^2$) | $\langle x^2 \rangle_s = \langle x^2 \rangle_i$, (Å$^2$) | $\langle x^2 \rangle_{bw}$, (Å$^2$) | $N_b$ | $N_f$ |
|---|---|---|---|---|---|---|---|---|
| Lysozyme | 0.05 | — | — | 0.05 | — | —— | — | — |
| | 0.2 | 0.2 | 0.8 | 0.03 | 0.16 | 0.5 | 180 | — |
| | 0.45 | 0.2 | 0.8 | 0.03 | 0.3 | 0.78 | 346 | 54 |
| Myoglobin | 0.1 | — | — | 0.1 | — | — | — | — |
| | 0.44 | 0.4 | 0.6 | 0.04 | 0.39 | 0.56 | 250 | 200 |
| | 0.7 | 0.4 | 0.6 | 0.04 | 0.64 | 0.64 | 350 | 350 |

displacement amplitude of the globule as a whole; $\langle x^2 \rangle_{bw}$ is the mean-square displacement amplitude of bound water, and $h$ is the hydration degree.

In the myoglobin macromolecule, up to 40% and 60% of the atoms are involved in complex cooperative (segmental) and individual motions respectively ($A = 0.4$, $B = 0.6$) (see Table 2). In lysozyme, only 20% of the atoms are involved in complex collective (segmental) motions ($A = 0.2$) whereas 80% take part in individual motions ($B = 0.8$). Means-square displacement amplitudes of either type in lysozyme and myoglobin are 0.3 and 0.65 Å respectively. Analysis of RSMR spectra indicates that myoglobin displays low-frequency segmental motions of alpha-helices with correlation times of hundreds of nanoseconds [22, 103]. In lysozyme, intragobular motions occur with correlation times of fractions of nanoseconds or smaller than that [22, 103]. Myoglobin (α-protein) in the native state is significantly different from native lysozyme, a (α + β)-protein, in that the intramolecular mobility in the former is much more pronounced than in the latter. Different spatio-temporal characteristics of specific protein motions are due to dissimilar structural organization of the two proteins [104, 105].

## 6. Proteins as complex structures

### 6.1 Energy landscape and its hierarchical organization

Amino acid residues are linked to each other in the main protein chain by single bonds. The degrees of freedom due to rotation around single bonds whose potential energy has several minima and maxima [3], account for the complicated shape of the potential energy surface even in the simplest proteins. In a protein molecule that consists of some 200 amino acid residues, each assuming two alternative configurations, the number of possible isoenergetic states is $2^{200}$ or almost $10^{60}$ — an astronomical number. All these states are inhabited, provided the macromolecule is subject to no steric limitations.

For this reason, proteins have the energy landscape or potential (conformational) energy hypersurface [6, 7, 75, 128], unlike simpler systems, such as atoms or stiff molecules, characterized by energy levels the characteristic cross section through the protein hypersurface by energy levels $E \cong NkT/2$ is altogether different in terms of topological structure from the analogous hypersurfaces of rigid molecules. There are numerous unbound regions that give rise to conformational substates (CS) or local energy minima.

In what follows, we discuss organization of CS in a MbCO macromolecule (Fig. 16) [75, 128]. Protein macromolecules have hierarchical structural organization (tertiary, secondary, and primary structures are distinguished). Studies of protein energy landscapes have rather a short history and provided sufficient experimental data for only one protein, myoglobin. This data suggests that the energy landscape of MbCO macromolecules is organized in a hierarchy [75, 128, 129].

Figure 16 is a schematic representation of the hierarchical organization of the energy landscape in a MbCO molecule. Conformational states $(CS)_i$ shown in the figure correspond to the $i$-th organizational level. Hierarchy of conformational states suggests that energy barriers of tier 0 are significantly higher than those within tier 1, etc. The upper lane in Fig. 16 gives a classical representation of the MbCO macromolecule with the unique native structure and the unique energy level.

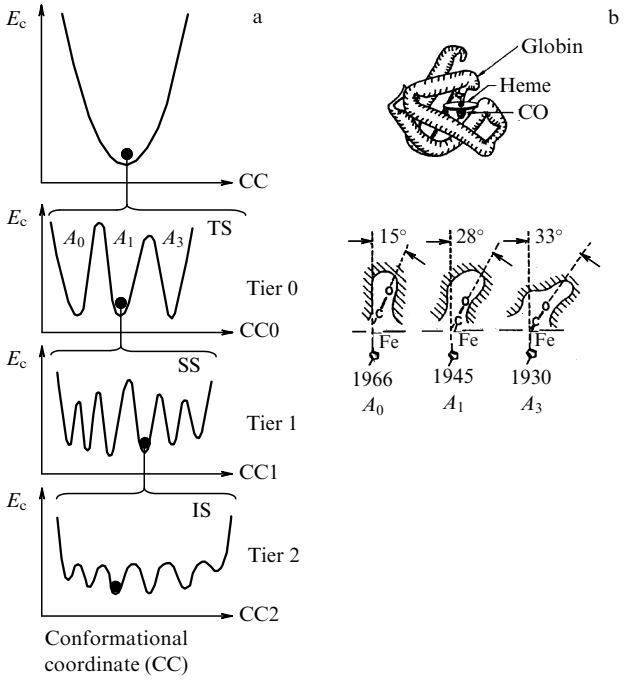


**Figure 16.** (a) Vastly oversimplified schematic representation of the hierarchical structure of MbCO macromolecule. Taxonomic substate (TS), statistical substates (SS), and individual substates (IS). (b) Illustration of the development of three substates of tier 0. See the text for details.

Results of infrared spectroscopy indicate that this representation is vastly simplified. The Mb-bound molecule of CO produces at least three absorption bands, each associated with a fairly distinct substate of tier 0. At least three such substates emerge from the experimental data. These substates differ in terms of orientation of CO binding to the heme surface (as shown in Fig. 16b). Also, they have different structures, dynamic and kinetic properties.

Because conformational states of tier 0 (CS0) are different and few in number (three of four according to different authors [75]), they are often referred to as taxonomic states. The difference between CS0 is of functional importance for MbCO, since it determines CO-myoglobin binding kinetics. The binding is especially fast in the $A_0$ substate but very slow in $A_3$. The existence of the next tier 1 of conformational substates (CS1) ensues from the non-exponential kinetics of CO rebinding to myoglobin (see Table 1, A1).

Experimental studies have demonstrated different CO binding kinetics in substates $A_0$, $A_1$, and $A_3$. Accordingly, the distribution functions $g(H)$ (see Table 1, A1) for each of these substates are also different. The presence of distributions $g(H)$ suggests a large number of conformational substates of tier 1. Moreover, the statistical approach to these substates appears relevant because the taxonomic approach is inapplicable. For this reason, CS1 of this tier are called statistical. It is believed that the available data on low-temperature heat capacity and laser hole burning (Table 1, A2 and A3) give evidence of the existence of tier 2 composed of conformational states (CS2). The appearance of a linear term in the low-temperature heat capacity is related to the phonon-assisted tunneling between CS2 of atomic groups. Tunneling between CS2 is possible only for small atomic groups, in the first place for those located on the periphery of the macromolecule where the number of CS2 is sufficiently large.

Evidently, the mobility of small peripheral atomic groups must have practically no effect on CO binding to the active center of the macromolecule. Ligand binding to the active center is regulated by the collective motions of large atomic groups (including the heme group) in the vicinity of the active center. Studies on low-temperature heat capacity and spectral diffusion reveal few conformational substates of tier 2. At low temperatures, a protein molecule is in one of the specific conformational substates of tier 2. At such temperatures, only small atomic groups are capable of tunneling between CS2. We call these passages individual motions of small atomic groups; hence, CS2 are referred to as individual substates. There are no transitions between CS of higher tiers. Activation transitions between principally accessible CS2 appear with increasing temperature. The term 'principally accessible CS' [24, 25] is used here on purpose, because steric limitations imposed by the high packing density of atomic groups in a protein macromolecule make some of its CS inaccessible at a given moment as being occupied by other atomic groups. This situation is reminiscent of the diffusion process in supercooled liquids [130].

The intensity of individual motions of small atomic groups increases dramatically at temperatures above 200 K; simultaneously, cooperative motions of large atomic groups become apparent between principally accessible conformational substates of tier 1 (CS1). In this process, individual motions serve as sort of 'lubricants' for large-scale cooperative motions. In other words, equilibrium fluctuations of a lower tier are 'lubricants' facilitating equilibrium fluctuations at the next (higher) hierarchical level. Thus, specific protein motions described in a previous paragraph occur at temperatures above 200 K. Many ideas underlying the exploration of hierarchically organized energy landscapes are and will be borrowed from physics of glasses and spin glasses [131, 132]. Figure 17a shows the same energy landscape as in Fig. 16 represented by means of a tree diagram [128].

### 6.2 Functionally important motions in proteins

Up to now, we considered equilibrium fluctuations. In this Section, emphasis is laid on functionally important motions.

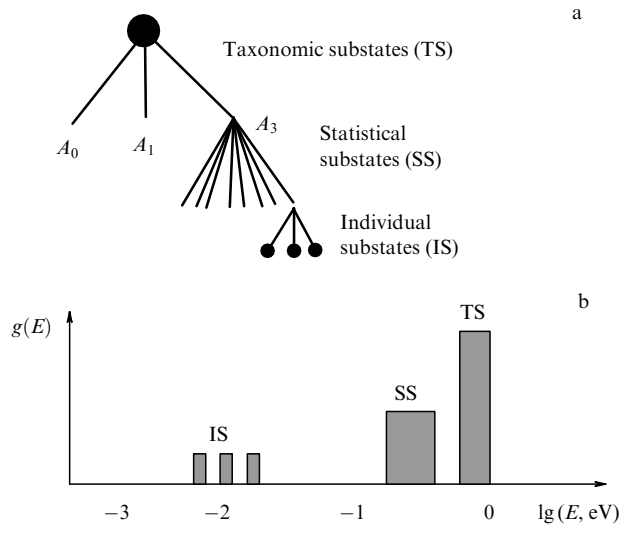


**Figure 17.** (a) Tree diagram showing energy landscape of a MbCO macromolecule (see Fig. 16) (b) approximate barriers between different substates.

By way of example, there is a MbCO dissociation reaction:

$$\mathrm{MbCO} + h\nu \rightarrow \mathrm{Mb}^* + \mathrm{CO} \rightarrow \mathrm{Mb} + \mathrm{CO}\,. \qquad (6.1)$$

The myoglobin molecule can exist in two equilibrium states differing in terms of Mb and MbCO conformation. Hence, there are two types of motion, equilibrium fluctuations (EF) and functionally important motions (FMI). Equilibrium fluctuations (EF) are motions within a given equilibrium state. They were considered at length earlier in this paper. Functionally important motions (FIM) serve for a protein to perform its biological function (in the case of myoglobin, conversion of MbCO to Mb and back) or to ensure transition from one equilibrium state to another [6]. Both EF and FIM need to be evaluated to better understand protein dynamic properties. Functionally important motions and equilibrium fluctuations in the simplest case of near-equilibrium systems are related by the fluctuation-dissipation theorem [70].

Reaction (6.1) appears to be very complicated as it goes in to details, but some of its features are perfectly clear even now. Photon absorption by the heme induces stress that is released after the bond between Fe and CO is broken. The energy binding Mb and the ligand is roughly equal to the protein denaturation energy, which accounts for the far-from-equilibrium state of the protein following the bond rupture [2, 129]. An elementary act of the simplest reaction under consideration consists of the conformational modification of the macromolecule, $\mathrm{MbCO} \Rightarrow \mathrm{Mb}$. The overall reaction rate depends on the rate of this conformational change [2] or relaxation rate $\mathrm{Mb}^* \Rightarrow \mathrm{Mb}$. Functionally important motions facilitate the relaxation of myoglobin from the excited state $\mathrm{Mb}^*$ to the ground state Mb.

Because a protein macromolecule in the excited state is far from equilibrium, the relationship between functionally important motions and equilibrium fluctuations is much more complicated than for near-equilibrium systems. During relaxation from the excited ($\mathrm{Mb}^*$) to ground (Mb) state, all types of motions are consecutively involved, from vibrational motions (e.g. occurring in solids) that are apt to significantly alter the heme-ligand binding rate, to individual motions of small atomic groups within tier 2 ('lubricants' facilitating collective motions of larger atomic groups of tier 1). As a result, the relaxation process starts at the active center and then expands over the periphery of the macromolecule.

Jortner and coworkers [133] have demonstrated that relaxation in hierarchical dynamic systems must exhibit oscillations noticeable in coordinates $\mathrm{d}\lg(f(t))/\mathrm{d}\lg(t)$ of time $t$, where $f(t)$ is the relaxation function. Anfinrud et al. [134] have studied myoglobin relaxation after photodissociation that extended over about six orders of magnitude in time. If the given relaxation curve is plotted in the above coordinates [128], the predicted oscillations can be observed; this also gives evidence of the hierarchy of myoglobin dynamical organization.

### 6.3 Complexity of proteins

Physicists have recently taken an increasing interest in the so-called 'complex' systems, in the first place glasses and spin glasses. These systems are characterized by the highly degenerate ground state and non-ergodicity. All that is

[6] Evidently, certain motions leading to the transition from one equilibrium state to another can not be regarded as functionally important.

known about proteins at large, including the presence of a hierarchically organized landscape gave authors of certain reviews reason to regard these substances as 'complex' systems just because they bear resemblances to glasses, spin glasses, and other materials with the disordered structure [7, 8, 135]. Intuitively, however, it is clear that such biological systems as the cell, brain, and organism are immeasurably more complicated than glasses and spin glasses. It is worthwhile to note that von Neiman predicted, as early as the middle of the last century, that the search for the sense of the notion of complexity would be a central problem of 20th century science as the search for the sense of energy and entropy was in the 19th century.

Nevertheless, many authors up to now use different definitions of 'complexity' and even put different sense into the term [136, 137]. The notion of 'complexity' is most explicitly introduced into condensed state physics, where a clear-cut distinction is made between 'simple' (crystals with a fixed atomic composition) and 'complex' (liquids, amorphous substances, glasses) systems. The 'complexity' of these systems arises from the disorderedness accounting for their properties. A measure of complexity also exists in the theory of information. An example is Shannon's entropy [38]. The canonical theory of information completely ignores information content. However, there are many scientific disciplines, such as biology, where the value of information is of paramount importance. According to Bongard, the value of information is described by formula [38]

$$V = \log_2 \left( \frac{p^1}{p} \right), \tag{6.2}$$

where $p$ and $p^1$ are the probabilities of attaining the goal before and after the relevant information is received, respectively. Redundant or repeated information has no value at all because it neither increases nor decreases the probability of attaining the goal. Hence, the value of information is interpreted as its nonredundancy and indispensability [38].

Under 'complexity' in the theory of information is understood a minimal number of binary data containing all information about a given object sufficient for its reproduction (decoding) [138, 140]. This definition of complexity is similar to the definition of the value of a message as its indispensability [38].

The genetic information is coded in the form of a four-letter alphabet in a strand of DNA. One gene contains all information necessary for the synthesis of one protein chain. In the 'postgenomic' era, genetic codes of many (almost 5,000,000) proteins became known including identical proteins of different species. This permits their evolution to be traced.

In the evolutionary context, many point mutations in which one amino acid residue is exchanged for another are neutral, that is they fail to modify protein properties. By comparing point mutations in a given protein, it is possible to say which amino acids are essential for its biological function and which are of minor importance. It has been shown that four conserved amino acid residues in cytochromes C are crucial for the self-organization of macromolecules in this protein family [141]. Five conserved amino acid residues are equally important for the functioning of myoglobin (see Fig. 14) [114]. Evidently, conserved amino acids are of special value in the primary sequence of a protein. The most important information is associated with the active center and conserved amino acid residues of each protein macromolecule.

Valuable genetic information is contained in the DNA chain. After its reception by a cellular system responsible for protein biosynthesis, the newly-produced macromolecules also acquire valuable information concentrated in the primary amino acid sequence close to the active center. This information-bearing structure determines both the spatial arrangement and the function of the protein macromolecule. Because the most valuable information is stored in and around the active center, its structural organization is characterized by a high degree of ordering. The spatial structure of other parts of the macromolecule is less ordered, and its ordering decreases with the distance from the active center due to the appearance of conformational substates, the number of which is highest on the periphery of the macromolecule. Hence, specificity of protein 'complexity' which is due not only to the presence of a complex hierarchical energy landscape (as in glasses and spin glasses) but also to informational 'complexity, i.e. storage of valuable information.

Unlike glasses and spin glasses, proteins are readily reproduced under laboratory conditions. Owing to this, a protein macromolecule is itself a unique laboratory for the study of 'complex' systems.

## 7. Conclusion and outlook

This review presents the emerging picture of protein dynamical organization wholly based on a limited volume of experimental data. To-day, it is sufficient to judge only about the structural arrangement of a very simple protein, myoglobin, and to a lesser extent of lysozyme. It may be supposed that the molecular organization of protein globules, as outlined in this paper, holds for proteins of different mass and structural complexity. More experimental and theoretical work is needed to clarify this issue. The main objective of experiments must be the elucidation of spatio-temporal characteristics of specific protein motions for a large number of proteins differing in mass and structural organization. Until quite recently, very few theoretical predictions and experimental studies of energy landscapes (hence, dynamic and functional properties of protein macromolecules) have been reported. Therefore, any advance in this field should be encouraged.

The authors are grateful to V N Fleurov, K V Shaitan, A Yu Grosberg, D S Chernavskii, M I Klinger, L A Blyumenfel'd, H Frauenfelder, and A B Rubin for many valuable comments and fruitful discussions.

The work was done with the financial support of the Russian Foundation for Basic Research (project 00-04-48161) and Program for the Support of Scientific Schools (project 00-15-97392).

## References

1. Schrödinger E *What is Life? The Physical Aspect of the Living Cell* (Cambridge: University Press, 1948) [Translated into Russian (Moscow: Atomizdat, 1972)]
2. Blyumenfel'd L A *Problemy Biologicheskoĭ Fiziki* 2nd ed. (Problems of Biological Physics) (Moscow: Nauka, 1977) [Translated into English (Berlin: Springer-Verlag, 1981)]
3. Vol'kenshtein M V *Biofizika* (Biophysics) (Moscow: Nauka, 1988)
4. Rubin A B *Biofizika* (Biophysics) (Moscow: Izd. Mosk. Univ., 1999)

5. Chernavskiĭ D S, Chernavskaya N M *Belok-Mashina*: *Biologicheskie Makromolekulyarnye Konstruktsii* (Protein-Machine: Biological Macromolecular Structures) (Moscow: Izd. Mosk. Univ., 1999)
6. Shaĭtan K V *Biofizika* **39** 949 (1994) [*Biophysics* **39** 993 (1994)]
7. Frauenfelder H, Wolynes P G *Phys. Today* **47** (2) 58 (1994)
8. Frauenfelder H, Wolynes P G, Austin R H *Rev. Mod. Phys.* **71** S419 (1999)
9. White A et al. *Principles of Biochemistry* 6th ed. (New York: McGraw-Hill, 1978) [Translated into Russian (Moscow: Mir, 1981)]
10. Ptitsyn O B, Finkelstein A V *Quart. Rev. Biophys.* **13** 339 (1980)
11. Finkel'shteĭn A V et al. *Biofizika* **44** 980 (1999) [*Biophysics* **44** 946 (1999)]
12. Finkelstein A V, in *Protein Folding, Evolution, and Design* (Proc. of the Intern. School of Physics 'Enrico Fermi', Course 165, Eds R A Broglia, E I Shakhnovich, G Tiana) (Amsterdam: IOS Press, 2001) p. 249
13. Huber R *Nature* **280** 565 (1979)
14. Richards F M *Annu. Rev. Biophys. Bioeng.* **6** 151 (1977)
15. Privalov P L *Biofizika* **32** 742 (1987)
16. Morozov V N, Morozova T Ya *Mol. Biologiya* **17** 577 (1983)
17. Abaturov L A, Lebedev Yu A, Nosova N T *Mol. Biologiya* **17** 543 (1983)
18. Doster W, Cusack S, Petry W *Nature* **337** 754 (1989)
19. Parak F, Frolov E N, Moessbauer R L, Goldanskii V I *J. Mol. Biol.* **145** 825 (1981)
20. Shaĭtan K V, Rubin A B *Biofizika* **30** 517 (1985)
21. Parak F, Heidemeier J, Knapp W, in *Biological and Artificial Intelligence Systems* (Eds E Clementi, S Chin) (Leiden: ESCOM, 1988) p. 23
22. Krupyanskiĭ Yu F et al. *Biofizika* **32** 761 (1987)
23. Krupyanskii Yu F et al. *Z. Naturforsch. C* **37** 57 (1982)
24. Goldanskii V I, Krupyanskii Y F, Fleurov V N *Phys. Scripta* **33** 527 (1986)
25. Goldanskii V I, Krupyanskii Yu F *Quart. Rev. Biophys.* **22** 39 (1989)
26. Krupyanskii Yu F et al. *Hyperfine Interact.* **53** 59 (1990)
27. Anfinsen C *Science* **181** 223 (1973)
28. Levinthal C *J. Chim. Phys.* **65** 44 (1968)
29. Shulz G E, Schirmer R H *Principles of Protein Structure* (New York: Springer-Verlag, 1979)] [Translated into Russian (Moscow: Mir, 1982) p. 181]
30. Govindarajan S, Goldstein R A *Proc. Natl. Acad. Sci. USA* **95** 5545 (1998)
31. Cooper A *Proc. Natl. Acad. Sci. USA* **73** 2740 (1976)
32. *II S'ezd Biofizikov Rossii, 23–27 Aug. 1999, Moscow. Tezisy Dokladov* (II Congress of Russian Biophysicists. Abstracts) (Ed. A B Rubin) (Pushchino: ONTI PNTs RAN, 1999)
33. *Proc. of the 3rd European Biophysics Congress, Munich, Germany, Sept. 9–13, 2000*; *Eur. Biophys. J.* **29** 239 (2000)
34. *4th Intern. Conf. on Biological Physics, ICBP-2001, Kyoto, Japan, July 30–Aug. 3, 2001*, Abstracts (2001)
35. Alberts B et al. *Molecular Biology of the Cell* (New York: Garland Publ., 1983) [Translated into Russian: Vol. 1 [Moscow: Mir, 1987)]
36. Khurgin Yu I, Chernavskiĭ D S, Shnol' S E *Biofizika* **32** 775 (1987)
37. Kastler H *Vozniknovenie Biologicheskoĭ Organizatsii* (Origin of Biological Organization) (Moscow: Mir, 1967)
38. Vol'kenshtein M V *Entropiya i Informatsiya* (Entropy and Information) (Moscow: Nauka, 1986)
39. Gol'danskiĭ V I, Krupyanskiĭ Yu F, Flerov V N *Dokl. Akad. Nauk SSSR* **272** 978 (1983)
40. Austin R H et al. *Biochemistry* **14** 5355 (1975)
41. Frauenfelder H, Petsko G A, Tsernoglou D *Nature* **280** 558 (1979)
42. Gol'danskiĭ V I, Krupyanskiĭ Yu F, Flerov V N *Mol. Biologiya* **17** 532 (1983)
43. Anderson P W, Halperin B I, Varma C M *Philos. Mag.* **25** 1 (1972)
44. Phillips W A *J. Low Temp. Phys.* **7** 351 (1972)
45. Grosberg A Yu, Khokhlov A R *Statisticheskaya Fizika Makromolekul* (Statistical Physics of Macromolecules) (Moscow: Nauka, 1989) [Translated into English (New York: AIP Press, 1994)]
46. Ptitsyn O B, Volkenstein M V *J. Biomol. Struct. Dyn.* **4** 137 (1986)
47. Klinger M I *Comments Cond. Matter Phys.* **16** 137 (1992)
48. *Fizicheskiĭ Entsiklopedicheskiĭ Slovar'* (Physical Encyclopedic Dictionary) (Ed.-in-Chief B A Vvedenskiĭ) (Moscow: Sovetskaya Entsiklopediya, 1960)
49. Vollmayr K, Kob W, Binder K *J. Chem. Phys.* **105** 4714 (1996)
50. Böttcher C J F, Bordewijk P *Theory of Electric Polarization* Vol. 2, 2nd ed. (Amsterdam: Elsevier Sci. Publ. Co., 1978)
51. Bässler H *Phys. Rev. Lett.* **58** 767 (1987)
52. Davidson D W, Cole R H *J. Chem. Phys.* **18** 1417 (1950)
53. Nienhaus G U, Parak F *Hyperfine Interact.* **90** 243 (1994)
54. Dobson C M, Ptitsyn O B *Curr. Opin. Struct. Biol.* **7** 1 (1997)
55. Ptitsyn O B *J. Protein Chem.* **6** 273 (1987)
56. Dobson C M, Karplus M *Curr. Opin. Struct. Biol.* **9** 92 (1999)
57. Fersht A R *Curr. Opin. Struct. Biol.* **7** 3 (1997)
58. Shakhnovich E I *Curr. Opin. Struct. Biol.* **7** 29 (1997)
59. Brooks C L III et al. *Proc. Natl. Acad. Sci. USA* **95** 11037 (1998)
60. Onuchic J N, Luthey-Schulten Z, Wolynes P G *Annu. Rev. Phys. Chem.* **48** 545 (1997)
61. Brockwell D J, Smith D A, Radford S E *Curr. Opin. Struct. Biol.* **10** 16 (2000)
62. Grosberg A Yu *Usp. Fiz. Nauk* **167** 129 (1997) [*Phys. Usp.* **40** 125 (1997)]
63. Finkel'shteĭn A V, Badretdinov A Ya *Mol. Biologiya* **31** 469 (1997) [*Mol. Biol.* **31** 391 (1997)]
64. Kim P S, Baldwin R L *Annu. Rev. Biochem.* **51** 459 (1982)
65. Bryngelson J D , Wolynes P G *Proc. Natl. Acad. Sci. USA* **84** 7524 (1987)
66. Go N, Abe H *Biopolymers* **20** 991 (1981)
67. Shakhnovich E I, Gutin A M *Biophys. Chem.* **34** 187 (1989)
68. Abkevich V I, Gutin A M, Shakhnovich E I *Biochemistry* **33** 10026 (1994)
69. Karplus M, Shakhnovich E, in *Protein Folding* (Ed. T E Greighton) (New York: W.H. Freeman and Co., 1992) p. 127
70. Landau L D, Lifshitz E M *Statisticheskaya Fizika* (Statistical Physics) (Moscow: Nauka, 1964) [Translated into English (Oxford: Pergamon Press, 1969)]
71. Ellis R J, Hartl F U *Curr. Opin. Struct. Biol.* **9** 102 (1999)
72. Feldman D E , Frydman J *Curr. Opin. Struct. Biol.* **10** 26 (2000)
73. Spirin A S, in *II S'ezd Biofizikov Rossii, 23–27 Aug. 1999, Moscow. Tezisy Dokladov* (II Congress of Russian Biophysicists. Abstracts) (Ed. A B Rubin) Vol. 1 (Pushchino: ONTI PNTs RAN, 1999) p. 5
74. Hardesty B, Tsalkova T, Kramer G *Curr. Opin. Struct. Biol.* **9** 111 (1999)
75. Frauenfelder H, Sligar S G, Wolynes P G *Science* **254** 1598 (1991)
76. Frauenfelder H et al. *Proc. Natl. Acad. Sci. USA* **98** 2370 (2001)
77. Frauenfelder H et al. *J. Phys. Chem.* **94** 1024 (1990)
78. Beece D et al. *Biochemistry* **19** 5147 (1980)
79. Rupley J A, Careri G *Adv. Protein Chem.* **41** 37 (1991)
80. Krupyanskiĭ Yu F et al. *Mol. Biologiya* **20** 1356 (1986)
81. DeVault D, Chance B *Biophys. J.* **6** 825 (1966)
82. Murzin A G, Finkelstein A V *J. Mol. Biol.* **204** 749 (1988)
83. Kendrew J C et al. *Nature* **181** 662 (1958)
84. Privalov P L et al. *J. Mol. Biol.* **190** 487 (1986)
85. Strambini G B, Gabellieri E *Biophys. J.* **70** 971 (1996)
86. Singh G et al. *Z. Phys. B* **55** 23 (1984)
87. Verkin B I et al. *Fiz. Nizk. Temp.* **10** 1225 (1984)
88. Yang I-S, Anderson A C, Preprint Univ. of Illinois, IL -(CM)-87-02 (1987)
89. Yang I-S, Anderson A C *Phys. Rev. B* **34** 2942 (1986)
90. Friedrich J *Methods Enzymol.* **246** 226 (1995)
91. Iben I E T et al. *Phys. Rev. Lett.* **62** 1916 (1989)
92. Barkalov I M, Bolshakov A I, Goldanskii V I, Krupyanskii Yu F *Chem. Phys. Lett.* **208** 1 (1993)
93. Sartor G et al. *J. Phys. Chem.* **96** 5133 (1992)
94. Sartor G, Hallbrucker A, Mayer E *Biophys. J.* **69** 2679 (1995)
95. Miyazaki Y, Matsuo T, Suga H *Chem. Phys. Lett.* **213** 303 (1993)
96. Miyazaki Y, Matsuo T, Suga H *J. Phys. Chem. B* **104** 8044 (2000)
97. Doster W et al. *Biophys J.* **50** 213 (1986)
98. Sochava I V, Smirnova O I *Food Hydrocolloids* **6** 513 (1993); *Biofizika* **36** 725 (1991)
99. Angell C A *Science* **267** 1924 (1995)
100. Leeson D T et al. *J. Phys. Chem. B* **101** 6331 (1997)
101. Van Hove L *Phys. Rev.* **95** 249 (1954)
102. Moessbauer R L *Hyperfine Interact.* **33** 199 (1987)
103. Krupyanskii Yu F, Goldanskii V I, in *Protein Folding, Evolution, and Design* (Proc. of the Intern. School of Physics "Enrico Fermi",

Course 165, Eds R A Broglia, E I Shakhnovich, G Tiana) (Amsterdam: IOS Press, 2001) p. 25
104. Krupyanskii Yu F et al. *J. Biol. Phys.* **28** 139 (2002)
105. Krupyanskii Yu F et al. *Khim. Fiz.* **22** 41 (2003)
106. Dwiwedi A, Petersen T, Debrunner P G *J. Phys. Colloq.* (Paris) **40** C2-531 (1979)
107. Wise H, Debrunner P, Wagner G, Preprint Univ. of Illinois, IL – (Ex) – 84 (1984)
doi 108. Chang I et al. *Chem. Phys.* **212** 221 (1996)
109. Krupyanskii Yu et al. *Eur. Biophys. J.* **26** 25 (1997)
110. Chernavskii D S et al. *Proc. Natl. Acad. Sci. USA* **77** 7218 (1980)
doi 111. Doster W, Cusack S, Petry W *Phys. Rev. Lett.* **65** 1080 (1990)
doi 112. Réat V et al. *Proc. Natl. Acad. Sci. USA* **95** 4970 (1998)
doi 113. Götze W, Sjogren L *Rep. Prog. Phys.* **55** 241 (1992)
doi 114. Frauenfelder H, McMahon B *Proc. Natl. Acad. Sci. USA* **95** 4795 (1998)
115. Parak F et al. *Eur. Biophys. J.* **15** 237 (1987)
116. Parak F, Frauenfelder H *Physica A* **201** 332 (1993)
117. Bialek W, Goldstein R F *Biophys. J.* **48** 1027 (1985)
118. Goldstein R F, Bialek W *Comm. Mol. Cell. Biophys.* **3** 407 (1986)
119. Bialek W, Onuchic J N *Proc. Natl. Acad. Sci. USA* **85** 5908 (1988)
120. Case D A *Curr. Opin. Struct. Biol.* **4** 285 (1994)
121. Seno Y, Go N *J. Mol. Biol.* **216** 111 (1990)
122. Kidera A, Go N *J. Mol. Biol.* **225** 457 (1992)
doi 123. Hayward S, Go N *Annu. Rev. Phys. Chem.* **46** 223 (1995)
doi 124. Zach C et al. *Hyperfine Interact.* **126** 83 (2000)
125. Melchers B et al. *Biophys. J.* **70** 2092 (1996)
doi 126. Kitao A, Hayward S, Go N *Proteins* **33** 496 (1998)
127. Krupyanskii Yu F et al. *Biofizika* **33** 401 (1988) [*Biophysics* **33** 430 (1988)]
doi 128. Frauenfelder H, McMahon B H *Ann. Phys.* (Leipzig) **9** 655 (2000)
129. Ansari A et al. *Proc. Natl. Acad. Sci. USA* **82** 5000 (1985)
130. Barlow A J, Lamb J, Matheson A J *Proc. R. Soc. London Ser. A* **292** 322 (1966)
131. Zallen R *The Physics of Amorphous Solids* (New York: Wiley, 1983)
132. Fischer K H, Hertz J A *Spin Glasses* (Cambridge: Cambridge Univ. Press, 1991)
doi 133. Metzler R, Klafter J, Jortner J *Proc. Natl. Acad. Sci. USA* **96** 11085 (1999)
doi 134. Jackson T A, Lim M, Anfinrud P A *Chem. Phys.* **180** 131 (1994)
135. Frauenfelder H *Nature Struct. Biol.* **2** 821 (1995)
136. Guerra F, Peliti L, Vulpiani A, in *Measures of Complexity*: *Proc. of the Conf., Rome, Sept. 30 – Oct. 2, 1987* (Lecture Notes in Physics, 314, Eds L Peliti, A Vulpiani) (Berlin: Springer-Verlag, 1988)
137. Parisi G, in *Measures of Complexity*: *Proc. of the Conf., Rome, Sept. 30 – Oct. 2, 1987* (Lecture Notes in Physics, 314, Eds L Peliti, A Vulpiani) (Berlin: Springer-Verlag, 1988)
138. Kolmogorov A N *Problemy Peredachi Informatsii* **1** (1) 3 (1965); in *Novoe v Zhizni, Nauke, Tekhnike* (Ser. Matematika, Kibernetika, Vyp. 1) [News in Life, Science, Technique (Ser. Mathematics, Cybernetics, Issue 1)] (Moscow: Znanie, 1991) p. 24
139. Chaitin G *J. Assoc. Comput. Mach.* **13** 547 (1966)
doi 140. Adami C, Ofria Ch, Collier T C *Proc. Natl. Acad. Sci. USA* **97** 4463 (2000)
doi 141. Ptitsyn O B *J. Mol. Biol.* **278** 655 (1998)