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Mechanical denaturation of globular protein in the solid state

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A method for taking stress-strain diagrams in microsamples prepared from glutaraldehyde-treated monocrystals and amorphous films of hen egg-white lysozyme has been developed. Analysis of the diagrams has shown that the deformation obeys Hooke's law within 0–2%. Upon further deformation of a crystalline sample (up to 6–10%), when a critical stress, σ_{cr}, is reached, the protein molecules in the sample denature and become greatly extended. Depending on the crystal type and crystallographic direction, the sample length increases 2–4 times. The critical stress is essentially dependent on the factors affecting intra- and intermolecular interactions: temperature, hydration level and urea concentration. Mechanisms for mechanical denaturation are proposed.

1. Introduction

Mechanical load and deformation are factors which proteins are subjected to both in living organisms and when used in biotechnological processes. To study how such factors influence the functional properties of enzymes, the latter have been subjected to deformation in a hydrodynamic field [1,2], or have been deformed in the immobilized state together with a polymer matrix [3,4]. These studies have established a strong reversible change in the chemical activity of enzymes in response to a mechanical influence, but there are some difficulties in interpreting this effect at the molecular level because of the absence of any uniformity in the arrangement of protein molecules in the samples and the uncertainty in distribution of stress and strain at the level of a protein globule. In this respect, protein monocrystals seem to be a more suitable material for mechano-chemical studies. The ordered packing of molecules in a crystal provides a uniform distribution of load between the molecules, thus permitting the force applied to each individual molecule to be calculated. The crystals permit investigation of the effects of deformation of protein molecules in different directions, thus affecting various degrees of freedom, detecting 'weak points' in the molecule, etc.

The present work is the first attempt to study the mechano-chemistry of enzymes using immobilized crystalline samples. Based on experience accumulated in studies of the viscoelastic properties of such objects [5,6], we developed a technique for taking stress-strain diagrams for microsamples composed of immobilized protein crystals. The behaviour of such samples subjected to strong loads was studied in order to estimate their stability limits, i.e., to determine the range of stresses over which protein molecules could be reversibly deformed in solid samples.

2. Materials and methods

2.1. Sample preparation

The preparation of hen egg-white lysozyme crystals and amorphous films cross-linked by

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glutaraldehyde vapour treatment have been described in ref. 5.

Plates 7–15 μm thick were cut from lysozyme crystals using a sliding microtome. Dumb-bell-shaped samples were then cut from the plates. Such a form ensured that the sample was safely fixed at the ends with only its narrow part being deformed. For cutting samples a piece of safety razor was used whose cutting edge was bent aside in a particular manner. The samples were cut off in a plastic dish filled with distilled water. Samples cut off in the [010] direction of monoclinic $P2_1$ crystals and in the [1$\bar{1}$0] and [001] directions of tetragonal $P4_32_12$ crystals were used. All operations for preparing the samples were carried out under a stereomicroscope.

2.2. Apparatus

The sample was fixed with pincers made from a piece of split tungsten wire [5] and placed in a special holder inside a thermostatted humid chamber (the construction is described in ref. 6). The free end of the sample was attached to a microdynamometer, also placed in the chamber. As a microdynamometer two quartz fibres were used with one end sealed into a steel tube with the use of sulphur. Prior to sample fixation, a drop of melted and then supercooled ultra-pure sulphur was applied to the free end of one of the quartz fibres. After the sample has been introduced into the sulphur drop, the sulphur was crystallized by touching it with a cotton fibre covered with sulphur powder. A general view of the sample is presented in fig. 1.

The force F was estimated from ΔL, the change in distance between the ends of the quartz fibres. Calculations were performed by using the formula $F = 3\pi D^4 E_q \Delta L / 64 a^3$ [7], where D is the diameter of the quartz fibre, $E_q = 7 \times 10^{10}$ N m^{-2} Young's modulus for quartz, and a the length of the quartz fibre. Direct calibration of the microdynamometer, by hanging on a wire pieces of definite weight, showed this formula to be valid within an accuracy of approx. 5%.

The sample deformation, $\epsilon = \Delta l / l_0$, was measured using a microscope equipped with an eyepiece micrometer. To facilitate measurements,

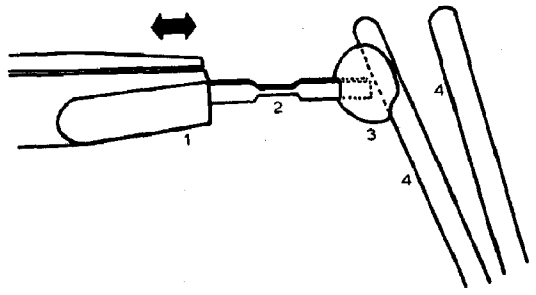

Fig. 1. Schematic representation of the fixed sample: (1) Pincers, (2) sample, (3) crystallized drop of sulphur, (4) end of the quartz fibre of the microdynamometer.

particles of carbon black were applied to the narrow region of the sample and served as reference points in length measurements. During the course of the experiment, the load applied to the sample was varied with a special micromanipulator which enabled the pincers to be shifted against the microdynamometer. For construction of the stress-strain diagram, ΔL and Δl were measured alternately under a microscope. Obtaining a pair of ΔL and Δl values normally took us about 2 min.

In all cases, unless otherwise stated, the required humidity inside the chamber was provided by placing at the bottom a drop of the buffer that was used to prepare the mother liquid for lysozyme crystallization: 50 mM acetate buffer (pH 4.5) with 2% $NaNO_3$ for monoclinic crystals and amorphous films and the same buffer (pH 4.7) with 5% NaCl for tetragonal crystals.

3. Results

3.1. Stress-strain diagram

Typical stress-strain curves for various lysozyme samples are presented in figs. 2 and 3. It is observed that at low loads the deformation obeys Hook's law up to $\epsilon = 1.5$–2%. Upon further increase in load, the deformation of the crystal sample deviates appreciably from linearity in the range 2–10%. When the sample is kept under loads corresponding to this range, a marked creep

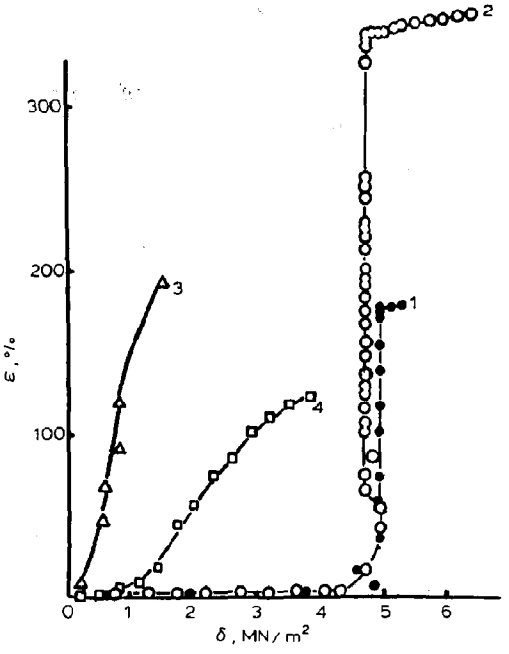

Fig. 2. Stress-strain diagrams for different crystalline lysozyme samples and for an amorphous one. (1) Tetragonal crystal, direction [001], $T = 22\,^{\circ}C$; (2) monoclinic crystal, direction [010], $T = 52\,^{\circ}C$; (3) monoclinic crystal, direction [010], $T = 92\,^{\circ}C$; (4) amorphous sample, $T = 31\,^{\circ}C$.

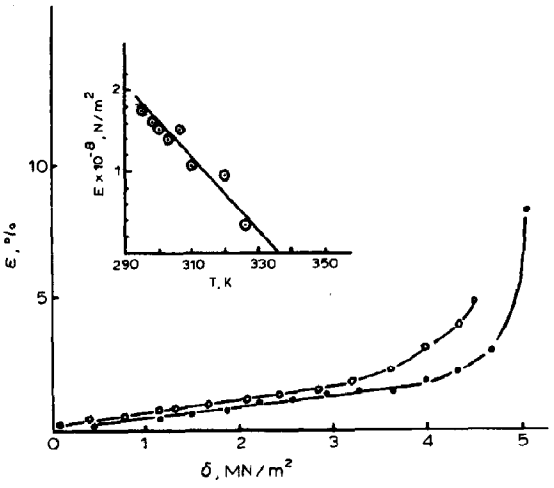

Fig. 3. Stress-strain diagrams for small deformations taken on a tetragonal crystal (direction [1$\bar{1}$0]) at 2-min (●———●) and 30–60-min intervals between measurements; $T = 22\,^{\circ}C$. (Inset) Temperature dependence of Young's modulus for these crystals.

is observed, whose rate strongly depends on temperature and load.

After reaching a particular critical tension, σ_{cr}, the sample extends drastically for a fixed load. This process begins with a large local decrease in cross-section of the sample so that a neck is formed. The neck then propagates along the sample under constant load. The sample volume considerably increases on stretching. In the case of monoclinic crystals, the relative volume increases about 1.6-times. Simultaneously, the direction of the optical axis in the crystal changes sharply. As seen from fig. 5, σ_{cr} varies for different crystal packings. After the neck has propagated throughout the narrow part of the sample the latter becomes more rigid and breaks on further loading.

When the tension is removed after the deformation at σ_{cr}, a large residual deformation is observed which gradually diminishes in the absence of load. The rate of such diminution can be substantially increased by raising the temperature. Upon a gradual increase in temperature from 22 to 57 °C for 30 min, the residual deformation of tetragonal crystals in the [1$\bar{1}$0] direction disappears completely (fig. 4). However, as shown by the diagram of the repeated loading (fig. 4, curve 3), the mechanical properties of the sample are not completely restored, σ_{cr} being considerably decreased. The small linear deformations ($\epsilon \leqslant 2\%$) are completely reversible and show no hysteresis, whereas non-linear deformations ($\epsilon = 2$–10%), although reversible, show substantial hysteresis in the strain-stress curve.

In amorphous films a marked deformation was observed at tensions which are less than σ_{cr} for crystalline samples, as demonstrated in fig. 2. Deformation in amorphous films is accompanied by a creep which substantially exceeds that of crystalline samples.

3.2. Dependence of σ_{cr} on temperature, urea concentration and humidity

On increasing the temperature, the tension-deformation diagram changes: the slope of the linear portion of the $\sigma(\epsilon)$ dependence increases manyfold. This corresponds to a decrease in Young's modulus, whose temperature dependence is de-

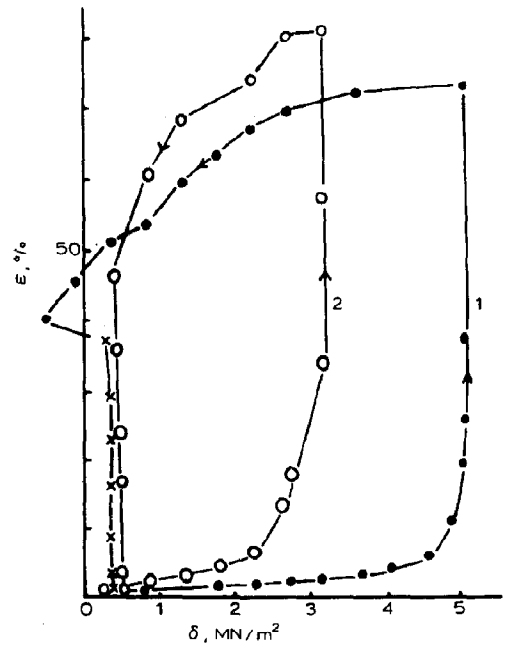

Fig. 4. Reversibility of stress-strain diagram. Consecutive diagrams marked by the figures were taken on a tetragonal crystal ([1$\bar{1}$0] direction) at 22° C except the points denoted by (×——×), where the temperature rose from 22 to 57° C over 30 min. Deformation directions are indicated by arrows.

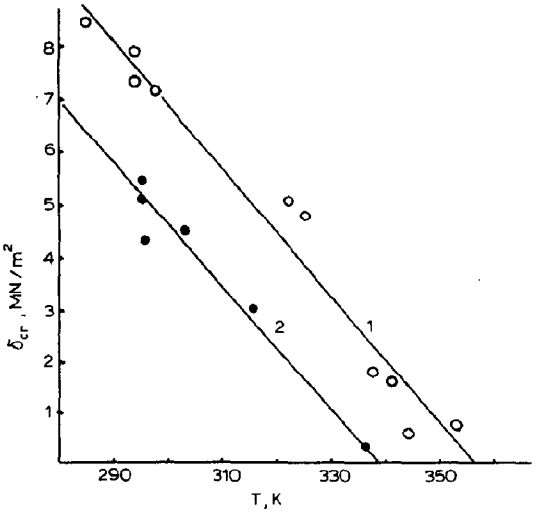

Fig. 5. Temperature dependence of critical tension σ_{cr}: (1) monoclinic crystals (direction [010]), (2) tetragonal crystals (direction [1$\bar{1}$0]).

picted in the inset to fig. 3. Similarly to Young's modulus, σ_{cr} decreases linearly with increasing temperature (fig. 5). Extrapolation of this dependence to $\sigma_{cr} = 0$ for both monoclinic and tetragonal samples yields a temperature which differs by no more than 2° C from the denaturation temperature, T_d, measured calorimetrically for each crystal (A.A. Makarov, D.R. Monacelidze and N.G. Esipova, unpublished results). For temperatures above T_d, the diagram changes markedly, as seen from fig. 2.

When soaked with 2 and 4 M urea solution, the tetragonal samples showed a 20 and 40% decrease at 24° C in σ_{cr}, respectively. For humidity $A = 80\%$ provided with $CaCl_2$ solution, the σ_{cr} value of tetragonal samples in the [1$\bar{1}$0] direction exceeded 4.5-times that for the humidity created by the buffer.

4. Discussion

The considerable reinforcement of the samples at $\sigma > \sigma_{cr}$ and the subsequent recovery of their initial length after annealing at elevated temperature suggest that no break-down of covalent bonds occurs. Since all the protein molecules in the crystal are cross-linked, any considerable rearrangement among them is unlikely to occur upon deformation. With this in mind, a many-fold increase in sample size at σ_{cr} may only be accounted for by strong structural changes within protein globules. The strong changes in specific volume of the protein provide further evidence of this. It can be considered that the maximal extension and strengthening of the sample are associated with unfolding of the peptide chain between covalent cross-links and with the subsequent deformation of covalent bonds in the stretched peptide chain, respectively. Analysis of the possible distribution of intermolecular cross-links attached to the ϵ-amino groups of lysine and the N-terminal amino group of the lysozyme peptide chain shows that the maximal distance between the sites of cross-links is about 130 Å, with four disulphide bonds being taken into account. As the mean size of the lysozyme molecule is about 30 Å, the degree of extension can reach 300%. This value truly reflects

the extent of the changes in length due to mechanical denaturation (see fig. 2).

The considerable amount of energy consumed in this process also points to great structural changes upon mechanical denaturation. In the case of monoclinic and tetragonal ([1$\bar{1}$0] direction) samples the mechanical work amounts to 103 and 36 kcal/mol, respectively, which substantially exceeds the difference in free energy between the native and denatured states, $\Delta G = 14$ kcal/mol [8]. Such a difference may be due to the non-equilibrium character of the mechanical denaturation and to significant differences between heat- and mechanically denatured protein states. Unlike thermal denaturation, which involves transition to the random-coil or 'molten-globule' state [9], mechanical denaturation may result in the formation of highly extended β-strands directed along the deformation. On thermal denaturation of lysozyme crystals their volumes increase by 10–15% (T.Ya. Morozova, personal communication) whereas on mechanical denaturation of monoclinic crystals the volume increases by 60%, thus indicating that a greater number of non-covalent bonds is broken in the latter case. It is interesting that such large changes in molecular structure seem to be almost reversible, since annealing at increased temperature leads to restoration of crystal length.

The external load is transmitted to each globule through non-valent interactions in the range of intermolecular contacts and through covalent glutaraldehyde cross-links. The following sequence of events on extension could be suggested: the loss of physical contact between globules makes the covalent cross-links strained, and then 'unwinding' of the polypeptide chain occurs. However, the state at which the globules lose the physical contacts and hang like beads on the covalent cross-links is not observed as a distinct step on the $\epsilon(\sigma)$ curve. Therefore, the conclusion can be drawn that at loads producing loss of physical contacts and stretching of cross-links destruction of the globule occurs just after the loss of contacts due to stress concentration at sites of attachment of cross-links, hence, the loss of contacts may be considered as being the initial mechanism of mechanical denaturation.

A simple model enables estimation of the energy required to break the physical contacts between the globules. Indeed, let us suppose that each globule in the sample can exist in two states, one of which is capable of resisting the load we call a 'native' one and the other state, a 'denatured' one we shall assume to have zero rigidity. The latter is in agreement with a nearly 1000-fold decrease in Young's modulus on denaturation of protein crystals [5]. Application of a load increases the free energy of the native state and decreases the difference in free energy between native and denatured states, thus causing the equilibrium portion of denatured molecules to increase. It can be shown that for a uniformly loaded layer of such molecules there is a critical σ value at which such a layer loses its ability to withstand a load. Let the modulus for the sample of native molecules be E_N and that for the sample of denatured molecules $E_D = 0$, the former being independent of load. In addition, assume that all the mechanical energy delivered to this layer is consumed in shifting the equilibrium between native and denatured molecules. We then have for the ratio of native and denatured molecules in the layer:

$$\frac{\alpha}{1-\alpha} = \exp\left[\frac{1}{RT}\left(\Delta G_0 - v\int_0^{\epsilon}\frac{\sigma}{\alpha}\,\mathrm{d}\epsilon\right)\right] \qquad (1)$$

where ΔG_0 is the difference in molar free energies for the denatured and native states in the absence of load, v the molar volume of protein in the native sample, T the absolute temperature and R the gas constant. The expression for mechanical work can be simplified taking into account that $E_D = 0$, $\sigma = \alpha E_N \epsilon$, then $v\int_0^{\epsilon}\sigma/\alpha\ \mathrm{d}\epsilon = \frac{1}{2}(\sigma^2 v/E_N\alpha^2)$. Substituting the latter into eq. 1 and differentiating the result with respect to α we can obtain an expression for parameters of the system in the critical point which is determined by $\mathrm{d}\sigma/\mathrm{d}\alpha = 0$. We now obtain for the critical tension:

$$\sigma_{cr} = E_N\epsilon_{cr}\left[1 - \frac{RT}{vE_N\epsilon_{cr}^2}\right] \qquad (2)$$

σ_{cr} is easily obtained from eq. 2. Substituting the experimental values of parameters, in particular, for the [110] direction of tetragonal crystals of lysozyme: $\sigma_{cr} = 5 \times 10^6$ N/m^2, $E_N = 1.6 \times 10^8$

N/m^2 at $T = 298$ K (see figs. 3 and 4), we obtain $\epsilon_{cr} = 4.9\%$ taking into account that $v = 0.0178$ m^3/mol [10]. This value is close to that experimentally observed, especially considering that the latter is affected by a creep.

Substituting the above values as well as $\alpha_{cr} = \sigma_{cr}/E_N\epsilon_{cr}$ into eq. 1 gives $\Delta G_0 = 1.1$ kcal/mol for the free energy difference. This value is in accordance with estimates of the energy of intermolecular contacts in protein crystals [11] and is one order of magnitude lower than $\Delta G = 14$ kcal/mol (thermodynamic stability of lysozyme in solution) [8]. The above agreement in energies supports the suggestion that the loss of the physical contact is the triggering mechanism of mechanical denaturation. Interestingly, the energy of contact interaction as well as σ_{cr} and Young's modulus for crystals correlate with the thermodynamic stability of the globule, since all factors tending to reduce it (temperature increase and urea addition) decrease the above values and vice versa: dehydration, which increases the denaturation temperature, also increases the values. The simplest explanation for this correlation would be the suggestion that the above-mentioned factors do no influence the intermolecular interactions any less than the intramolecular ones or that their action is realized through the globular surface.

It is of interest to compare the tensile strength of protein crystals with that of native supramolecular structures exposed to stress in living organisms. The greatest stress, σ_{max}, applied to an actin filament in cross-striated muscle can be readily estimated if we take into account that the ultimate stress developed by the muscle is between 2 and 5×10^5 N m^{-2} [12] and the density of actin filaments in the muscle cross-section is about 2×10^{15} m^{-2} [13]. Assuming a rectangular cross-section of 55×110 Å for helical actin filaments [14] we obtain $\sigma_m = 1.6\text{–}4 \times 10^6$ N m^{-2} which is close to σ_{cr} of lysozyme crystals at room temperature. If the crystals are good models for actin filaments (both objects have nearly the same value of Young's modulus [5,15] the ultimate strength of actin filaments must be close to the above-estimated σ_m. This means that the sarcomere structure and σ_{max} may be determined by the ultimate strength of actin filaments.

In fact, the ultimate stress in the intermolecular contacts of a protein crystal, σ'_{cr}, should be an order of magnitude greater than the experimentally found value, σ_{cr}, due to the 10-fold ratio between the largest cross-section of the protein molecule and that of the intermolecular contact [16]. The value $\sigma'_{cr} = 5\text{–}8 \times 10^7$ N m^{-2} obtained in such a way is very close to the ultimate tensile stress of glassy polymers, $\sigma_{cr} = 7\text{–}10 \times 10^7$ N m^{-2} [17], and biological materials, viz., collagen, $\sigma_{cr} = 5\text{–}10 \times 10^7$ N m^{-2} [18] and bone, $\sigma_{cr} = 10 \times 10^7$ N m^{-2} [19]. This means that the strength of interglobular contacts is comparable to that of polymer materials. It is high enough to deliver reversibly about 1 kcal elastic energy per mol protein molecules before the crystal loses its stability. The deformation of protein molecules cannot exceed 6–10% under this condition.

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