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Application of Large-Angle X-ray Diffuse Scattering to Studies of Globular-Protein Structure in Solution

BY BORIS A. FEDOROV AND ALEXANDER I. DENESYUK

Institute of Protein Research, Academy of Sciences of the USSR, Poustchino, Moscow Region, USSR

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Theoretical indicatrices of large-angle X-ray diffuse scattering covering the region of Bragg distances of 30–7 Å have been obtained and analysed for some globular proteins. The scattering curves given in the paper for sperm whale myoglobin, lamprey globin, ribonuclease S, hen-egg lysozyme, subtilysine, horse cytochrome C, lactate dehydrogenase and α-chymotrypsin display essential differences in the given interval of Bragg distances which testify to a considerable sensitivity of large-angle X-ray scattering to the internal structure of globular proteins. An 'express' method for calculating diffuse scattering curves has been worked out. It is shown by this method that the curve profile of scattering by globular proteins is determined to a comparable degree by all the three main elements of the protein structure: its shape, the course of the backbone chain and the character of distribution of side groups. The structural mechanism of differences of sperm whale myoglobin and lamprey globin indicatrices of scattering has been considered in detail. An apparently most effective way of applying large-angle X-ray diffuse scattering to studies of globular-protein structure in solution is substantiated. The essence of the method is that it does not analyse the protein structure itself but its change (or preservation) which can take place, for example, at the transition of a molecule from the crystal into solution. To determine these changes it is necessary: (*a*) to know the native structure in detail; (*b*) to calculate the large-angle scattering indicatrix based on it; (*c*) to obtain in a wide range of angles the experimental curve of scattering by the protein investigated in solution.

Introduction

Large-angle X-ray diffuse scattering is attracting greater attention in studies of the structure of globular proteins in solution. This is connected both with its fundamental possibilities in studies of the internal structure of macromolecules and with the considerable progress made recently in the development of this method. Up to the present, several important stages have been covered in the theoretical and experimental development of large-angle X-ray scattering by protein solutions, including the following.

(*a*) Direct experimental evidence has been obtained for the solvent influence on the course of the indicatrix of large-angle scattering by globular proteins (Stuhrmann, 1970). It was theoretically substantiated that this influence has, mainly, a non-specific nature (Fedorov, Ptitsyn & Voronin, 1972; Fedorov, 1976*a*), *i.e.* the curve profile of large-angle scattering depends only on the average electron density of the solvent and not on its real structure. Thus, to describe large-angle scattering it is sufficient to use the simplest model of the solvent, namely, a homogeneous continuum with a constant electron density, which allowed the mathematical formalism to be greatly simplified.

(*b*) Some theoretical methods (Fedorov, Ptitsyn & Voronin, 1972, 1974; Stuhrmann, 1973) have been worked out, permitting the calculation of the large-angle diffuse scattering indicatrices from the atomic coordinates of macromolecules by taking correct account of the solvent influence. These methods were used to obtain theoretical X-ray scattering curves in solutions of some globular proteins with a known spatial structure. Taking the sperm whale myoglobin solution as an example, it was shown that these curves are in satisfactory agreement with the experimental scattering indicatrices throughout almost all the accessible interval of scattering angles (up to ~20° for Cu $K\alpha$ radiation) (Oberthür, Stuhrmann & Fedorov, 1977).

(*c*) On the basis of a theoretical and experimental investigation of scattering indicatrices of some globular proteins the high sensitivity of large-angle X-ray scattering was demonstrated (Fedorov, 1976*a*,*b*) in the region of scattering angles $\mu \sim 0{\cdot}2$–$0{\cdot}8$ Å^{-1} [$\mu = (4\pi/\lambda) \sin\theta$; λ is the X-ray wavelength, 2θ is the scattering angle] which corresponds to the Bragg distances of ~40 to 8 Å.* This sensitivity is displayed by a noticeable change of the scattering-curve profile not only at a transition of proteins with a tertiary structure to proteins with a quaternary structure, but at times at comparatively small changes of the tertiary structure itself.

(*d*) An analysis was done of the indicatrix of 'supra-large-angle' diffuse scattering corresponding to the Bragg distances of 8 to 4 Å ($2\theta \sim 12$–$22°$). A

* For Cu $K\alpha$ radiation this region corresponds to the scattering angles of $2\theta \sim 3$–$12°$. Here, and below, the term 'large-angle X-ray scattering' will refer to this region.

particular study was also made of the nature of the 4·5 Å maximum observed on all the curves of scattering by globular proteins (Riley & Arndt, 1953; Echols & Anderegg, 1960; Grigoryev, Volkova & Ptitsyn, 1971*b*) and by structurized polypeptides (Grigoryev, Volkova & Ptitsyn, 1971*a*; Ptitsyn, Fedorov & Voronin, 1974). It was shown (Fedorov & Ptitsyn, 1977) that this maximum is connected with numerous van der Waals distances appearing between atoms not bound covalently at a tight packing of the polypeptide chain. Thus, this maximum is not specific to the details of packing of a polypeptide chain and therefore cannot provide any additional information on the secondary or tertiary structure of a globular protein.

The indicated achievements in the development of large-angle X-ray diffuse scattering in macromolecule solutions permit us to raise the general question of what is the most effective application of this method to structural studies of globular proteins in solution. What are the practical possibilities of this method? Is it possible by using the high sensitivity of large-angle scattering to attempt to some extent the inverse task of structural analysis, *i.e.* to determine the nature or type of polypeptide-chain packing in proteins from the course of the scattering curve, or is it the 'trial and error' method that is best suited for the application of large-angle scattering? If only the latter holds true, we must determine what questions of a structural nature this method can answer.

Method of calculating scattering indicatrices

Two methods were used to calculate the scattering indicatrices presented in the paper. The first is a combination of methods described earlier (Fedorov *et al.*, 1974) and takes into account the solvent influence by introducing effective atomic factors $A_i(\mu)$ as

$$A_i(\mu) = f_i(\mu) + n_i f_H(\mu) - \rho_0 P_i(\mu) K, \qquad (1)$$

where $f_i(\mu)$ and $f_H(\mu)$ are the atomic factors of the ith atom and H, respectively, n_i is the number of hydrogens directly bound to the ith atom, ρ_0 is the solvent electron density, and

$$P_i(\mu) = 4\pi \frac{\sin \mu R_i - \mu R_i \cos \mu R_i}{\mu^3} \qquad (2)$$

is the scattering amplitude of a sphere with radius R_i, corresponding to the van der Waals radius of the ith atom and H atoms bound to it. Coefficient K from equation (1) is a factor compensating for the difference between the volume of the whole macromolecule and the sum of the van der Waals volumes of all its atoms. To calculate the scattering intensity by a macromolecule in solution the scattering amplitude of an atomic system forming a macromolecule was determined,

$$\varphi(\mu) = \sum_{i=1}^{N} A_i(\mu) \exp(i\mu . \mathbf{r}_i); \qquad (3)$$

the scattering intensity was calculated at the point μ of reciprocal space

$$I(\mu) = |\varphi(\mu)|^2 \qquad (4)$$

with a subsequent averaging of $I(\mu)$ over the surface of a sphere with radius $|\mu| = \mu$. A choice of ~200 points on the sphere provided reasonable precision.

This method of calculating the scattering curves, which will be called the 'combination method', ensures a considerable time saving in comparison with calculations according to the Debye formula (Guinier & Fournet, 1955), and can be used to obtain theoretical scattering indicatrices up to $\mu \sim 0{\cdot}8$ Å^{-1}. Using this method we obtained the scattering curves for a number of proteins (in aqueous solution) with a known spatial structure. Fig. 1 represents the scattering curves for ribonuclease S (Wyckoff, Tsernoglou, Hanson, Knox, Lee & Richards, 1970),* lysozyme (Imoto, Johnson, North, Phillips & Rupley, 1972), subtilysine (Alden, Birktoft, Kraut, Robertus & Wright, 1972) and Fig. 2 represents those for cytochrome C (Dickerson,

* Here and below the reference following the protein indicates the source of the protein atom coordinates.

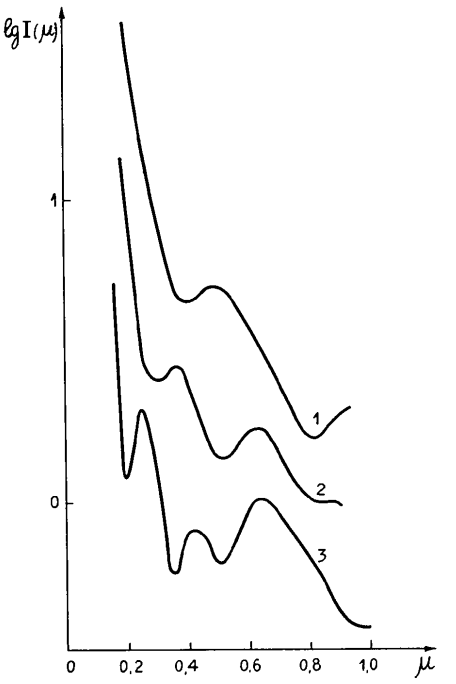

Fig. 1. Large-angle scattering indicatrices calculated by the 'combination' method for (1) ribonuclease S, (2) hen-egg lysozyme, (3) subtilysine.

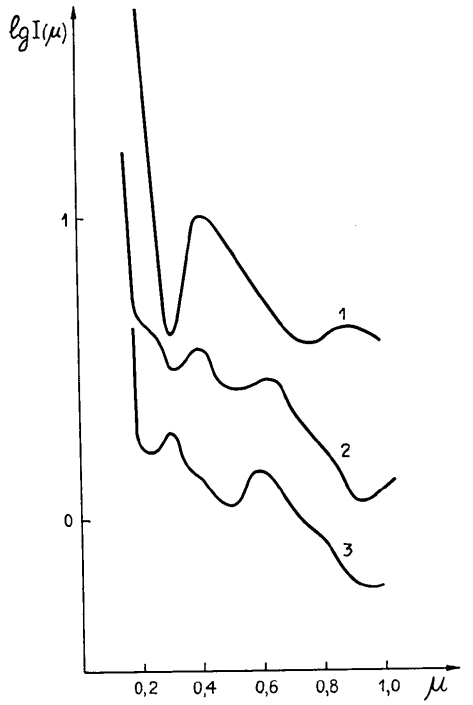

Fig. 2. Large-angle scattering indicatrices calculated by the 'combination' method for (1) horse cytochrome C, (2) lactate dehydrogenase, (3) α-chymotrypsin.

Takano, Eisenberg, Kallai, Samson, Cooper & Margoliash, 1971), lactate dehydrogenase (Adams, Ford, Liljas & Rossmann, 1973), α-chymotrypsin (Birktoft & Blow, 1972) as an example of the sensitivity of large-angle diffuse scattering to the structure of the protein molecule. However, the application of this method showed that the time saved is insufficient to solve the problem posed in the present paper, *i.e.* the detailed analysis of the connexion between the structure of a globular protein and its indicatrix of large-angle scattering. Consequently, an 'express' method for calculating diffuse scattering curves was introduced. It is based on calculating the distance distribution function $D(r)$ by atom coordinates as a sum of products of effective atomic factors (1) for all i and j atoms separated by the distance r:

$$D(r) = \sum_{i,j} A_i(0)A_j(0). \qquad (5)$$

The intensity of diffuse scattering for the given macromolecule can be calculated as (Guinier & Fournet, 1955)

$$I(\mu) = \int_0^\infty D(r)\frac{\sin \mu r}{\mu r}\,dr. \qquad (6)$$

Unlike the method described above, the express method neglects the dependence of effective atomic factors $A_i(0)$ [and, consequently, also $D(r)$] on μ.

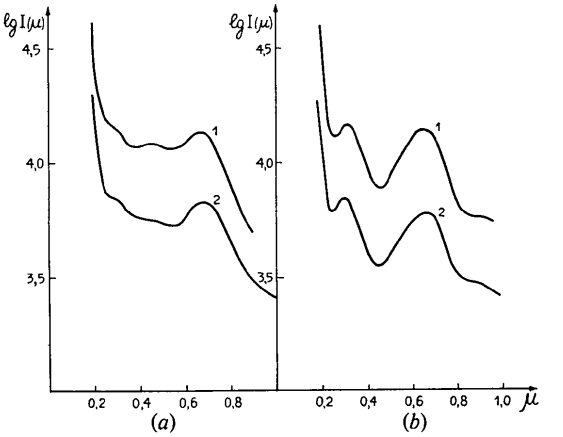

Fig. 3. Theoretical scattering curves calculated by the 'combination' (curves 1) and 'express' (curves 2) methods for (*a*) Mb-SW and (*b*) Gb-Lp.

However, in the interval of scattering angles which we consider (up to $\mu \sim 0{\cdot}8$ Å^{-1}), this dependence is relatively weak, and the scattering indicatrices calculated by the express method preserve all the qualitative features inherent in the curves obtained by more precise methods. Fig. 3 presents an example of theoretical indicatrices of scattering by sperm whale myoglobin (Mb-SW) (Watson, 1969) and lamprey globin (Gb-Lp) (Hendrickson, personal communication) in an aqueous solution, calculated by the two methods considered. As is seen, in the case of Gb-Lp both curves have two distinctly pronounced maxima, while both Mb-SW scattering curves display an extensive plateau. Thus, the express method, providing a reasonable precision of calculation, can serve as a basis for further analysis.

Comparative influence of the shape and internal heterogeneities of globular proteins on their large-angle scattering curve

To estimate the possibilities of solving the inverse problem of structural analysis of globular proteins from large-angle diffuse scattering data, it is necessary first of all to ascertain what structural factors, besides the mutual packing of the polypeptide backbone chain, affect the course of the scattering indicatrix. From this point of view the relative influence of the external and internal protein structure was studied, *i.e.* the comparative contribution of the macromolecule shape and its inner heterogeneities to the formation of the scattering curve. To determine these contributions separately one can use the above mentioned (Fedorov, 1976*b*) possibility of representing the distance distribution function $D(r)$ as a product of two functions

$$D(r) = D_h(r)D_{\text{inh}}(r), \qquad (7)$$

one of which, $D_h(r)$, depends on the particle shape only and the other, $D_{inh}(r)$, on the internal distribution of heterogeneities. Representation of the function $D(r)$ in the form of (7) permits detailed investigation of various features of the large-angle scattering indicatrix, *i.e.* to find with which of the functions, $D_h(r)$ or $D_{inh}(r)$, this feature is chiefly connected. As an example, let us consider the nature of the differences between the large-angle scattering indicatrices of Mb-SW and Gb-Lp of globular proteins with homologous spatial structures. With what structural factors are these differences mainly connected? With a change of the macromolecule overall shape or with the different distribution of internal heterogeneities? In other words, which of the two functions, $D_h(r)$ or $D_{inh}(r)$, is responsible for the scattering indicatrix profile in the region of $\mu \sim 0{\cdot}3$–$0{\cdot}8$ Å^{-1}? To find the answer the 'hybrid' distribution functions $D(r)$ were composed:

$$D_1(r) = D_h^{\text{Mb-SW}}(r).D_{inh}^{\text{Gb-Lp}}(r)$$
and (8)
$$D_2(r) = D_h^{\text{Gb-Lp}}(r).D_{inh}^{\text{Mb-SW}}(r),$$

and on their basis the scattering intensities were obtained according to (6). The results of this calculation and also the 'real' scattering indicatrices for Mb-SW and Gb-Lp calculated by the express method are presented in Fig. 4. It is seen that the differences in the Mb-SW and Gb-Lp scattering curves in the region of angles $\mu \sim 0{\cdot}3$–$0{\cdot}8$ Å^{-1} are determined only by the functions $D_{inh}(r)$; *i.e.* the differences are connected with the different distribution of electron density inside protein molecules and not with the differences in their overall shape. This does not necessarily mean that the shape of the protein globule does not affect the scattering indicatrix in the indicated region of scattering angles. From a comparison of the scattering curves (Fig. 4), one can draw the conclusion that the internal heterogeneity distribution function makes a noticeable contribution to the formation of the large-angle scattering indicatrix. To determine whether the macromolecule shape affects the scattering curve in this region of angles, the hybrid functions $D(r)$ were composed for some globular proteins with a known spatial structure. The scattering intensities calculated on their basis were analysed. In a number of cases the macromolecule shape also appeared to affect considerably the profile of the large-angle scattering indicatrix. Fig. 5 presents the real and hybrid scattering intensities for Mb-SW and α-chymotrypsin (Ch) calculated by the express method. In this case the Ch macromolecule shape is responsible for the appearance of a sharp maximum at $\mu \sim 0{\cdot}3$ Å^{-1}.

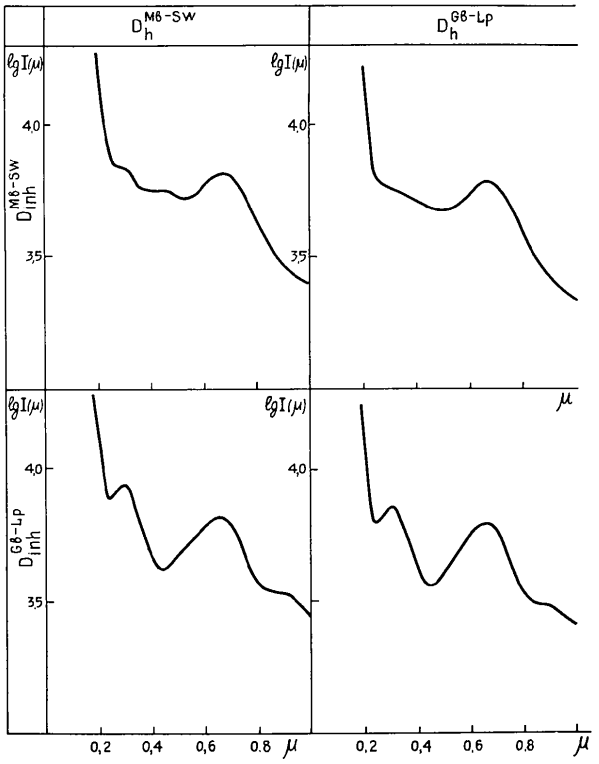

Fig. 4. Intensities of scattering obtained by the 'express' method from 'hybrid' and 'real' functions of distribution $D(r) = D_h(r).D_{inh}(r)$ taken for Mb-SW and Gb-Lp.

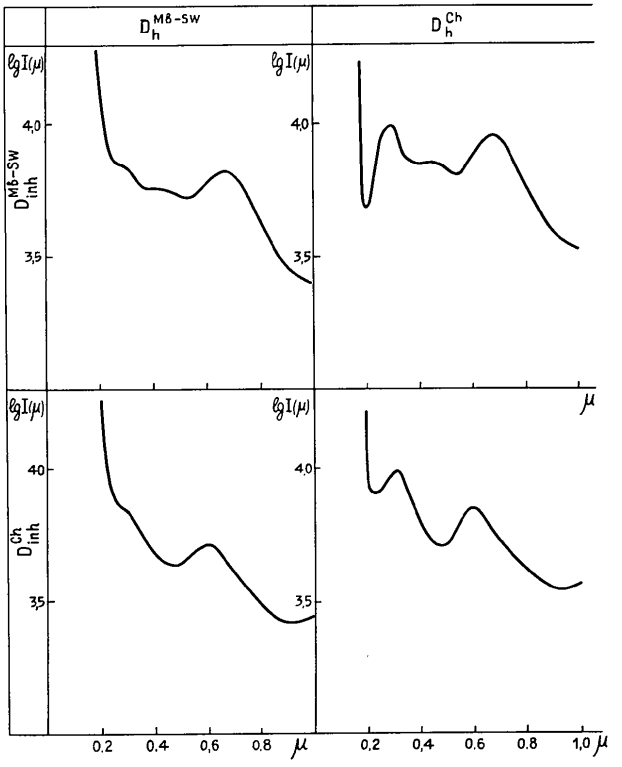

Fig. 5. Intensities of scattering obtained by the 'express' method from 'hybrid' and 'real' functions of distribution $D(r) = D_h(r).D_{inh}(r)$ taken for Mb-SW and Ch.

Thus, the described analysis shows that both the macromolecule shape and the type of internal heterogeneity can make a noticeable and comparable contribution to the large-angle scattering indicatrix.

The comparative influence of the polypeptide backbone chain and side groups on the large-angle scattering curve

The internal heterogeneity function $D_{inh}(r)$ of a protein molecule is determined both by the type of packing of the main chain and by side groups. To estimate the comparative influence of these two factors on the scattering indicatrix, the latter was divided into two parts: the first, I_b, represented the part of general intensity connected with scattering from atoms of the backbone chain only, while the second, $I_s = I - I_b$, is the intensity resulting from scattering from atoms of side groups and the interference of waves scattered by side and backbone chains. The intensities I_b and I_s were calculated for some globular proteins. Fig. 6 presents these intensities for Mb-SW and Gb-Lp. The curves I_b for both proteins differ essentially from their 'total' scattering intensities. A comparison of the indicatrices in Figs. 6 and 3 shows that side groups do not only make a considerable contribution to the formation of the scattering-curve profiles, but also play a noticeable role in the appearance of differences between Mb-SW and Gb-Lp scattering indicatrices.

The structural basis of differences in Mb-SW and Gb-Lp scattering indicatrices

The analysis presented above indicates that all the three main elements of globular protein structure (particle shape, backbone chain course, side group distribution)

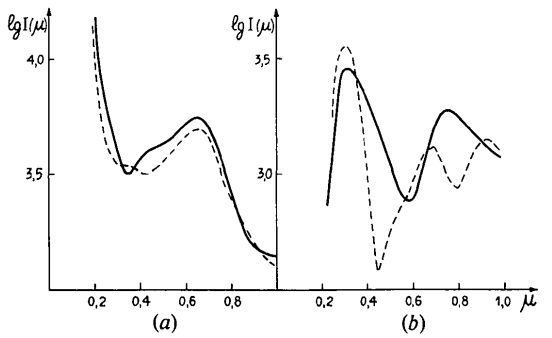

Fig. 6. Contributions to the large-angle scattering indicatrix of Mb-SW (——) and Gb-Lp (– – –) calculated by the 'express' method: (*a*) contribution of the backbone chain to scattering (distances 'backbone–backbone' chains); (*b*) contribution of the remaining elements to scattering (distances 'side–side' and 'side–backbone' chains).

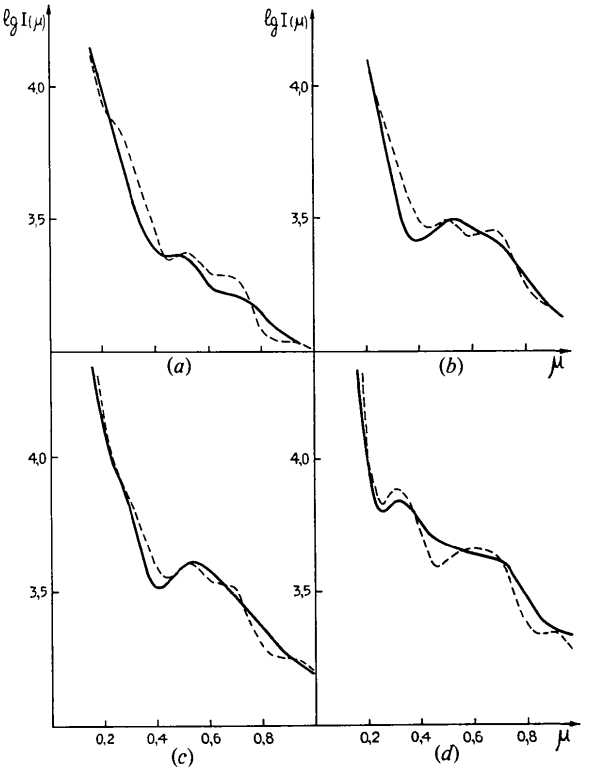

Fig. 7. Theoretical scattering curves by fragments Mb-SW (——) and Gb-Lp (– – –) obtained by the 'express' method: (*a*) the fragment including the helices *A–B–C–D*; (*b*) the fragment including the helices *A–B–C–D–E*; (*c*) the fragment including the helices *A–B–C–D–E–F*; (*d*) the fragment including the helices *A–B–C–D–E–F–G*.

can have comparable effects on the profile of the indicatrix of scattering in the region of large angles. This fact of course makes it impossible to solve in general the inverse problem of structural analysis, namely, the determination of the type of packing of a polypeptide chain from the diffuse scattering curve. Consequently, the question then arises of the most appropriate application of the trial and error method.

Proceeding from this, an analysis of the Mb-SW and Gb-Lp scattering indicatrices was done to describe in detail the concept of 'high sensitivity' of large-angle X-ray scattering, with these two proteins as examples, and to determine its application for solving globular-protein structural problems. As noted above, Mb-SW and Gb-Lp have homologous spatial structures but display an essential difference in their indicatrices of large-angle scattering. To determine the concrete structural mechanism of these differences the following approach was used: the scattering intensities were calculated by the express method from analogous Mb-SW and Gb-Lp structural fragments, the length and localization of which varied in a wide range to find the structural element most responsible for the differences in the scattering curves.

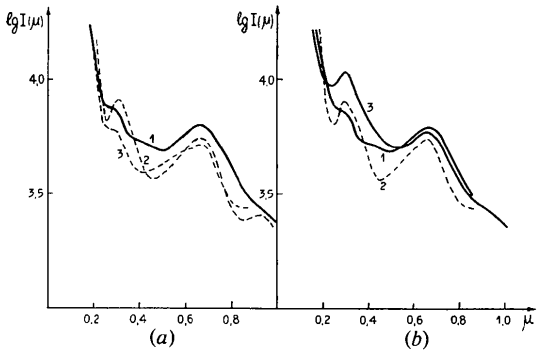

Fig. 8. Theoretical scattering curves for Mb-SW (——) and Gb-Lp (– – –) obtained by the 'express' method: (*a*) Mb-SW (1), Gb-Lp (2), Gb-Lp modified molecule (3); (*b*) Mb-SW (1), Gb-Lp (2), Mb-SW modified molecule (3).

One of the numerous methods employed for splitting the proteins into fragments and isolating the structural elements led to the answer. The key element of the structure of the indicated proteins proved to be the *G* helix. Indeed, if we combine the fragments Mb-SW and Gb-Lp by adding the number of helices, *i.e.* if we consider the fragments Mb-SW and Gb-Lp, consisting of helices *A*, *A–B*, *A–B–C*, *A–B–C–D*, *etc.*, then, as seen in Fig. 7, the indicatrices of scattering by these fragments for Mb-SW and Gb-Lp are more or less similar up to the fragment *A–B–C–D–E–F* (Fig. 7*a*,*b*,*c*). But the addition of the *G* helix changes the picture considerably (Fig. 7*d*): the scattering curves of both proteins take the profile characteristic of whole protein molecules, *i.e.* have two sharp maxima in the case of Gb-Lp and a plateau in the case of Mb-SW. A visual analysis of Gb-Lp and Mb-SW three-dimensional models actually showed that in these proteins the position of the *G* helix in relation to the other helices is essentially different. The *G* helix in Mb-SW is at a noticeable angle to the *B* helix while in Gb-Lp both helices are practically parallel.

The structure of both proteins was modified to provide final evidence of the dominant role of the *G* helix in the origin of differences between the Mb-SW and Gb-Lp indicatrices of scattering. In the case of Mb-SW we 'turned' the *G* helix by 20° relative to its *C* end, placing it almost parallel with the *B* helix, while for Gb-Lp the *G* helix was turned by 30° in the opposite direction relative to the same *C* end (the difference in the angles is due to the different length of the *G* helices in Mb-SW and Gb-Lp). The scattering intensities were calculated by the express method for both the modified structures (Fig. 8). It is seen from Fig. 8(*a*) that the curve of scattering by modified Gb-Lp is much closer to the Mb-SW indicatrix of scattering, while the curve of scattering by modified Mb-SW is closer to the scattering indicatrix of Gb-Lp (Fig. 8*b*).

Discussion: the trial and error method in large-angle diffuse scattering

The presented analysis of the Mb-SW and Gb-Lp indicatrices of scattering testifies that in the given case we are dealing with 'supersensitivity' of large-angle X-ray scattering. This is apparently due to the *G* helix being in the 'focus' of interference effects which considerably depend on the localization of the *G* helix. Whatever is the physical basis of this phenomenon, the analysis undertaken indicates that in a number of cases large-angle scattering curves are highly sensitive to comparatively small *local* rearrangements of the internal structure of protein.

It was found earlier (Timchenko, Ptitsyn, Serdyuk, Fedorov & Kravchenko, 1976) that the curves of large-angle scattering by globular proteins are also noticeably sensitive to minor *large-block* rearrangements in the protein structure. Fig. 9 shows the theoretical indicatrix of scattering by hen-egg lysozyme in an aqueous solution illustrating this property (Timchenko *et al.*, 1976). A slight moving away of the two lysozyme parts by 5° (Fig. 9, II) or 10° (Fig. 9, III) led to noticeable changes of the curve profile in the region of the $\mu \sim 0{\cdot}3$ $Å^{-1}$ minimum.

The sensitivity of the indicatrix of scattering to local and large-block rearrangements in the protein structure

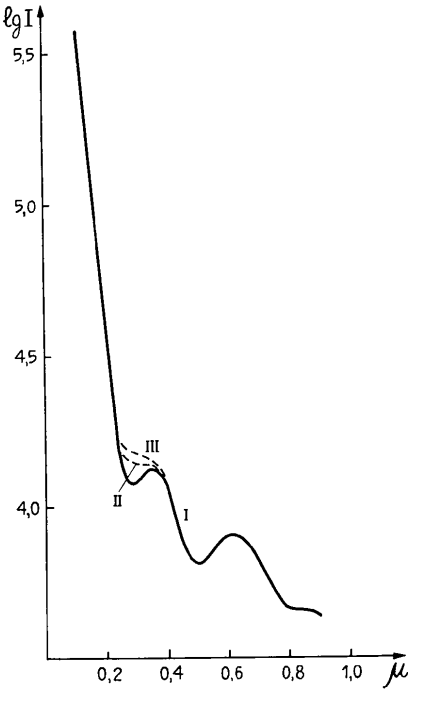

Fig. 9. Theoretical scattering curves for hen-egg lysozyme taken from the paper by Timchenko *et al.* (1976): for the native protein (I), and when its substrate-binding slit is widened to 5° (II) and to 10° (III).

permits a specific formulation of what seems to be the most effective approach to the application of the trial and error method at large-angle diffuse scattering. In the large-angle analysis the method does not consider the protein structure *itself* as an object of investigation, but its *change* (or preservation) which may occur either at a transition of the molecule from crystal into solution or at a realization of its biological functions. The basis of such a consideration must be: (*a*) a known protein structure (*e.g.* in crystal form); (*b*) the large-angle X-ray indicatrix of scattering calculated for this structure, taking into account the influence of the solvent; (*c*) the experimental scattering curve for the given protein in solution measured over a wide range of scattering angles. If the theoretical and experimental curves do not coincide, the protein structure can be modified within certain limits using physical and biological considerations to attain a better agreement between the indicatrix of scattering calculated for the modified structure and the experimental curve. This technique has already been tested in our laboratory in studying lysozyme structure in solution (Timchenko *et al.*, 1976) and has given the first encouraging results.

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