DETERMINATION OF THE DISTANCE BETWEEN HEAVY-ATOM MARKERS IN HAEMOGLOBIN AND HISTIDINE DECARBOXYLASE IN SOLUTION BY SMALL-ANGLE X-RAY SCATTERING

B. K. VAINSHTEIN, L. A. FEIGIN, Yu. M. LVOV, R. I. GVOZDEV+, S. A. MARAKUSHEV+ and G. I. LIKHTENSHTEIN+

Institute of Crystallography, USSR Academy of Sciences, Moscow, 117333, Leninsky pr. 59 and *+Institute of Chemical Physics, USSR Academy of Sciences, Chernogolovka (Moscow region), 142432, USSR*

Received 21 April 1980

1. Introduction

A method was proposed [1,2] for determination of the distance between heavy-atom markers in macromolecules in solution by means of small-angle X-ray scattering. The intensity of scattering by a macromolecule (with mean electron density ρ) with the attached heavy atoms (with density ρ_M) in solution can be written as:

$$I(s) = \overline{F[(\rho + \rho_M) * (\rho + \rho_M)]}$$
$$= \overline{F(\rho * \rho)} + \overline{F(\rho_M * \rho_M)} + 2\overline{F(\rho * \rho_M)}$$
$$= I_1(s) + I_2(s) + I_3(s) \quad (1)$$

where $I_1(s)$ is the intensity of scattering by the macromolecule without markers, $I_2(s)$ is the scattering by markers and $I_3(s)$ is the cross term; $s = 4\pi \sin\theta/\lambda$. One of the possibilities of determining the distance between heavy-atom markers is based on the separation of the term $I_2(s)$ from experimental scattering curves. Such experiments were carried out for solutions of gramicidin S (mol. wt 1200) [2,3].

We have carried out the analysis of expression (1) in the case of small-angle X-ray scattering by proteins with heavy-atom markers in solution and estimated the relation of the existing accuracy of experiment to the minimal value of electrons in a marker from which the scattering can be still detected [4]. Also we have found the condition under which $I_3(s)$ is much less than $I_2(s)$ in the region of the scattering curve subsidiary maxima [4]. These conditions are responsible for $N_M > 4 \times 10^{-3} N$ where N_M and N are the numbers of electrons in a heavy-atom marker and in a protein molecule, respectively. In other words for protein with mol. wt ~100 000 the number of electrons in the marker must be >200. Otherwise, the marker must contain <3 heavy atoms with atomic number 80–90. This conclusion is in full agreement with the results of computer model calculations carried out for protein-like particles with strongly scattering markers [4].

Here, the groups of 4 mercury atoms were used as heavy atom markers. Owing to the 'increase in the weight' of the marker, it is possible to attempt to determine the distance between the heavy-atom markers in proteins in solutions, i.e. in their native state.

2. Materials and methods

2.1. *Heavy-atom marker*

As the heavy-atom marker we used the organomercury compound, mercarbide marker (4 mercury atoms) [5] which was synthesized as in [6]. The mercury concentration capable of forming mercaptides with thiols was estimated by direct or reverse spectrophotometric titration of solutions following Ellman [7]. The mercarbide marker solution was prepared by the method in [7].

2.2. *Measurements of the intensity of small-angle X-ray scattering*

The measurements of the intensity of small-angle X-ray scattering by solutions of proteins were performed by the automatic small-angle slit diffractometer AMUR (Inst. Crystallog., USSR Acad. Sci.), CuKα

radiation, in the range of scattering angles 2θ: 0.1°–6° (s: 0.07–4.3 nm^{-1}). The collimation correction for the slit height was made to obtain the small-angle scattering curves which represent the difference between the specimen and the solvent scattering curves [8].

3. Results and discussion

3.1. *Experiment with haemoglobin*

The mercarbide marker solution was added to human haemoglobin (met-form) solution in the amount of 2 mol/mol protein, the markers were linked to the SH-groups of the amino acid 93 residues (cysteine) in each of the two β-chains of haemoglobin. In the initial solution the marker was 10^{-4} M and therefore after addition of the markers to the protein, the protein solution was concentrated by ultrafiltration through a PM-10 membrane.

The curves $I(s)$ and $I_1(s)$ differ in the range of scattering angles s: 0.7–4.3 nm^{-1} (fig.1). The difference curve $\Delta I(s) = I(s) - I_1(s)$ is given in fig.2; it displays oscillations, the maxima appear at $s = 2.21$ and $s = 3.35$ nm^{-1}.

As follows from eq. (1) such a difference curve comprises the terms $I_2(s) + I_3(s)$; the term $I_2(s)$ may be calculated according the following equation:

$$I_2(s) = f_M^2 \left(1 + \frac{\sin sr_0}{sr_0}\right) \qquad (2)$$

(f_M is the scattering factor of a marker) and gives information about the distance between the markers

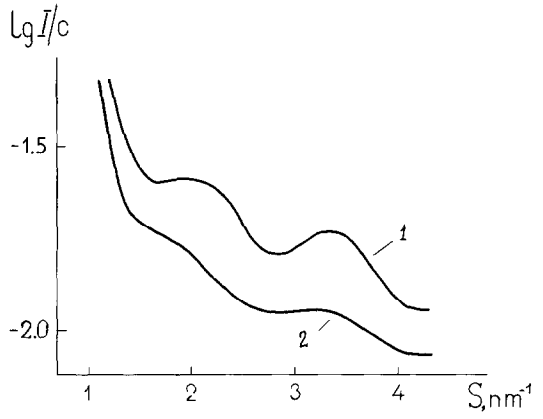

Fig.1. Small-angle X-ray scattering curves for met-haemoglobin with heavy-atom markers, $I(s)$ (1) and for native met-haemoglobin, $I_1(s)$ (2).

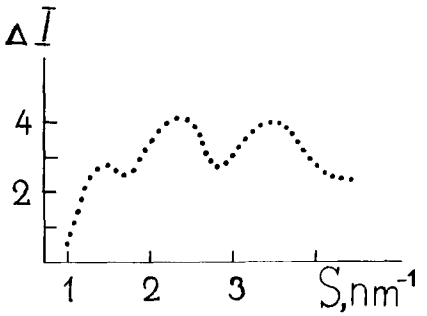

Fig.2. The difference curve for met-haemoglobin $\Delta I(s) = I(s) - I_1(s)$.

r_0. The term $I_3(s)$ ('marker–protein globule' interference) in a certain sense 'hinders' the extracting of such information. As $I_3(s) \ll I_2(s)$ the positions of the maxima and the minima on the experimental difference curve correspond to the function $I_2(s)$ and we arrive at $r_0 = (3.8 \pm 0.2)$ nm as the distance between the markers associated with the amino acid residue 93 of the β-chains of haemoglobin. This value characterizes the distance between centers of the electron density of the markers.

It is of interest to compare this distance with those obtained by other methods. From the X-ray crystallographic determination of the structure of deoxyhaemoglobin [9] (its structure closely corresponds to that of met-haemoglobin) one can find that the distance between the mercury atoms (paramercury benzoate) attached to residue 93 of the β-chain is 3.76 nm. This distance in met-haemoglobin is the same [9] and coincides with our value. The precision of the value is restricted by the following factors (apart from experimental error):

(i) The marker contains 4 mercury atoms and its dimensions (from stereochemical considerations) are estimated to be 0.4 × 0.4 × 0.5 nm, i.e. the center of electron density may be shifted by 0.1–0.2 nm from the site of the marker linkage to the cysteine residue;
(ii) Neglecting cross term $I_3(s)$ in the analysis.

3.2. *Experiment with histidine decarboxylase*

The histidine decarboxylase from *Micrococcus* spn., an enzyme of mol. wt 100 000, was another protein object. The purification and preparation of solutions of native histidine decarboxylase was effected as in [10]. The concentrations of the specimens were determined with an SF-4 spectrophotometer, $E_{280}^{1\%} = 16$.

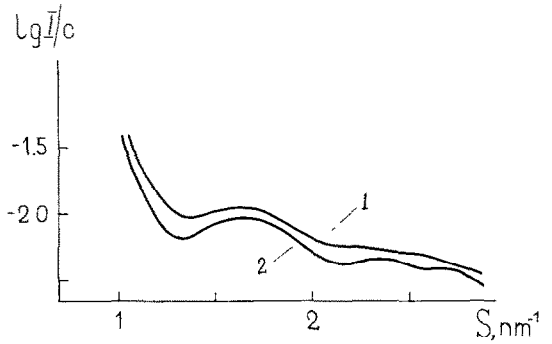

Fig.3. Small-angle X-ray scattering curves for histidine decarboxylase with heavy-atom markers $I(s)$ (1) and for native histidine decarboxylase $I_1(s)$ (2).

The mercarbide marker solution was added to the enzyme solution to 3 mol/mol protein. The histidine decarboxylase molecule contains 3 rapidly titratable cysteine residues. The mercury markers were linked to the cysteine SH-groups.

Two experimental methods for finding the difference scattering curve were used.

(1) The curves of small-angle X-ray scattering by histidine decarboxylase (with markers) and by native enzyme (without markers) (fig.3-1,2) were obtained after subtraction of the intensity of scattering by the solvent. Thereafter the difference between these curves, $\Delta I(s) = I(s) - I_1(s)$ (fig.4-1) was plotted.

(2) Enzyme solution was prepared without markers (native) and with markers of the same concentration (24.7 mg/ml) and in the same buffer. Then it became possible to exclude the measurement of scattering by buffer and thereby to reduce substantially the time of measurements. The difference curve (fig.4-2) was obtained by subtracting the experimental curve of scattering by a solution of histidine decarboxylase from the experimental curve of scattering by histidine decarboxylase solution with markers (without any account of the background scattering by solvent).

The positions of the maxima on the curve obtained by these two methods are very close to each other. Thus the presence of the maxima on the difference curves of small-angle scattering of histidine decarboxylase as well as their position associated with the scattering by heavy-atom markers in histidine decarboxylase may be regarded as reliably determined. Neglecting the influence of the term $I_3(s)$, we suppose that the

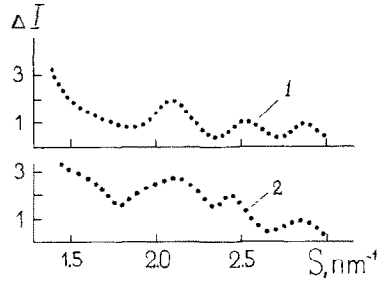

Fig.4. Difference curves $\Delta I(s)$ for histidine decarboxylase obtained following the first method (1) and the second method (2).

difference curve $\Delta I(s)$ corresponds to the curve $I_2(s)$ – interference between heavy markers. So, we have determined from eq. (2) the distance between the markers as being 6.9 ± 0.3 nm. For such a value of the position of the interference maxima in the calculated scattering function $I_2(s)$ appears to be equal to 1.14, 2.06 and 2.88 nm^{-1}; all these maxima are found to be present on the experimental difference curves $\Delta I(s)$ (fig.4). The maximum at $s = 2.49$ nm^{-1} still remains to be explained.

Thus now there is a possibility to measure the distances between heavy-atom markers in proteins in solution by means of small-angle X-ray scattering.

Acknowledgements

The authors are indebted to N. I. Sosfenov for help in experiments and to N. A. Gonchar (Lab. Enzymol., USSR Acad. Med. Sci.) for preparing histidine decarboxylase specimens.

References

[1] Kratky, O. and Worthmann, W. (1947) Monatsh. Chem. 76, 263–281.

[2] Vainshtein, B. K., Sosfenov, N. I. and Feigin, L. A. (1970) Dokl. Akad. Nauk SSSR 190, 574–577 (Sov. Phys. Dokl. (1970) 15, 12–14).

[3] Feigin, L. A., Sosfenov, N. I. and Vainshtein, B. K. (1969) VIII Gen. Assemb. Int. Cong., USA, 1969, abst. p. 218.

[4] Feigin, L. A., Gonchar, N. A., Likhtenshtein, G. I., Lvov, Yu. M. and Marakushev, S. A. (1978) Kristallografiya 23, 749–755 (Sov. Phys. Crystallog. (1978) 23, 420–424).

[5] Likhtenshtein, G. I. (1979) Multinuclear redox metalloenzymes, Nauka, Moscow.
[6] Hoffman, K. A. (1900) Ber. Deutsch. Chem. Ges. 33, 1328–1332.
[7] Likhtenshtein, G. I., Levchenko, L. A., Raevskii, A. V., Sadkov, A. P., Pivovarov, T. S. and Gvozdev, R. I. (1973) Dokl. Akad. Nauk SSSR 213, 1442–1446.
[8] Shchedrin, B. M. and Feigin, L. A. (1966) Kristallografiya 11, 159–163 (Sov. Phys. Crystallog. (1966) 11, 166–169).
[9] Muirhead, H., Cox, J. M., Mazzarella, L., Perutz, M. F. (1967) J. Mol. Biol. 28, 117–156.
[10] Prozorovskii, V. N., Alekseeva, A. E., Nikolaev, A. A. and Mardashev, S. R. (1970) Biokhimiya 35, 759–763.