DISTANCE BETWEEN TWO BINDING SITES OF THE SAME ANTIBODY MOLECULE

A neutron small-angle scattering study of pig anti-Dnp antibody complexed with mono-Dnp-dextran

L. CSER, F. FRANĚK+, I. A. GLADKIKH, R. S. NEZLIN*, J. NOVOTNÝ+ and Yu. M. OSTANEVICH

Laboratory of Neutron Physics, Joint Institute for Nuclear Research, Dubna, USSR, +Institute of Molecular Genetics, Czechoslovak Academy of Sciences, 160 20 Prague 6, Czechoslavakia and **Institute of Molecular Biology, USSR Academy of Sciences, 117 312 Moscow, USSR*

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1. Introduction

Although the general features of the antibody molecules are known, considerable uncertainty still exists as to their shape when present in immune complexes. Small-angle X-ray scattering analysis employed [1,2] showed that the radii of gyration of rabbit antibodies decrease upon binding low-molecular weight haptens. With the use of neutron small-angle scattering, we have shown [3] that pig antibodies which occur early and late during an immune response to Dnp-antigens and differ by their ability to precipitate antigen [4], differ by their radii of gyration as well; when reacting with a low-molecular weight hapten, the radii of gyration of the early and late antibodies decrease by 4.6% and 7.5%, respectively.

This paper reports on neutron small-angle scattering experiments aimed at determining the distance between the two binding sites of the same antibody molecule employing complexes of anti-Dnp antibody with an antigenically univalent, high molecular weight ligand. The contrast variation method offers a possibility to measure the distance between two ligands bound to the same molecule when the condition is fulfilled that the ligands have a sufficiently large volume and that their neutron scattering density differs from that of the antibody. To this end, a high molecular weight antigen, a mono-Dnp derivative of dextran (mol. wt 40 000), was synthesized and its complexes with pig antibodies were examined in various H_2O–D_2O mixtures. Although the distance values could be determined only with a large statistical error, the data allow the conclusion that the geometrical parameters of the complexes formed with the early (i.e., precipitating) antibody are significantly different from those of the complexes formed with the late (i.e., non-precipitating) antibody. The data suggest that the precipitating antibody complexed with a high molecular weight antigen assumes an extended shape with an antigen to antigen distance of 35.8 ± 1.3 nm.

Abbreviations: Dnp, 2,4-dinitrophenyl; Fab, antigen binding fragment of antibody

2. Materials and methods

Pig anti-Dnp antibodies were the same preparations as those used in [3]. 8-(2,4-dinitrophenyl)-5,8-Diaza-4-oxooctanoic acid was synthesized as in [3]. Dextran T-40 (mol. wt 40 000) was purchased from Pharmacia (Uppsala, Sweden).

For the synthesis of the mono-Dnp-dextran (hereinafter referred to as 'antigen'), a mixed anhydride [5–7] of ethyl chloroformate and Dnp- diazaoxooctanoic acid* was added to a precooled solution

* A solution of 81 mg (0.25 mmol) Dnp-diazaoxooctanoic acid and 0.5 ml triethylamine in 2 ml anhydrous dimethylformamide was cooled to −20°C and mixed with 0.3 ml ethyl chloroformate cooled to the same temperature; this mixture was used without delay

of 2 g dextran (50 μmol) in 6 ml alkaline pyridine (0.1 M sodium bicarbonate–pyridine 1:1) and stirred vigorously for 5–10 min. Then, a crude dextran product (0.3–0.4 mol Dnp/mol dextran) was separated from the low-molecular weight reactants on a column of Sephadex G-25 (2.6 × 36 cm equilibrated with 0.2% formic acid) and freeze-dried. This product, 500 mg, was dissolved in 50 ml phosphate buffer (0.02 M phosphate + 0.1 M NaCl, pH 6.4) and applied onto a column of Sepharose-bound anti-Dnp antibodies [8] equilibrated with the same buffer. The column (4.6 × 14 cm) was washed with 200 ml phosphate buffer and the main Dnp-dextran fraction was eluted by 150 ml 2 M potassium thiocyanate in phosphate buffer. The tightly adsorbed Dnp-dextran derivatives were removed from the anti-Dnp antibody by 3 M potassium thiocyanate and discarded. Following gel filtration on Sephadex G-25 equilibrated in 0.2% formic acid the main Dnp-dextran fraction was freeze-dried and analyzed spectrophotometrically for the number of Dnp-groups using molar absorption coefficient 1.74×10^4 $M^{-1}cm^{-1}$ at 360 nm. Preparations that had 0.95–1.09 Dnp-groups/molecule dextran were pooled and used for further work. Mono-Dnp-dextran prepared in this way was found to be liable to hydrolysis, at pH 7; it is sufficiently stable, however, at pH 6.4 (half-life of hydrolysis $(t_{1/2})_{6.4}$ ~30 days).

The molecular weight was determined by the method of Chervenka [9] in a Spinco Model E ultracentrifuge. For scattering experiments, 2% (w/v) antibody solutions as well as 2% (w/v) antibody solutions with added mono-Dnp-dextran (molar ratio dextran–antibody 2.2:1) were prepared in H_2O and D_2O buffers containing 0.1 M sodium phosphate of app. pH 6.4 and centrifuged at 6000 × *g* for 20 min. The H_2O and D_2O solutions were then mixed together to give the H_2O:D_2O ratio desired. To determine the molecular volume and scattering power density of the antigen, 2% (w/v) solutions were prepared both in H_2O and D_2O buffers at pH 6.4. The scattering experiments were performed at the D 11 facility, Institut Max von Laue-Paul Langevin, Grenoble [10] and at the time-of-flight small-angle scattering facility, Neutron Physics Laboratory, Joint Institute for Nuclear Research, Dubna [11].

3. Results and discussion

3.1. *Characterization of antigen–antibody complexes*

The sedimentation patterns of antigen–antibody complexes showed one major and one minor peak in all samples. In H_2O, the uncorrected sedimentation coefficients (at a conc. 0.65%) of the major peaks were 6.70 S and 6.65 S for the early and late complexes, respectively. In D_2O, the corresponding uncorrected values were 4.49 and 4.46 S. The minor peaks (uncorrrected sedimentation coefficient 2.85 S) obviously represented the antigen that had been added in slight excess; the sedimentation coefficient of mono-Dnp-dextran alone was 2.60 S. These experiments did not rule out the presence of a certain amount of aggregates, which sedimented to the bottom of the cell before the full speed was reached.

An aliquot of the H_2O solution of the late antibody–antigen complex was subjected to gel chromatography on a column of Sepharose 6B. The major part (75–80%) of the material eluted in a symmetrical peak the elution volume of which corresponded to the expected elution volume of the complex, and the rest of the material was mostly present as uncomplexed antigen. The stoichiometry of the main peak component, according to A_{280} and A_{360} values, was 1.95 antigen molecules/antibody molecule. This material was mol. wt 200 000 ± 10 000; the value is in reasonable agreement with the expected molecular weight of an antibody–antigen complex (i.e., 150 000 + 2 × 40 000) if partial dissociation of the complex at a very low concentration (0.02%) is taken into account.

3.2. *Scattering data*

The radii of gyration of the antigen (table 1) indicate that the shape of the antigen molecule differs slightly from the sphere. A linear plot of square root intensity at zero momentum transfer, $\sqrt{I(0)}$ versus D_2O concentration [12] shows that the scattering density of the antigen equals to that of 50% D_2O; this gives a molecular volume of 39.3 nm^3.

The scattering data of H_2O solutions of antigen–antibody complexes were transformed to yield the Guinier plots. The plots gave straight lines typical of monodisperse solutions thus permitting the radii of gyration to be calculated (table 1). The scattering curves obtained from H_2O–D_2O mixtures showed

Table 1
Radii of gyration derived from scattering curves[a]

Sample	R_g (nm)	Data source
(Early anti-Dnp antibody) –(mono-Dnp-dextran)$_2$ complex	11.48 ± 0.60	this paper
Early anti-Dnp antibody	6.74 ± 0.08	[3]
Early anti-Dnp antibody with hapten	6.42 ± 0.09	[3]
(Late anti-Dnp antibody) –(mono-Dnp-dextran)$_2$ complex	8.87 ± 0.98	this paper
Late anti-Dnp antibody	6.16 ± 0.07	[3]
Late anti-Dnp antibody with hapten	5.69 ± 0.08	[3]
Mono-Dnp-dextran (mol. wt 40 000) in D_2O	2.16 ± 0.2	this paper
Mono-Dnp-dextran (mol. wt 40 000) in H_2O	1.89 ± 0.2	this paper

[a] Data given without correction for resolution

the presence of high-molecular weight aggregates. Consequently, the scattering data could not be interpreted in terms of radii of gyration.

At 41% D_2O, the scattering density of the solvent equals that of the antibody [12] and the scattering curve observed is expected to be due to the dextran moiety of the complex. In order to eliminate the effect of density fluctuations of the antibody, the background spectrum of antibody alone in 41% D_2O was measured and subtracted from the spectrum of

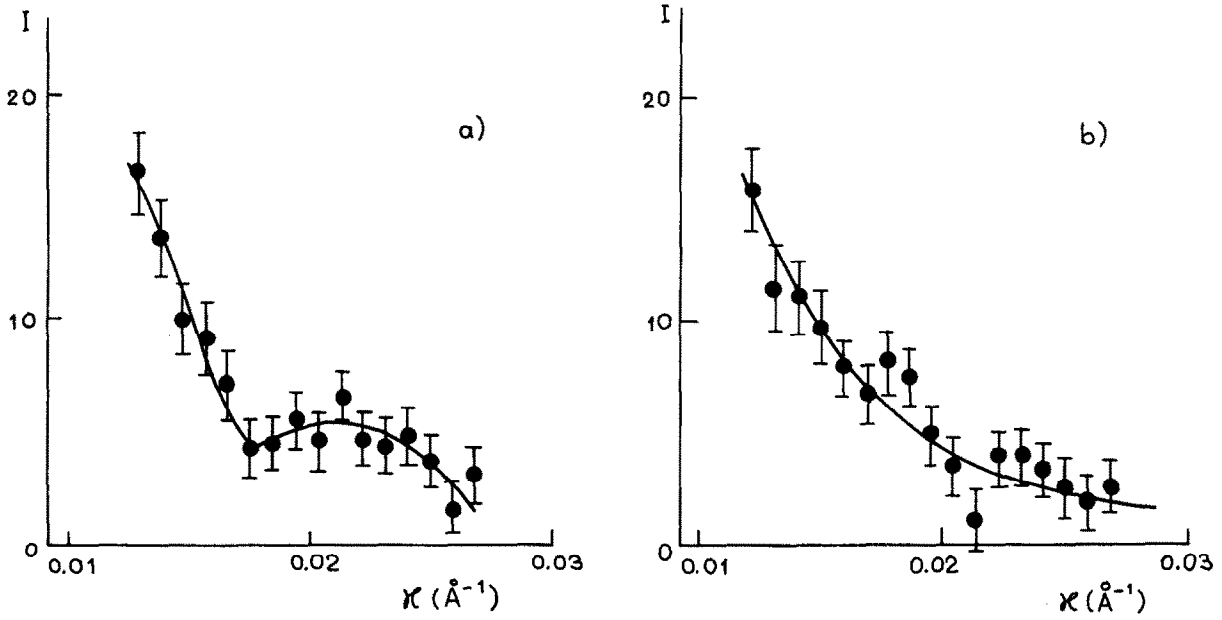

Fig.1. Neutron small-angle scattering curve of antibody–antigen complexes obtained in 41% D_2O. Horizontal axis: the value of transferred momentum, κ. Vertical axis: intensity scattered, I, in arbitrary units. Smoothed solid curves were prepared for the Fourier transformation of data. (a) Early antibody–antigen complex; (b) late antibody–antigen complex.

the complex. The curve of the early antibody–antigen complex obtained at 41% D_2O (fig.1) displayed a weak subsidiary maximum apparently representing the scatter on two dextran molecules kept at a fixed distance. The shape of the subsidiary maximum, however, was distorted due to the presence of aggregates. No subsidiary maximum could be observed with the late antibody–antigen complex (fig.1).

3.3. *Interpretation of the scattering data and computation of the antigen–antigen distance*

A comparison of the radii of gyration of the early and late antibody–antigen complexes (table 1) together with the difference of the scattering curves obtained at 41% D_2O suggest that the complexes of the early and late antibody have different shapes and dimensions. The data were obtained only with a moderate statistical accuracy. Fortunately, for the early antibody–antigen complex, the distance between the centres of gravity of the two antigens bound to the same antibody molecule could be estimated using two independent approaches. In the case of the late antibody–antigen complex, this possibility did not exist and, therefore, the confidence level of the latter value is rather low.

The position of the subsidiary maximum (fig.1a) may serve to calculate the average distance between the centres of gravity of the antigens using the commonly used Fourier transformation [13]. The numerical value of this distance is 35.7 ± 1.5 nm.

Alternatively, one can take advantage of the fact that a complex particle composed of two sub-particles (each of which possesses it own radius of gyration, R_1 and R_2, respectively), is characterized by the radius of gyration $R_g = (f_1R_1^2 + f_2R_2^2 + f_1f_2a^2)^{1/2}$ [12]. Particularly, for the early antibody–antigen complex it holds that:

$$R_C^2 = f_AR_A^2 + f_BR_B^2 + f_Af_Ba^2 \qquad (1)$$

where R_C, R_A and R_B stand for the radii of gyration of the complex, antibody and a fictitious dumb-bell-like dextran body, respectively; a is the distance between the centres of gravity of the two subparticles, f_A and f_B are the relative scattering powers, having values of 0.7 and 0.3, respectively. The antigen–antigen distance is then estimated as $2R_B$. Calculations based on several tentative models have shown that the $f_Af_Ba^2$ term is never higher than 1.4 nm^2 and can be neglected. Using the data for the radii of gyration of the antibody–antigen and antibody–hapten complexes listed in table 1, the calculation according to eq. (1) led to the distance value of 36.0 ± 2.5 nm.

Two independent approaches yielded very similar values of antigen–antigen distance in the early antibody–antigen complexes. By averaging the two estimated values a mean value of the antigen–antigen distance of 35.8 ± 1.3 nm is obtained.

Analogous calculations using eq. (1) for the late antibody–antigen complex gave a value of 27.3 ± 3.7 nm for the antigen–antigen distance. (Note: for this distance value the position of the subsidiary maximum should be outside the investigated range of the transferred momentum κ).

To estimate the distance between the binding sites of the early antibody molecule, we have to subtract from the observed antigen–antigen distance the distance between the centre of the antigen molecule and its Dnp-moiety; this was taken to be 4.1 nm. The resulting value, 27.6 ± 1.5 nm, lies within the range typical of extended models of antibody molecules [2]. Thus, the early antibody, when complexed with a high-molecular weight antigen, exists obviously in an extended conformational state (with Fab arms and elbows stretched). The scattering data obtained permit us to conclude that, with respect to the same high-molecular weight antigen, the late antibody behaves differently than the early antibody, giving rise to complexes in which the distance between the two binding sites is likely to be smaller. The difference is compatible with the previous observation [14] that the early antibody possesses a higher segmental flexibility than the late antibody.

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References

[1] Pilz, I., Kratky, O., Licht, A. and Sela, M. (1973) Biochemistry 12, 4998–5005.
[2] Pilz, I., Kratky, O. and Karush, F. (1974) Eur. J. Biochem. 41, 91–96.
[3] Cser, L., Franěk, F., Gladkikh, I. A., Nezlin, R. S., Novotný, J. and Ostanevich, Yu. M. (1977) FEBS Lett. 80, 329–331.
[4] Franěk, F., Doskočil, J. and Šimek, L. (1974) Immunochemistry 11, 803–809.
[5] Boissonas, R. A. (1951) Helv. Chim. Acta 34, 874–879.
[6] Vaughan, J. R., jr (1951) J. Am. Chem. Soc. 73, 3547.
[7] Wieland, T. and Bernhard, H. (1951) Liebig's Ann. Chem. 572, 190–194.
[8] Franěk, F. (1971) Eur. J. Biochem. 19, 176–183.
[9] Chervenka, C. H. (1970) Anal. Biochem. 24, 24–29.
[10] Schmatz, W., Springer, T., Schelten, J. and Ibel, K. (1974) J. Appl. Cryst. 7, 96–116.
[11] Gladkikh, I. A., Kunchenko, A. B., Ostanevich, Yu. M. and Cser, L. (1978) JINR Commun. P3-11487.
[12] Cser, L., Gladkikh, I. A., Kozlov, Zh. A., Nezlin, R. S., Ogievetskaya, M. M. and Ostanevich, Yu. M. (1976) FEBS Lett. 68, 283–287.
[13] Guinier, A., Fournet, G. (1955) Small-Angle Scattering of X-Rays, John Wiley, New York.
[14] Dudich, E. I., Nezlin, R. S. and Franěk, F. (1978) FEBS Lett. 89, 89–92.